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JCI Insight. 2020;5(2):e133501. https://doi.org/10.1172/jci.insight.133501.

Clinical Medicine Immunology Oncology

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We investigated levels of plasma metabolites and T cell properties, including energy metabolism markers, in the blood of patients with non-small cell lung cancer before and after treatment with nivolumab (n = 55). Predictive values of combination markers statistically selected were evaluated by cross-validation and linear discriminant analysis on discovery and validation cohorts, respectively. Correlation between plasma metabolites and T cell markers was investigated.

The 4 metabolites derived from the microbiome (hippuric acid), fatty acid oxidation (butyrylcarnitine), and redox (cystine and glutathione disulfide) provided high response probability (AUC = 0.91). Similarly, a combination of 4 T cell markers, those related to mitochondrial activation (PPAR γ coactivator 1 expression and ROS), and the frequencies of CD8⁺PD-1^{hi} and CD4⁺ T cells demonstrated even higher prediction value (AUC = 0.96). Among the pool of selected markers, the 4 T cell markers were [...]



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BACKGROUND. Current clinical biomarkers for the programmed cell death 1 (PD-1) blockade therapy are insufficient because they rely only on the tumor properties, such as programmed cell death ligand 1 expression frequency and tumor mutation burden. Identifying reliable, responsive biomarkers based on the host immunity is necessary to improve the predictive values.

METHODS. We investigated levels of plasma metabolites and T cell properties, including energy metabolism markers, in the blood of patients with non-small cell lung cancer before and after treatment with nivolumab (*n* = 55). Predictive values of combination markers statistically selected were evaluated by cross-validation and linear discriminant analysis on discovery and validation cohorts, respectively. Correlation between plasma metabolites and T cell markers was investigated.

RESULTS. The 4 metabolites derived from the microbiome (hippuric acid), fatty acid oxidation (butyrylcarnitine), and redox (cystine and glutathione disulfide) provided high response probability (AUC = 0.91). Similarly, a combination of 4 T cell markers, those related to mitochondrial activation (PPAR γ coactivator 1 expression and ROS), and the frequencies of CD8⁺PD-1^{hi} and CD4⁺ T cells demonstrated even higher prediction value (AUC = 0.96). Among the pool of selected markers, the 4 T cell markers were exclusively selected as the highest predictive combination, probably because of their linkage to the abovementioned metabolite markers. In a prospective validation set (*n* = 24), these 4 cellular markers showed a high accuracy rate for clinical responses of patients (AUC = 0.92).

CONCLUSION. Combination of biomarkers reflecting host immune activity is quite valuable for responder prediction.

FUNDING. AMED under grant numbers 18cm0106302h0003, 18gm0710012h0105, and 18lk1403006h0002; the Tang Prize Foundation; and JSPS KAKENHI grant numbers JP16H06149, 17K19593, and 19K17673.

Introduction

Programmed cell death 1 (PD-1) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4) are the critical players in maintaining immune tolerance during tumor growth (1–4). Blocking of molecules individually or both together rejuvenates CD8⁺ T cells in the dormant or reversible exhaustion stage and can induce strong antitumor activity in mice and humans (1–3, 5, 6). CD8⁺ T cells, once activated by recognition of tumor antigens, proliferate and attack tumors. Activated T cells with chronic stimulation eventually express PD-1 and CTLA-4 and go into the reversible exhaustion stage to avoid excessive proliferation and the generation of autoimmunity. Some of the activated CD8⁺ T cells may go into the

Authorship note: RH and KC equally contributed to this work.

Conflict of interest: TH has received personal fees from Bristol-Myers Squibb, grants from Ono Pharmaceutical Company, grants from Sysmex Corporation, and grants from Shimazu Corporation and has a patent (2019-000181). KC has received personal fees from Bristol-Myers Squibb and grants from Sysmex and has a patent (2019-000181). FM has a patent (2019-000181).

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Submitted: September 16, 2019 Accepted: December 10, 2019 Published: January 30, 2020.

Reference information: *JCI Insight.* 2020;5(2):e133501. https://doi.org/10.1172/jci. insight.133501. irreversible exhaustion stage, resist rejuvenation, and are defined by PD-1^{hi}, CTLA-4^{hi}, T cell immunoglobulin and mucin domain–containing molecule 3–positive (Tim-3⁺), Ki-67⁺, and others (7). Immune tolerant individuals who carry tumors are likely to contain variable proportions of CD8⁺ T cells with reversible and irreversible exhaustion stages.

Although antibodies against CTLA-4, PD-1, or its ligand, PD-L1, have been approved for treatment of various human cancers and demonstrated the impressive clinical efficacy (4), a significant portion of cancer patients remain less responsive (4–6). Therefore, predictive biomarkers to distinguish responders and non-responders are desperately required to save cost and time for those patients.

Effective cancer immunotherapy depends on the cancer-immunity cycle, in which naive T cells are primed in draining lymph nodes (DLNs) and differentiated to effector T cells that egress into circulation and traffic to tumor sites, guided by a gradient of chemokines released from tumor sites to attack tumor cells (8). Tumor antigens are captured by dendritic cells, which in turn traffic to DLNs and stimulate naive T cells again. To distinguish responders from nonresponders, it is critical to evaluate how many CD8⁺ T cells are in the stage of reversible or irreversible exhaustion in patients by monitoring peripheral blood mononuclear cells (PBMCs), while the actual battles of the immune system against cancer take place in tumor sites and DLNs.

When CD8⁺ T cells are activated by antigenic stimulation, such as tumor antigens, they mobilize various intracellular signaling pathways to generate abundant energy and anabolic reaction substrates required for proliferation (9). Fagarasan's group previously showed that proliferation of the antigen-stimulated T cell population drastically changes the systemic metabolites in mouse blood (10). We also confirmed similar drastic change in serum metabolites due to the antitumor immune reactions in a PD-1 blockade cancer therapy model (11). Activated tumor-reactive CD8⁺ T cells were also shown to carry mitochondria with higher reactive oxygen species (ROS) detected by mitochondrial dye staining. ROS signaling links to the regulation of mTOR/AMPK phosphorylation, which subsequently activates the PPAR γ coactivator 1 α (PGC-1 α) pathway. This feed-forward pathway for mitochondrial biogenesis enhances CD8⁺ T cell activation (11, 12). We further showed that PGC-1 α /PPAR complex activation enhances the efficacy of the PD-1 blockade therapy by enhancing fatty acid oxidation (FAO) in CD8⁺ T cells, which leads to longevity of effector CD8⁺ T cells (13). Therefore, these T cell metabolism-associated markers may serve as predictive or early monitoring biomarkers in the PD-1 blockade therapy.

It is also known that gut microbiota and immune activity mutually affect each other as Fagarasan and coworkers have demonstrated using IgA-deficient or PD-1–deficient mouse models (14, 15). In the case of tumor immunity, recent reports also suggested that microbiota and/or their metabolites are related to the efficacy of immune checkpoint inhibitors (16–18). Especially, certain microbiota, *Akkermansia muciniphila*, and diversity of flora are shown to correlate to the responsiveness to the PD-1 blockade therapy (18). However, it is still elusive whether any of the microbiota-associated factors could be responder biomarkers for the PD-1 blockade therapy.

Currently, PD-L1^{hi} expression on histological tumor tissue samples is used as a responder predictive marker for non-small cell lung cancer (NSCLC) (19, 20). The FDA has recently approved microsatellite instability–high or mismatch repair deficiency as common responder biomarkers for various solid tumors (21). These markers, however, cannot cover all the responsive patients, probably because the responses of tumor-reactive CD8⁺ T cells are affected not only by tumor properties but also by host immune activity (21). Several groups have identified candidates for responder biomarkers by analysis of various immune cell compartments at tumor sites or in peripheral blood. The proposed markers include the frequencies of CD8⁺ T cells, CD4⁺ T cells, eosinophils, neutrophils, subsets of suppressive macrophages, and subsets of T cells (16). Immune regulators, such as certain cytokines or chemokines, were also listed as candidates of biomarkers (16). However, the predictive value of each of these single markers for host immunity is not sufficient for clinical usage.

In the present studies using blood samples from 55 patients with NSCLC, we demonstrated that a combination of several plasma metabolites and/or T cell markers could serve as good responder biomarkers (AUC = 0.96 by cross-validation). The metabolite markers include those related to microbiota (hippuric acid), FAO (butyrylcarnitine), and redox (cystine and glutathione disulfide). The cellular markers of T cells in responders contain those associated with suppressive function (the PD-1^{hi} population) and mitochondrial activities (PGC-1 and ROS expression) in CD8⁺ T cells. These T cell markers are linked with the metabolite markers described above. Therefore, we propose that the combinatorial quantitation of either the specific plasma metabolites or PBMC T cell markers will be a highly predictive biomarker of the PD-1 blockade therapy for clinical usage.

Results

Microbiota metabolism- and energy metabolism-related metabolites correlate with responsiveness to the PD-1 blockade cancer immunotherapy. Accumulating evidence indicates there is a considerable association between immune responses and the metabolome (22). However, it remains unknown whether particular metabolites can serve as predictive biomarkers for the PD-1 blockade therapy in humans. In this study, we identified plasma metabolites and T cellular markers from 55 NSCLC patients (discovery cohort) and validated the markers in 24 patients (validation cohort) (Figure 1). To investigate how metabolites are associated with antitumor immunity first, we analyzed metabolites and T cell functional markers in plasma and PBMCs, respectively, in 55 patients with NSCLC before and after nivolumab treatment (Figure 2A and Supplemental Tables 1–4; supplemental material available online with this article; https://doi.org/10.1172/jci. insight.133501DS1). Blood was harvested just before the injection of nivolumab at 0, 2, and 4 weeks, and we designated these samples as the 1st, 2nd, and 3rd samples, respectively (Figure 2A). We defined responsive and unresponsive patients based on the criteria of progression-free survival (PFS) more than 3 months or PFS no more than 3 months according to the PFS data of phase III clinical studies of NSCLC patients where around 40% to 50% of patients died within 3 months (Supplemental Figure 1A and refs. 23, 24). Although PFS more than 6 months has often been used as a responder criterion, both criteria (PFS > 3 months and PFS > 6 months) provided similar overall survival (OS) curves in the present study (Supplemental Figure 1, B and C). Moreover, PD-L1 tumor proportion score (TPS) at tumor sites could not discriminate between responders and nonresponders clearly in the present study (Supplemental Figure 1, D and E). We measured 247 metabolites in 55 patients, of whom 8 had to stop the therapy because of severe side effects and other reasons, leaving us with the data of 47 patients for analysis (Figure 1 and Figure 2A). Volcano plot analysis of these metabolites based on fold change and P value demonstrated that hippuric acid in the 1st samples and hippuric acid, indoxyl sulfate, 4-cresol, and glutathione disulfide (GSSG) in the 3rd samples were significantly elevated in responders compared with nonresponders (Figure 2B and Table 1). On the other hand, the levels of α -ketoglutaric acid and butyrlcarnitine in the 3rd samples were lower in responders, but there were no items with significant differences between responders and nonresponders in the 2nd samples (Figure 2B and Table 1). Hippuric acid, indoxyl sulfate, and 4-cresol are reported to be almost exclusively produced by microbiota in mammals (25), which is consistent with the finding that patients treated with antibiotics within 3 months before the nivolumab treatment had lower levels of these 3 metabolites (Supplemental Figure 2A). Importantly, responsive patients had higher levels of the microbiota-derived metabolites (indoxyl sulfate and 4-cresol) than unresponsive patients, indicating that stronger antitumor immune responses are associated with the gut microenvironment (Figure 2C and Supplemental Figure 2B). We did not exclude those patients pretreated with antibiotics from this study because there were no differences in survival between patients treated with and without antibiotics at any time within 3 months before nivolumab injection (Supplemental Figure 2C). GSSG levels were higher in responders than in nonresponders, especially in the 3rd samples (Figure 2, B and D, and Table 1). GSSG is an oxidized form of glutathione, which controls the ROS levels appropriately in cells (26). Butyrylcarnitine levels were higher in nonresponders than in responders (Figure 2, B and D, and Table 1). Butyrylcarnitine, the 4-carbon acylcarnitine, serves as a fatty acid transporter into mitochondria to generate ATP. Acylcarnitine species with various amounts of carbon are released from cells once the function of FAO is attenuated (27-29). It should be noted that butyrylcarnitine and other acylcarnitine species (isovalerylcarnitine and hexanoylcarnitine) had a trend to increase in the later phase of therapy in nonresponders (Supplemental Figure 2D). There was a trend of lower α -ketoglutaric acid in responders than in nonresponders (Figure 2, B and D, and Table 1). In the tricarboxylic acid cycle in mitochondria for ATP production, α -ketoglutaric acid is a core metabolite and is reduced in the blood because of consumption by activated T cells (10, 11). Therefore, these data indicate that antitumor immune responses to the PD-1 blockade therapy are linked to microbiota and energy metabolism.

A combination of plasma metabolites can be a predictive biomarker. We addressed the probability of each predictive biomarker candidate selected above (Figure 2B and Table 1) by receiver operating characteristic (ROC) curve analysis with logistic regression. The AUC of each candidate was not high enough for prediction (right column in Table 1), though their AUCs were higher than that of PD-L1 TPS (0.66).



Figure 1. CONSORT flow diagram. irAE, immune-related adverse event.

Therefore, we next used stepwise discriminant analysis to elucidate superior biomarkers based on a combination of metabolites. We first selected those with a significant difference between responders and nonresponders in their levels or in their ratio (fold change) of the different time points among 1482 items in total (247 items × 3 time points + 247 items × 3 ratios) (Supplemental Table 5). A stepwise Akaike's information criterion (AIC) regression procedure against the markers listed in Supplemental Table 5 demonstrated that metabolite combinations I, II, and III were most predictive for the 1st, 1st + 2nd, and 1st + 2nd + 3rd samples, respectively (Table 2 and Supplemental Figure 3). Linear discriminant analysis (LDA) demonstrated that metabolic combination I distinguished between responders and nonresponders with a 23% error rate for 1st samples (Figure 3A). To test the reliability of metabolite combination I, we assigned 47 samples to LDA-R or LDA-NR based on the LDA cutoff value (Figure 3A). As shown in Figure 3B, prediction markers of metabolite combination I could significantly discriminate LDA-R from LDA-NR in PFS. We then found that metabolite combination II distinguished responders and nonresponders with a 22% error rate, and there was a significant difference in both PFS and OS between LDA-R and LDA-NR (Figure 3, C and D). Finally, we found that metabolite combination III discriminated responders and nonresponders with the lowest error rate, 19.6% (Figure 3, E and F). To calculate AUCs of metabolite combinations by discriminant models, we conducted 5-fold cross-validation with logistic regression. In the 5-fold cross-validation, we split the cohort into 5 folds, took the first 20% fold as test data, and trained the prediction model with the remaining 80% to predict into the test data. We iterated this procedure 5 times for each fold and evaluated the model performance with the AUC. As a result, the calculated AUC values were 0.77, 0.83, and 0.91 for the metabolite combinations I, II, and III, respectively, suggesting combination III better discriminated responders and nonresponders (Figure 3G).

Considering that patients are administered nivolumab 6 times over 3 months in the current clinical protocol for NSCLC, metabolite combinations I and II are useful as predictive biomarkers, while metabolite combination III may be less valuable for clinical use despite having the highest reliability.

A combination of cellular markers including mitochondrial activities of CD8⁺ T cells can distinguish between responders and nonresponders. Our previous reports have shown that mitochondrial activation and energy

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Figure 2. Particular plasma metabolites are associated with nivolumab treatment response. (**A**) A schematic diagram of this study. GC-MS/ LC-MS, gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. (**B**) Comparison of 247 metabolites between nonresponders and responders at each time point was summarized in volcano plots. Metabolites with log₂ [fold change] greater than 1.0 and $-\log_{10}$ (*P* value) greater than 1.3 were considered significant. Ten metabolites with significant difference between responders and nonresponders are listed in Table 1. (**C**) The peak areas measured by GC-MS or LC-MS of each microbiota-related metabolite in nonresponders (NR) and responders (R). (**D**) The peak areas of redox/energy metabolism-related metabolites. Each dot represents 1 patient. Error bars show median and interquartile range. **P* < 0.05; ***P* < 0.01 by Kruskal-Wallis test followed by Dunn's multiple-comparisons test (**C** and **D**).

metabolism in T cells are strongly associated with the response to the PD-1 blockade therapy (11, 13). Therefore, we simultaneously investigated cellular markers for effector function, energy metabolism, mitochondrial status, and immune activation in CD8⁺ T cells of the patients' PBMCs. Among the 52 markers shown in Supplemental Table 4 and their ratios between each time point, we found 26 items

Number in Figure 2B	Metabolite name	Timing	Changes in responders relative to nonresponders	Platform ^A	<i>P</i> value ^B	Fold change	AUC ^c
1	Hippuric acid	1st	Higher	LC	0.0056	2.3	0.74
2	Hippuric acid	1st	Higher	GC	0.0062	2.74	0.73
3	Hippuric acid	Зrd	Higher	GC	0.0002	2.89	0.81
4	Hippuric acid	3rd	Higher	LC	0.0003	2.3	0.81
5	Indoxyl sulfate	Зrd	Higher	LC	0.0011	2.19	0.78
6	Indoxyl sulfate	3rd	Higher	GC	0.0049	2.08	0.74
7	4-Cresol	Зrd	Higher	GC	0.0023	2.41	0.76
8	GSSG	3rd	Higher	LC	0.0068	2.1	0.73
9	α -Ketoglutaric acid	Зrd	Lower	GC	0.0238	0.42	0.69
10	Butyrylcarnitine	Зrd	Lower	LC	0.0015	0.43	0.77

Table 1. Ten metabolites with significant difference between responders and nonresponders shown in Figure 2B

^AThe analytical platform (GC-MS or LC-MS) used for metabolite measurement. ^B*P* value for distinction between responders and nonresponders (Wilcoxon's rank-sum test). ^CThe area under the curve (AUC) of each metabolite in relation to responsiveness was calculated from univariate logistic regression analysis.

with significant differences in the expression levels between responders and nonresponders (Supplemental Table 6). We further selected the best combination of predictive biomarkers from these 26 items using the stepwise method as described for metabolites. As shown in Table 3, the best combinations to predict responders in the 1st, 1st + 2nd, and 1st + 2nd + 3rd samples were designated as cellular marker combinations I, II, and III containing 2, 4, and 4 items, respectively. The marker of responsive patients having a lower PD-1^{hi} population among CD8⁺ T cells in pretreated samples (in the 1st samples) appeared in all 3 cellular marker combinations, I, II, and III (Figure 4A and Table 3). The cutoff for PD-1^{hi} was defined by the 97th percentile of PD-1 expression intensity (Supplemental Figure 4A). It is important to note that there was no difference between responders and nonresponders in the frequency of total PD-1⁺CD8⁺ T cells (Supplemental Figure 4B). Although the PD-1^{hi}CD8⁺ T cell population appears to vigorously proliferate as the frequency of Ki-67 was higher than PD-1¹⁰ or PD-1⁻CD8⁺ T cells, it produced less granzyme B and IFN- γ (Supplemental Figure 5). In addition, in PD-1^{hi}CD8⁺ T cells, T-box expressed in T cells (T-bet) expression was lower, but eomesodermin (EOMES) expression and intensity of Mito SOX were higher than in PD-11º or PD-1-CD8+ T cells (Supplemental Figure 5). The Ki-67⁺ T cells increased after treatment (2nd + 3rd) compared with pretreatment (1st) in both PD-1^{hi} and PD-1¹⁰ subpopulations, which corresponds to the previous report (Supplemental Figure 5 and ref. 30). The PD-1^{lo} population showed stronger effector phenotypes, such as higher production of granzyme B, IFN- γ , and T-bet, while the PD-1^{hi} population showed lower production of these markers. These data indicate that after PD-1 blockade, highly proliferated T cells express more PD-1 and go into a severe exhaustion state, whereas moderately proliferated T cells express less PD-1 and keep a greater capacity to revive to effector T cells. Indeed, the transcriptional expression array analysis in peripheral CD8⁺ T cells demonstrated that the frequency of PD-1^{hi} in CD8⁺ T cells correlated well with the expression levels of CTLA4 and TIM3, known as exhaustion markers (Supplemental Figure 6A). The frequency of subpopulations among PD-1^{hi}CD8⁺ T cells showed no significant differences between responders and nonresponders, indicating that quantity rather than quality of CD8+PD-1hi T cells was associated with responsiveness (Supplemental Figure 6B). To test the correlation between the PD-1^{hi}CD8⁺ T cells and tumor antigen load, we also examined the tumor burden as previously described (31). Patients with only nonmeasurable lesions, including 2 responders and 5 nonresponders, were excluded from this analysis of tumor burden. As shown in Supplemental Figure 7A, there was no correlation between PD-1hi frequency and tumor burden. Notably, the tumor burden did not correlate to responsiveness to the PD-1 blockade therapy (Supplemental Figure 7B), suggesting that the tumor burden may not be a determinant of the severity of exhaustion in patients with NSCLC in our study.

Mitochondrial ROS, which is measured by a dye called Mito SOX, is one of the mitochondrial activation indicators (11). We found that the ratio of Mito SOX levels in CD8⁺ and CD4⁺ T cells (Mito SOX CD8/CD4) was higher in pretreated responders (1st samples), and this marker was shared by all marker

Metabolite combination	Selected metabolite (time point)	Change in responders relative to nonresponders	Samples	Platform	Data in Supplemental Figure 3
1	Cystine (1st)	Higher	1st	GC	А
	Unk8 (1st)	Lower		GC	А
	Hippuric acid (1st)	Higher		LC	А
II	Arabinose (2nd)	Higher	1st + 2nd	GC	В
	Arginine (2nd)	Higher		LC	В
	Butyrylcarnitine (2nd)	Lower		LC	В
III	Hippuric acid (1st)	Higher	1st + 2nd + 3rd	LC	А
	Cystine (2nd)	Higher		GC	С
	GSSG (3rd)	Higher		LC	С
	Butyrylcarnitine (3rd)	Lower		LC	С

Table 2. The best predictive combination of metabolites selected by AIC regression procedure

combinations as with the PD-1^{hi}CD8⁺ T cell marker (Table 3 and Figure 4B). These results indicate that higher mitochondrial activation status in CD8⁺ T cells when normalized by that of CD4⁺ T cells before treatment is important for a better response (Figure 4B). Because PGC-1 is a master regulator of mitochondrial biogenesis and mitochondrial metabolic pathways, such as oxidative phosphorylation and FAO (32, 33), we addressed PGC-1 expression using a polyclonal antibody that recognizes both PGC-1 α and PGC-1 β (PGC-1 $\alpha\beta$ hereafter). Whereas the PGC-1 $\alpha\beta$ expression in CD8⁺ T cells decreased in responders between the 1st and 2nd samples, it increased between the 2nd and 3rd samples (Table 3 and Figure 4C). Therefore, the ratio of PGC-1 $\alpha\beta$ expression in the 2nd relative to 1st samples was lower in responders but higher in the 2nd relative to 3rd samples (Figure 4C). To clarify the mechanism of the quick decline of PGC-1 $\alpha\beta$ expression following the first nivolumab injection in responders, we investigated the PGC-1aß expression in each population of CD8⁺ T cells. As shown in Supplemental Figure 8A, CCR7⁻CD8⁺ (effector) T cells have higher PGC-1αβ levels than CCR7⁺CD8⁺ (naive) T cells. Importantly, the frequency of CCR7⁻CD8⁺ in PBMCs decreased after the first shot of nivolumab in responders (Supplemental Figure 8B). Therefore, the quick decline is presumably because the frequency of CCR7⁻CD8⁺ T cells, which have higher PGC-1αβ, decreased in the peripherally circulating PBMCs in responders after nivolumab injection. This interpretation will be discussed later. As other groups have already reported, we also found that the frequency of CD4⁺ T cells was increased after nivolumab treatment in responders (Table 3, Figure 4D, and ref. 34). Further analysis revealed that nivolumab treatment increased the population of CD4+CD45RO+CCR7+ (central memory) in responders and decreased CD4+CD45RO-CCR7- (terminally differentiated effector memory CD45RA+ T cells) (Supplemental Figure 9).

A combination of the cellular markers associated with suppressive state and mitochondrial status in T cells is highly predictive. We assessed the error rate of cellular marker combinations I, II, and III by the method described above. LDA demonstrated clear separation between responders and nonresponders with an error rate of 19.1% for cellular combination I (Figure 5A). If we define responders and nonresponders based on the LDA criteria as we did in the metabolic markers, this classification significantly discriminated LDA-R and LDA-NR in both PFS and OS (Figure 5B). The cellular markers in combination I showed higher AUC in a single or combined mode than the PD-L1 TPS usually used in the clinic, indicating the importance of the markers derived from the immune properties of patients rather than those from tumors (Supplemental Table 7, Figure 5B, and Supplemental Figure 1, D and E). LDA of cellular marker combinations II and III predicted both LDA-R and LDA-NR with 4.3% error rates, and there were significant differences (P < 0.05) between LDA-R and LDA-NR in both PFS and OS (Figure 5, C-F). AUCs for cellular marker combinations I, II, and III were 0.85, 0.96, and 0.93, respectively, by 5-fold cross-validation with logistic regression within the same cohort (Figure 5G). These data indicate that a combination of cellular biomarkers obtained before the second therapy is sufficient to discriminate between responders and nonresponders. Although it has been reported that patients with EGFR mutations show a different response to the PD-1 blockade therapy (24), those patients could be correctly

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Figure 3. A combination of plasma metabolites predicts responsiveness to nivolumab treatment. (A) Linear discriminant analysis (LDA) was used to evaluate the accuracy of metabolite combination I as a predictive biomarker. Canonical plot of LDA for determination of responders (LDA-R) and nonresponders (LDA-NR). Each dot represents 1 patient. The vertical dotted line indicates the cutoff value. (B) Kaplan-Meier plots of PFS and OS of LDA-R (solid line) and LDA-NR (dotted line) determined by combination I. (C) Canonical plot of LDA based on metabolite combination II. (D) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR in combination II. (E) Canonical plot for LDA based on metabolite combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR in combination III. (F) Canonical plot for LDA based on metabolite combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Canonical plot for LDA based on metabolite combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Canonical plot for LDA based on metabolite combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plots of PFS and OS of LDA-R in combination III. (F) Kaplan-Meier plot for LDA based on metabolite combination III. (F) Kaplan-Meier plot for LDA based on metabolite Combination III. (F) Kaplan-Meier plot for LDA based on the combination III. (F) Kaplan-Meier plot for LDA based on the combination III. (F) Kaplan-Meier

determined using our cellular marker combination II (Supplemental Figure 10), suggesting that these combination markers based on immune properties may be more useful than tumor property-derived markers alone.

To prospectively validate cellular marker combination II, we measured the 4 cellular markers in a new cohort (validation cohort) of 24 patients (Figure 1). The LDA criteria of cellular marker combination II based on the discovery cohort correctly identified responders and nonresponders with an 8.3 % error rate and AUC was 0.919 with LDA (Figure 5H).

Cellular marker combination	Selected cellular marker (time point)	Change in responders relative to nonresponders	Samples	Data in Figure 4
I	% of PD-1 ^{hi} among CD8 ⁺ T cells (1st)	Lower	1st	А
	Mito SOX CD8/CD4 (1st)	Higher		В
П	% of PD-1 ^{hi} among CD8⁺ T cells (1st)	Lower	1st + 2nd	А
	Mito SOX CD8/CD4 (1st)	Higher		В
	PGC-1αβ of CD8⁺ T cells (2nd/1st)	Lower		C
	% of CD4 ⁺ T cells among PBMCs (2nd/1st)	Higher		D
III	% of PD-1 ^{hi} among CD8+ T cells (1st)	Lower	1st + 2nd + 3rd	А
	Mito SOX CD8/CD4 (1st)	Higher		В
	PGC-1αβ of CD8⁺ T cells (3rd/2nd)	Higher		C
	% of CD4 ⁺ T cells among PBMCs (2nd/1st)	Higher		D

Table 3. The best combination of cellular markers for prediction selected by AIC regression

The metabolic and cellular markers are linked. We next carried out the stepwise method to select the best combination of markers among the total metabolic and cellular markers with significant differences between responders and nonresponders (Supplemental Tables 5 and 6). Surprisingly, all selected markers were derived from cellular markers, resulting in the same combination and results as shown in Table 3. Therefore, we hypothesized that there might be a linkage between responsible metabolic and cellular markers, which might lead us to exclude the metabolic markers. Because cellular combination II was appropriate for prediction and practical for clinical use as described above, we focused on cellular marker combination II and investigated which metabolic markers correlated with each of the cellular markers in the combination. Spearman's correlation coefficients (r) were used to measure the association between the cellular and metabolic markers. Generally, |r| of more than 0.4 in Spearman's is considered to have a modest to strong correlation. We found that PGC-1 $\alpha\beta$ expression in CD8⁺ T cells correlated with the microbiota-related metabolic markers, the frequency of PD-1^{hi}CD8⁺ T cells correlated with the FAO-related metabolic marker, and the T cell Mito SOX marker was correlated with redox-related metabolic markers (Figure 6A). Note that cystine and pyroglutamic acid are components of glutathione, as shown in Supplemental Figure 11. We further examined the correlation between the frequency of PD-1^{hi}CD8⁺ T cells and FAO-related gene expression in CD8⁺ T cells. Importantly, the frequency of PD-1^{hi} (1st) was negatively correlated with the transcriptional expression of carnitine palmitoyltransferase 1B (CpTIB) (2nd) (r = -0.44), which transports acylcarnitine from the cytoplasm into the mitochondria (27–29), suggesting the reduced function of acylcarnitine transportation in PD-1^{hi} T cells (Supplemental Figure 12). Corresponding to this mechanism, in these patients, the frequency of PD-1^{hi} population also correlated with butyrylcarnitine 3rd/1st and hexanoylcarnitine 2nd/1st (Figure 6A and Supplemental Figure 12), demonstrating that the frequency of the PD-1^{hi} marker correlated with plasma increases in these acylcarnitine families after nivolumab treatment. This interpretation will be discussed later.

Cluster analysis showed a relative correlation weight between cellular combination II and metabolic markers, which can be classified into the 3 groups of (a) microbiota-related metabolites, (b) FAO-related metabolites, and (c) redox-related metabolites (Figure 6B). Details of the correlation between cellular markers and metabolite markers are summarized in Table 4. In conclusion, stepwise discriminant analysis among all markers excluded the responsible metabolic markers because they were closely linked with particular cellular markers that had slightly more predictive value than the metabolic markers. The current data support a link between microbiota activity and T cell energy metabolism, both of which contribute to the power of antitumor immunity and responsiveness to the PD-1 blockade immunotherapy.

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Figure 4. Particular cellular markers, including mitochondria status, were selected to make up a combinatorial predictive

marker. (A) Two representative NSCLC samples (nonresponder and responder) showing PD-1 and CCR7 positivity after gating on CD8+ PBMCs (left). Frequency of PD-1^{hi}CD8⁺ T cells in nonresponders and responders in the 1st samples (right). (B) Representative histograms of Mito SOX on gated CD4+ (black) and CD8+ (red) T cells (left). Ratio of Mito SOX levels in CD8⁺ and CD4⁺ T cells (Mito SOX CD8/CD4) for nonresponders and responders in the 1st samples (right). (**C**) PGC-1 $\alpha\beta$ of the 1st (black), 2nd (red), and 3rd (blue) samples among CD8⁺ PBMCs (upper left). MFI of PGC-1 $\alpha\beta$ between the 1st, 2nd, and 3rd samples (upper right). The solid line and dotted line represent responders and nonresponders, respectively. Fold change of PGC-1 $\alpha\beta$ expression between nonresponders and responders in the 2nd relative to 1st samples (lower left) and the 3rd relative to 2nd samples (lower right). (D) Frequency of CD4⁺ T cells among PBMCs in the 1st and 2nd samples (left). The solid line and dotted line represent responders and nonresponders, respectively. Fold change of CD4⁺ T cell frequency in the 2nd relative to 1st samples between nonresponders and responders (right). Each dot represents 1 patient. Error bars show median and interquartile range. *P < 0.05; **P < 0.01; ****P < 0.0001 by Wilcoxon's rank-sum test.

Discussion

In this study, we first demonstrated that the combination of several cellular markers of T cell activation status, including T cell mitochondrial condition, could effectively discriminate responders from nonresponders. The correlation analysis revealed that the functional activity of CD8⁺ T cells was related to a complex network of different higher-order function systems, such as the microbiome and energy metabolism. The advantages of our findings are summarized as follows: (a) we showed that each AUC of our immune property–based markers is higher than that of the hitherto clinically approved biomarker, PD-L1 TPS; (b) blood-based tests to examine

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Figure 5. A combination of cellular markers could predict survival more precisely. (**A**) LDA evaluated accuracy of cellular marker combination I. Canonical plot of LDA for determination of LDA-R and LDA-NR. Each dot represents 1 patient. The vertical dotted line indicates the cutoff value. (**B**) Kaplan-Meier plots of PFS and OS of LDA-R (solid line) and LDA-NR (dotted line) on cellular marker combination I. (**C**) Canonical plot for LDA based on cellular marker combination II. (**D**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR (dotted line) on cellular marker combination II. (**E**) Canonical plot for LDA based on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Kaplan-Meier plots of PFS and OS of LDA-R and LDA-NR on cellular marker combination III. (**F**) Canonical plot for LDA based on cellular marker combination for cellular marker combinations I, II, and III. (**H**) Canonical plot and ROC curve for prospective validation cohort with LDA based on cellular marker combination II.

immune cell properties and/or metabolites would be much simpler and more patient-friendly because testing phenotypes of tumors usually puts a huge burden on patients; and (c) combination of immune property–based biomarkers is more powerful than several single immune property–based biomarkers so far proposed (16).

One of the microbiota-derived metabolites, hippuric acid, which was higher in the pretreated samples of responders, could be an indicator of microbiome diversity and is produced preferentially by *Clostridiales* (35, 36). Diversity of microbiota and the ratio of *Clostridiales* are reported to be higher in PD-1 blockade therapy responders, which supports our results that hippuric acid is higher in responders. So far, identification of particular strains of microbiota involved in antitumor activation is still quite difficult because of numerous species of microbiota in the gut (17). However, measuring a few common metabolites, such as hippuric acid, indoxyl sulfate, and 4-cresol, produced by numerous kinds of microbiota used the quick, comprehensive evaluation of microbiota status without deep sequencing of microbiota 16s rRNA. Considering that the levels of microbiota-derived metabolites correlated with PGC-1 $\alpha\beta$ expression levels in CD8⁺ T cells, some components of microbiota regulation of peripheral CD8⁺ T cells remains largely unknown (18).

We showed that the temporal reduction of PGC-1 $\alpha\beta$ may be due to the decrement of effector CD8⁺ T cells (CCR7⁻ population) from the blood, which express higher PGC-1 $\alpha\beta$ than CD8⁺ naive T cells (CCR7⁺ population). We and other groups previously indicated the cancer-immunity cycle theory is critical for robust antitumor immunity where effector T cells traffic to the tumor site, guided by the gradient of IFN- γ -induced chemokines released from the tumor site during the PD-1 blockade therapy (8, 11). Therefore, it is anticipated that PD-1 blockade triggers the trafficking of peripheral effector T cells to the tumor site preferentially in the responders. Given that microbiota affect the activation status of effector T cells (17, 35, 36), it would be reasonable that plasma hippuric acid levels and PGC-1 $\alpha\beta$ expression in the peripheral CD8⁺ T cells than naive CD8⁺ T cells.

We previously reported that FAO is important to produce sufficient ATP, which is required for longevity in effector T cells (13). When FAO is promoted, acylcarnitines are substantially transported into the mitochondrial matrix, resulting in the reduction of acylcarnitine levels in the plasma (27–29, 37, 38). Therefore, plasma levels of acylcarnitine species could be an indicator of robustness in mitochondrial function or FAO usage in inflammation mediated by immunity (39–41). In the present study, acylcarnitines (butyrylcarnitine, isovalerylcarnitine, and hexanoylcarnitine) were elevated in nonresponders, especially in the latter seconnd and third phases, suggesting that the function of FAO in CD8⁺ T cells is attenuated in nonresponders (38). The substantial correlation between higher acylcarnitine levels and higher frequency of PD-1^{hi}CD8⁺ T cells suggests the presence of a large number of severely exhausted CD8⁺ T cells, which have a weaker FAO function in nonresponders. Indeed, the frequency of PD-1^{hi} negatively correlated with the transcriptional expression of *CpT1B* in CD8⁺ T cells, which serves as a transporter of acylcarnitine families from cytoplasm into mitochondria. Given that (a) PD-1^{ho}CD8⁺ T cells proliferated less and retained the effector function and (b) PD-1^{ho}CD8⁺ T cells had lower levels of CTLA-4 and Tim-3 based on correlation analysis, it is likely that PD-1^{hi}CD8⁺ T cells are in irreversible exhaustion states associated with the FAO defect in the periphery. However, the precise mechanistic linkage of FAO defect and T cell dysfunction is largely unknown.

Although we recruited 55 and 26 patients for the discovery and validation cohorts, respectively, the cohorts are rather small to obtain high reliability. However, validation is difficult in a larger cohort under the same conditions because the use of PD-1 blockade antibody single therapy is currently very rare because of the clinical strategy of combination therapy in patients with NSCLC. It will be important to test our biomarkers for the combination therapy as well in the future. Considering the convenience of specific metabolite measurement and the difficulties in technical variations for cellular marker measurements between different facilities, the combination of particular metabolites might be more practical in the clinic. Our new insight sheds light on the use of combinatorial biomarkers for cancer immunotherapy, which would provide nonresponders another opportunity to have immunotherapy and improve therapeutic efficacy.

JCI insight



Figure 6. Modest correlation between particular cellular and metabolite markers excludes metabolite makers from the combinatorial candidate biomarker. (A) Scatter plots between cellular markers (*x* axis) and metabolite markers (*y* axis). The dots represent the responders and the circles indicate the nonresponders. *r*, Spearman's correlation coefficients. Generally, |r| of more than 0.4 in Spearman's is considered to have a modest to strong correlation. (**B**) A clustered heatmap of absolute correlation coefficients over all marker pairs detected in **A** (using Spearman's correlation distance and complete linkage). Dark denotes higher correlation (|r| close to 1) and light lower correlation (|r| close to 0). The markers clustered into 3 groups, which were designated as metabolic categories I, II, and III.

Methods

Study design and participants. The study subjects, patients with NSCLC receiving nivolumab (anti-PD-1 blocking antibody) at Kyoto University Hospital, consented to the collection and storage of blood samples during treatment and allowed review of their medical records for past medical history, cancer tumor

Cellular markers (Change in responders relative to nonresponders)	Metabolite markers (Change in responders relative to nonresponders)	Metabolite category
PGC-1 $lphaeta$ of CD8 ⁺ T cells (2nd/1st) (lower)	Indoxyl sulfate (3rd) (higher)	(I) Microbiome-related metabolites
	Hippuric acid (3rd) (higher)	
	Hippuric acid (3rd/1st) (higher)	
Percentage of PD-1 ^{hi} among CD8⁺ T cells (1st) (lower)	Butyrylcarnitine (3rd/1st) (lower)	(II) FAO-related metabolites
Mito SOX CD8/CD4 (1st) (higher)	Cystine (2nd) (higher)	(III) Redox-related metabolites
	Pyroglutamic acid (2nd/1st) (higher)	

Table 4. Correlation between cellular and metabolite markers

type, toxicity assessments, and clinical response, survival, and laboratory data. We enrolled 55 patients with NSCLC, all of whom had previously received other chemotherapy. This study included 39 male and 16 female patients, aged between 31 and 83 years (median 68). Forty-three patients (78.2% among all patients) were diagnosed with adenocarcinoma, including 9 patients with EGFR mutation (17.3% among 52 patients) and 3 patients with anaplastic lymphoma kinase translocation (6.8% among 44 patients) (Supplemental Table 1). Patients received nivolumab (3 mg/kg) through i.v. infusion every 2 weeks (1 cycle) until disease progression or the emergence of an unacceptable side effect. At a median follow-up time of 455 days (range, 29 to 861 days), the median PFS and OS were 147 days (95% CI, 76 to 300 days) and 629 days (95% CI, 408 days to not reached), respectively (Supplemental Figure 1A). Among 55 patients enrolled, 8 patients had to stop the therapy because of severe side effects and other reasons (Figure 1). Blood samples were collected just before the first, second, and third nivolumab injection. Tumor size was measured by CT and evaluated for response using Response Evaluation Criteria in Solid Tumors 1.1. Total measurable tumor burden was defined as the sum of the long axis of all measurable lesions of the pretreatment CT as previously described (31). To assess PD-L1 expression, we performed PD-L1 IHC using the PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies) as previously described (42). TPS was defined as tumor PD-L1 expression frequency. PD-L1 TPS was classified into PD-L1 negative, weakly positive, or strongly positive (<1%, 1%–49 %, and \geq 50%, respectively) (42).

For the prospective validation study, 26 patients with NSCLC who had received nivolumab or pembrolizumab at Kyoto University Hospital were independently enrolled. Of these 26, 2 had to stop the therapy because of severe side effects, leaving us with the data of 24 patients for analysis (Figure 1). For functional analysis of the PD-1^{hi}CD8⁺ T cell population, we assessed 16 patients among 24 patients enrolled for the validation.

Thirty healthy blood donors were recruited at the Medical Examination Center at Takeda Hospital. Their age was between 46 and 78 (mean 66) years, and they included 22 male and 8 female donors.

Sample preparation for plasma metabolome measurement. Peripheral blood samples were collected in 7-mL EDTA vacutainers (Venoject II, VP-NA070K), immediately stored in a CubeCooler (Forte Grow Medical Co. Ltd.), and kept at 4°C until centrifugation at 4°C at 1800 g for 15 minutes. All the harvested plasma samples were then stored at -80° C until analysis. For GC-MS analysis, 50 µL of plasma was mixed with 256 µL of a solvent mixture (methanol/water/chloroform = 2.5:1:1) containing 2.34 µg/mL of 2-isopropylmalic acid (MilliporeSigma), which was used as an internal standard. The obtained mixture was shaken at 1200 rpm for 30 minutes at 37°C (Maximizer MBR-022UP, Taitec). After centrifugation at 16,000 g for 5 minutes at 25°C, 150 µL of supernatant was collected and mixed with 140 µL of purified water followed by vortex mixing for 5 seconds. After centrifugation at 16,000 g for 5 minutes at 25°C, 180 µL of supernatant (CVE-3100, Tokyo Rikakikai Co. Ltd.). The dried sample was dissolved in 80 µL of methoxyamine solution (20 mg/mL in pyridine, MilliporeSigma) and shaken at 1200 rpm for 30 minutes at 37°C. Forty microliters of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide solution (GL Sciences) was added for trimethylsilyl derivatization, followed by agitation at 1200 rpm for 30 minutes at 37°C. After centrifugation, 50 µL of supernatant was transferred to a glass vial and subjected to GC-MS measurement. For LC-MS analysis, the metabolite extraction protocol was slightly changed. Fifty microli-

ters of plasma was mixed with 256 μ L of methanol and shaken at 1200 rpm for 10 minutes at 37°C. After centrifugation at 16,000 g for 30 minutes at 25°C, 150 μ L of supernatant was mixed with 90 μ L of 1% acetic acid in water and 120 μ L of chloroform, followed by vortex mixing for 15 seconds. After centrifugation at 2000 g for 10 minutes at 25°C, 150 μ L of the upper layer was dried and solubilized in 50 μ L of 0.1% formic acid in water and then subjected to LC-MS analysis.

Plasma metabolome analysis. GC-MS analysis was performed with a GCMS-QP2010 Ultra (Shimadzu). The derivatized metabolites were separated on a DB-5 column ($30 \text{ m} \times 0.25 \text{ mm}$ inner diameter, film thickness 1.0 µm, Agilent Technologies). The helium carrier gas was set at a flow rate of 39 cm/s. The inlet temperature was 280°C and the column temperature was first held at 80°C for 2 minutes, then raised at a rate of 15°C/min to 330°C and held for 6 minutes. One microliter of the sample was injected into the GC-MS in the split mode (split ratio 1:3). The mass spectra were obtained under the following conditions: electron ionization (ionization voltage 70 eV), ion source temperature 200°C, interface temperature 250°C, and full scan mode in the range of m/z 85 to 500 and scan rate 0.3 s/scan. Identification of chromatographic peaks was performed using the National Institute of Standards and Technology Research Library or Shimadzu GC/ MS database and further confirmed with authentic commercial standards. For semiguantitative analysis, the area of each metabolite peak was calculated and divided by the area of the internal standard peak. LC separation was conducted on a Shim-pack GIST C18-AQ column (3 µm, 150 mm × 2.1 mm id, Shimadzu GLC) with a Nexera UHPLC system (Shimadzu). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was as follows: 0 to 3 minutes, 0% B; 3 to 15 minutes, linear gradient to 60% B; 15.0 to 17.5 minutes, 95% B; 17.5 to 20.0 minutes, linear gradient to 0% B; hold for 4 minutes; flow rate, 0.2 mL/min. The column oven temperature was maintained at 40°C. The LC system was coupled with a triple-quadrupole mass spectrometer, LCMS-8060 (Shimadzu). LCMS-8060 was operated with the electrospray ionization and multiple reaction monitoring mode. All ion transitions and collision energies were optimized experimentally by using authentic standards of each metabolite. Three microliters of the sample was injected into the LC-MS system. Quality control (QC, pooled plasma) samples were subjected to the same preparation protocol and injected every 10 and 5 samples for GC-MS and LC-MS analysis, respectively. Each metabolite's signals were normalized with a QC-based correction method using the smooth-spline algorithm (43-45). Information on all the measured metabolites, including retention time, m/z, and ion transitions, is summarized in Supplemental Tables 2 and 3.

Flow cytometry. Fresh PBMCs were isolated by Ficoll density gradient centrifugation. PBMCs were immediately stained using the following antibodies: anti-CD8a (RPA-T8, TONBO), -CD8 (SK1, TON-BO), -CD4 (RPA-T4, SK3, TONBO), -CD45RA (HI100, TONBO), -CD45RO (UCHL1, BioLegend), -CCR7 (3D12, eBioscience), -PD-1 (EH12.2H7, BioLegend), -Tim3 (F38-2E2, BioLegend), -KLRG1 (13F12F2, eBioscience), -CD25 (BC96, BioLegend), -CXCR3 (G025H7, BioLegend), -CCR6 (G034E3, BioLegend), -T-bet (4B10, BioLegend), -EOMES (WD1928, eBioscience), -Ki-67 (SolA15, eBioscience), -CTLA-4 (BNI3, TONBO), -p-mTOR (MRRBY, eBioscience), -p-Akt1 (Ser473) (SDRNR, eBioscience), –granzyme B (GB11, BioLegend), –IFN-γ (4S.B3, BD Biosciences), and -FOXP3 (236A/E7, BD Biosciences). PGC-1 expression was detected by anti-PGC-1αβ (rabbit polyclonal, Abcam, ab72230), which recognizes both PGC-1 α and PGC-1 β , followed by secondary staining with goat anti-rabbit IgG (Santa Cruz Biotechnology, sc-3739). Live/dead cell discrimination was performed using 7-AAD staining solution (TONBO, 13-6993). Intracellular staining was performed using a FOXP3 fixation kit (eBioscience). For assessment of intracellular phosphoproteins, cells were permeabilized with 0.5% Triton X-100 and fixed with 1.5% paraformaldehyde before staining. Acquisition of samples was carried out on the BD FACSCanto II cell analyzer (BD Biosciences). Data were collected using the BD FACSDiva Software version 6.1.3 and further analyzed with FlowJo 10.4 (Tree Star Inc.). Data were gated on live (7-AAD⁻) and single cells. Determination of mitochondrial mass, membrane potential, mitochondrial superoxide, and cellular ROS was performed using MitoTracker Green, MitoTracker Deep Red, Mito-SOX Red, and CellROX Green reagents, respectively (all from Life Technologies). These dyes were added to cells and incubated at 37°C in a 5% CO, humidified incubator for 30 minutes, followed by surface staining. Intracellular staining for granzyme B and IFN-y was performed following treatment with platebound anti-CD3 (OKT3, TONBO) and anti-CD28 (55725, BD Pharmingen) for 6 hours in the presence of Brefeldin A (eBioscience) and Monensin (eBioscience) for the last 4 hours. After nivolumab treatment, anti-PD-1 (EH12.2H7, APC conjugated, BioLegend) antibody was added to cells and incubated at 37°C in a 5% CO₂ humidified incubator for 60 minutes, followed by other surface staining.

Microarray analysis of patients' $CD8^+T$ cells. We isolated peripheral CD8⁺ T cells from the same cohorts available (1st samples, n = 33; 2nd samples, n = 27). CD8⁺ T cells were purified from patients' PBMCs by AutoMACS according to the manufacturer's instructions (Miltenyi Biotec, 130-045-201).

Total RNA was isolated with RNeasy Micro Kit (Qiagen). The quality of extracted RNA was analyzed using TapeStation (Agilent Technologies). Five nanograms of total RNA was labeled using the GeneChip WT Pico Reagent Kit (Thermo Fisher Scientific) and hybridized to GeneChip Clariom D Assay, Human (Thermo Fisher Scientific). The array data were analyzed using Signal Space Transformation-Robust Multi-Chip Analysis and Sketch-Quantile normalization (Expression Console Software). Then we applied the Linear Models for Microarray Analysis (limma) package of Bioconductor software (46) and obtained differentially expressed genes. Functional analysis of the decreased gene expression was performed using the Database for Annotation, Visualization and Integrated Discovery (http://david.ncifcrf.gov) (47). Microarray data have been deposited under Gene Expression Omnibus accession number GSE141479.

Statistics. Data are reported as the median and interquartile range. A Wilcoxon rank-sum test was conducted to compare the 2 groups. A Kruskal-Wallis test followed by Dunn's test for multiple comparisons were conducted to compare the difference across independent groups. The stepwise AIC regression procedure was performed to select the best marker combination. Then LDA was performed by using the estimated biomarker combination to predict the reliability and failure rate. To adjust the cutoff value to 0, we changed the constant value of the equation of LDA as shown in Supplemental Table 8. For the validation study, we used the same equation as LDA combination II. The prediction model was evaluated with 5-fold cross-validation and LDA to calculate AUCs for the discovery and validation cohorts, respectively. The survival rates of different groups of patients were calculated with the Kaplan-Meier method and presented graphically as a survival curve. A comparison of survival curves between 2 groups was tested by log-rank test. Spearman's or Pearson's correlation coefficient was used to calculate the association between the cellular markers, the metabolic markers, and gene expression levels. JMP software (version 12.0.0; SAS Institute Inc.), R software (version 3.4.4), DataRobot (version 4.3.0), and Prism software (version 6.0h; GraphPad Software) were used for data management and statistical analyses. Significance levels were set at P < 0.05 for all tests.

Study approval. All patient and donor samples were obtained from subjects who provided informed consent for blood use in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of Kyoto University (G1012).

Author contributions

RH, KC, KS, SK, MA, FM, and T. Honjo analyzed and interpreted data. RH, KC, YHK, HY, HO, YS, IM, and T. Hirai collected clinical samples and analyzed clinical data. RH, KT, SK, YO, and FM performed statistical analysis. KC, SF, and T. Honjo supervised the study. RH, KC, KS, KT, SK, and T. Honjo prepared the manuscript. YHK, SF, YO, FM, IM, and T. Hirai critically reviewed the manuscript.

Acknowledgments

We thank Y. Kitawaki, M. Al-Habsi, R.M. Menzes, T. Ito, T. Atsuta, and M. Hori for assistance in sample preparation. We thank Bristol-Myers Squibb for the collaboration.

This work was supported by AMED under grant numbers 18cm0106302h0003 (T. Honjo), 18gm0710012h0105 (SF), and 18lk1403006h0002 (KC); the Tang Prize Foundation (T. Honjo); and JSPS KAKENHI grant numbers JP16H06149 (KC), 17K19593 (KC), and 19K17673 (RH). Analyses were supported by Cell Innovator Inc.

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Supplementary Fig. S1. Survival rates are compared between sorted groups based on different criteria. A) Kaplan–Meier plots of PFS and OS of all patients enrolled in this study. B) Kaplan–Meier plots of PFS and OS of patients sorted by the criteria of PFS > 3 months (solid line) and PFS \leq 3 months (dotted line). C) The solid line and dotted line show patients with PFS > 6 months and PFS \leq 6 months, respectively. *****p* < 0.0001 by Log-rank test. D) Kaplan–Meier plots of PFS and OS of patients sorted by frequency of PD-L1 expression on tumors. The dashed line, solid line, and dotted line show patients with high PD-L1 expression (greater than 50%), low PD-L1 expression (1–50%), and rare PD-L1 expression (less than 1%), respectively. E) The solid line and dotted line show patients with positive expression (greater than 1%) and negative expression (less than 1%) of PD-L1, respectively.



Supplementary Fig. S2. Behaviors of microbiota-derived metabolites and acylcarnitine species. A) The peak area measured by GC- or LC-MS of each microbiome related metabolites in patients without pre-antibiotics treatment (ATB(-)) and with pre-antibiotics treatment (ATB(+)) are shown. These graphs display the data of the 1st + 2nd + 3rd samples. **p < 0.01, ****p < 0.0001 by Wilcoxon rank sum test. **B**) The peak areas measured by GC-MS of hippuric acid and indoxyl sulfate in non-responders (NR) and responders (R) are shown. Each dot represents one patient. Error bars show median and interquartile range. *p < 0.05, **p < 0.01, ***p < 0.001 by Kruskal–Wallis test followed by Dunn's multiple comparisons test. **C**) Kaplan–Meier plots of PFS and OS of patients with (solid line) and without ATB (dotted line) within 3 months prior to the first nivolumab injection. **D**) The peak area detected by LC-MS of acylcarnitines between 1st, 2nd and 3rd samples are shown. The solid line and dotted line represent responders, respectively. *p < 0.05, **p < 0.01 by Wilcoxon rank sum test.



Supplementary Fig. S3. Detailed data of metabolite markers selected by stepwise analysis. **A–C**) Graphs show comparison of peak areas of metabolic markers selected by stepwise analysis between non-responders and responders. Each dot represents one patient. Error bars indicate the median and interquartile range. *p < 0.05, **p < 0.01 by Wilcoxon rank sum test.

А





* r values by Pearson

Supplementary Fig. S4. Definition of PD-1^{high}. A) Overlay histograms of PD-1 intensity gated on CD8⁺ T cells from age-matched 30 healthy donors. Lines show the 50th, 90th, 97th, and 99th percentile of PD-1 intensity averaged with the donors. The correlation r values between % of PD-1^{high} based on each percentile and gene expression of exhaustion markers (CTLA-4, Tim-3 and Lag-3) in the patients' CD8⁺ T cells are shown in the table. Since r values are highest in 97th percentile, the 97th percentile was used as cut-off in this study. **B**) Graph shows total PD-1 positive frequency among peripheral CD8⁺ T cells in non-responders (NR) and responders (R) at the pre-treatment (1st) point.

Supplementary Fig. S5



Supplementary Fig. S5. Detailed phenotypes of PD-1 positive subsets in CD8⁺ T cells. FACS data show the expression levels of PD-1, Ki-67, Granzyme B, IFN- γ , T-bet, EOMES, and Mito SOX among CD8⁺ T cell in PBMC. Representative FACS plots of NSCLC samples are depicted (left panels). Indicated parameters between PD-1^{hi}, PD-1^{low}, and PD-1 negative CD8⁺ T cells are shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by Kruskal–Wallis test followed by Dunn's multiple comparisons test.



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Marker name among CD8⁺ PD-1 ^{high}	p-value*	Time point	Number of patients analyzed
Ki67+ (%)	0.31	1 st	16
Ki67+ (%)	0.19	2 nd	16
Ki67+ (%)	0.91	3 rd	13
Granzyme B ⁺ (%)	0.18	1 st	16
Granzyme B ⁺ (%)	0.85	2 nd	16
Granzyme B ⁺ (%)	0.25	3 rd	13
IFN-γ+ (%)	0.43	1 st	16
IFN-γ+ (%)	0.51	2 nd	16
IFN-γ+ (%)	0.65	3 rd	13
T-bet⁺ (%)	0.32	1 st	16
T-bet⁺ (%)	0.32	2 nd	16
T-bet⁺ (%)	0.71	3 rd	13
EOMES ⁺ (%)	0.09	1 st	16
EOMES ⁺ (%)	0.83	2 nd	16
EOMES ⁺ (%)	0.15	3 rd	13

*: p-value for distinction between R and NR (Wilcoxon rank sum test).

Supplementary Fig. S6. Characteristics of PD-1^{high} **CD8**⁺ **T cells. A)** Scatter plots between frequency of PD-1^{high} (X-axis) and exhaustion marker gene expression in purified CD8⁺ T cells from the same cohort (Y-axis). r: Pearson correlation coefficients. Generally, |r| of more than 0.6 in Pearson are considered to have strong correlation. B) p-values of each frequency among CD8⁺ PD-1^{high} T cells between non-responders and responders are listed in the table.



Supplementary Fig. S7. Correlation analysis between PD-1^{high} **CD8**⁺ **T cells and tumor burden. A)** Scatter plots between frequency of PD-1^{high} (X-axis) and tumor barden (Y-axis). The dots and the circles represent the responders and the non-responders, respectively. r: Spearman correlation coefficients. **B)** Graphs show comparison of tumor burden between non-responders and responders. Each dot represents one patient. Error bars indicate the median and interquartile range.



Supplementary Fig. S8. CCR7⁻ CD8⁺ T cells with higher PGC-1 $\alpha\beta$ expression decreased by PD-1 blockade.

A) PGC-1 $\alpha\beta$ expression (MFI) between CCR7⁻ and CCR7⁺ T cells in the 1st (left), 2nd (middle), and 3rd samples (right) were shown. Each dot represents one patient. Error bars indicate the median and interquartile range. **p* < 0.05 by Wilcoxon rank sum test. **B)** Frequency of CCR7⁻ among peripheral CD8⁺ T cells in the 1st, 2nd, and 3rd samples (left). The solid line and dotted line represent responders and non-responders, respectively. Fold change (2nd/1st) of CCR7⁻ frequency among CD8⁺ T cells in non-responders and responders (right). **p < 0.01 by Wilcoxon rank sum test.



Supplementary Fig. S9. Change of CD4⁺ T cell subset ratios by PD-1 blockade. CD4⁺ T cells are divided into naïve, Tcm, Tem, and Temra subsets according to the expression of CD45RO and CCR7 (left panel). The frequency of CD4⁺ Tcm and CD4⁺ Temra in non-responders and responders is shown (middle and right panels). **p < 0.01 by Wilcoxon rank sum test.

Cellular marker combination II (NSCLC patients with EGFR mutation, error rate: 0%)



Supplementary Fig. S10. Results of cellular marker combination II in patients with EGFR mutation. Canonical plot for NSCLC patients with EGFR mutation based on cellular marker combination II.



Supplementary Fig. S11. Schema showing biosynthesis and metabolism of glutathione.

L-Glutamate and Cysteine are combined to form L- γ -Glutamylcysteine, which is then combined with glycine to yield GSH. GSH is oxidized into its oxidized form (GSSG) after reaction with reactive oxygen species (ROS).



Supplementary Fig. S12. Modest correlation between % of PD-1^{high} **among CD8**⁺ **T cells and FAOrelated factors.** Scatter plots between frequency of PD-1^{high} (X-axis) and the FAO-related gene (left panel) or metabolite marker (right panel) (Y-axis). r: Spearman correlation coefficients. |r| of more than 0.4 in spearman are considered to have modest to strong correlation.

Patient demographics (discovery cohort).

	All patients	responders	non-responders	dropout
Number	55	25	22	8
Age (range)	31-83	48-83	33-81	31-79
Age (median)	68	70	64.5	70
Male	39 (70.9%)	19 (76.0%)	15 (68.2%)	5 (62.5%)
Adenocarcinoma	43 (78.2%)	18 (72.0%)	18 (81.8%)	7 (87.5%)
EGFR mutation	9 (17.3%)	1 (7.0%)	7 (35.0%)	1 (12.5%)
ALK translocation	3 (6.8%)	0 (0%)	2 (7.1%)	1 (14.3%)
Antibiotics treatment	17 (30.9%)	6 (24.0%)	8 (36.4%)	3 (37.5%)

Metabolites measured by GC-MS.

Name	Kegg ID	HMDB ID	Methyloxime/TMS derivative	m/z	Retention	Name	Kegg ID	HMDB ID	Methyloxime/TMS derivative	m/z	Retention
1.5-Anhydro-D-sorbitol	C07326	HMDB0002712	1.5-Anhydro-D-sorbitol-4TMS	259.0	14.77	Hypotaurine	C00519	HMDB0000965	Hypotaurine-3TMS	188.1	12.92
1.6-Anhydroglucose	-	HMDB0000640	1.6-Anhvdro-beta-D-glucose-3TMS	204.0	13.70	Hypoxanthine	C00262	HMDB0000157	Hypoxanthine-2TMS	265.0	14.50
1-Hexadecanol	C00823	HMDB0003424	1-Hexadecanol-TMS	299.0	15.37	Indoleacetic acid	C00954	HMDB0000197	Indol-3-acetic acid-2TMS	202.0	15.68
2-Aminoadipic acid	C00956	HMDB0000510	2-Aminoadipic acid-3TMS	260.0	13.62	Indolelactic acid	C02043	HMDB0000671	Indolelactic acid-3TMS	202.0	17.08
2-Aminobutyric acid	C02356	HMDB0000452	2-Aminobutyric acid-2TMS	130.0	8.69	Indoxyl sulfate	-	HMDB0000682	Indoxyl sulfate-2TMS	277.0	13.84
2-Aminoethanol	C00189	HMDB0000149	2-Aminoethanol-3TMS	174.0	9.77	Isocitric acid	C00311	HMDB0000193	Isocitric acid-4TMS	245.0	14.41
2-Deoxytetronic acid	-	HMDB0000337	2-Deoxytetronic acid-3TMS	321.0	11.20	Isoleucine	C16434	HMDB0000172	Isoleucine-2TMS	232.0	9.99
2-Hydroxybutyric acid	C05984	HMDB000008	2-Hydroxybutyric acid-2TMS	131.0	8.17	Kynurenine	C00328	HMDB0000684	Kynurenine-3TMS	307.0	17.07
2-Hydroxyisobutyric acid	-	HMDB0000729	2-Hydroxyisobutyric acid-2TMS	131.0	7.46	Lactic acid	C00186	HMDB0000190	Lactic acid-2TMS	219.0	7.39
2-Hydroxyisovaleric acid	-	HMDB0000407	2-Hydroxyisovaleric acid-2TMS	145.0	8.61	Lauric acid	C02679	HMDB0000638	Lauric acid-TMS	257.0	13.11
2-Hydroxypyridine	C02502	HMDB0013751	2-Hydroxypyridine-TMS	152.0	7.22	Leucine	C00123	HMDB0000687	Leucine-2TMS	232.0	9.74
2-Oxobutyric acid	C00109	HMDB0000005	2-Oxobutyric acid-methyloxime-TMS	188.0	7.86	Linoleic acid	C01595	HMDB0000673	Linoleic acid-TMS	337.0	17.06
2-Oxoglutaric acid (α- Ketoglutaric acid)	C00026	HMDB0000208	2-Oxoglutaric acid-methyloxime-2TMS	198.0	12.47	Lysine	C00047	HMDB0000182	Lysine-4TMS	174.0	15.23
2-Oxoisocaproic acid	C00233	HMDB0000695	2-Oxoisocaproic acid-methyloxime-	200.0	9.10	Malic acid	C00711	HMDB0000156	Malic acid-3TMS	233.0	11.73
3-(3-Hydroxyphenyl)-3-			3-(3-Hydroxyphenyl)-3-	267.0	14 69	Maltose	C00208	HMDB0000163	Maltose-methyloxime-8TMS	204.0	20.64
hydroxypropionic acid	-	HMDB0002643	hydroxypropionic acid-3TMS	207.0	14.00	Mannose	C00159	HMDB0000169	Mannose-methyloxime-5TMS	319.0	15.05
3-Aminoisobutyric acid	C05145	HMDB0003911	3-Aminoisobutyric acid-3TMS	304.0	11.67	Margaric acid	-	HMDB0002259	Margaric acid-TMS	117.0	16.58
3-Hydroxybutyric acid	C01089	HMDB0000357	3-Hydroxybutyric acid-2TMS	191.0	8.51	Methionine	C00073	HMDB0000696	Methionine-2TMS	250.0	12.14
3-Hydroxyisobutyric acid	C06001	HMDB0000023	3-Hydroxyisobutyric acid-2TMS	177.0	8.51	myo-Inositol	C00137	HMDB0000211	myo-Inositol-6TMS	432.0	16.54
3-Hydroxyisovaleric acid	-	HMDB0000754	3-Hydroxyisovaleric acid-21MS	131.0	9.09	Myristic acid	C06424	HMDB0000806	Myristic acid-IMS	285.0	14.60
3-Indolepropionic acid	-	HMDB0002302	3-Indolepropionic acid-21MS 3-Methyl-2-oxobutyric acid-	202.0	16.50	Octanoic acid	C06423	HMDB0000482	Octanoic acid-TMS	201.0	9.62
3-Methyl-2-oxobutyric acid	C00141	HMDB0000019	methyloyime-TMS	89.0	7.96	Oleic acid	000712		O DL _ L _ L ATMC	339.0	17.08
3-Methyl-2-oxovaleric acid	C00671	HMDB0000491	3-Methyl-2-oxovaleric acid- methyloxime-TMS	200.0	8.97	O-Phosphoethanolamine	C00346		O-Phosphoethanolamine-41MS	299.0	14.29
3-Methylhistidine	C01152	HMDB0000479	3-Methylhistidine-2TMS	168.0	15.01	Ornithine Ovalic acid	C00077		Ovalic acid-2TMS	420.0 210.0	14.52 8.13
4-Cresol	C01468	HMDB0001858	4-Cresol-TMS	180.0	8.56	Palmitic acid	C00249	HMDB0002323	Palmitic acid TMS	313.0	15.95
4-Hydroxyproline	C01157	HMDB0000725	4-Hydroxyproline-3TMS	140.0	12.15	Palmitoleic acid	C08362	HMDB0003229	Palmitoleic acid-TMS	311.0	15.85
Acetoacetic acid	C00164	HMDB0000060	Acetoacetic acid-methyloxime-TMS	203.0	8.01	Paraxanthine	C13747	HMDB0001860	Paraxanthine-TMS	237.0	15.77
Aconitic acid	C02341	HMDB0000958	Aconitic acid-3TMS	375.0	13.81	Phenol	C00146	HMDB0000228	Phenol-TMS	166.0	7.45
Alanine	C00041	HMDB0000161	Alanine-2TMS	218.0	7.91	Phenylalanine	C00079	HMDB0000159	Phenylalanine-2TMS	192.0	13.12
Alanine	C00041	HMDB0000161	Alanine-3TMS	188.0	10.77	Phosphogycerol	C03189	HMDB0000126	Phosphogycerol-4TMS	445.0	14.00
Allose	C01487	HMDB0001151	Allose-methyloxime-5TMS	205.0	15.00	Phosphoric acid	C00009	HMDB0002142	Phosphoric acid-3TMS	225.0	9.76
Arabinonic acid	-	HMDB0000539	Arabinonic acid-5TMS	292.0	14.23	Pipecolinic acid	C00408	HMDB0000070	Pipecolinic acid-TMS	156.0	10.44
Arabinose	C00259	HMDB0000646	Arabinose-methyloxime-4TMS	307.0	13.26	Proline	C00148	HMDB0000162	Proline-2TMS	216.0	10.12
Arabitol	C01904	HMDB0000568	Arabitol-5TMS	217.0	13.75	Pyroglutamic acid	C01879	HMDB0000267	5-Oxoproline-2TMS	258.0	12.21
Asparagine	C00152	HMDB0000168	Asparagine-3TMS	231.0	13.32	Pyrophosphate	-	HMDB0000250	Pyrophosphate-4TMS	451.0	13.32
Aspartic acid	C00049	HMDB0000191	Aspartic acid-3TMS	232.0	12.03	Pyruvic acid	C00022	HMDB0000243	Pyruvic acid-methyloxime-TMS	174.0	7.23
Benzoic acid	C00180	HMDB0001870	Benzoic acid-TMS	179.0	9.60	Ribitol	C00474	HMDB0000508	Ribitol-5TMS	319.0	13.81
beta-Alanine Baria agid	C10099	HMDB0000056	Deta-Alanine-31MS	290.0	11.31	Ribose	C00121	HMDB0000283	Ribose-methyloxime-4TMS	307.0	13.38
Caffeine	C12400		Caffeine	104.0	15.05	Ribulose	C00309	HMDB0000621	Ribulose-methyloxime-4TMS	263.0	13.38
Canrois acid	C01585	HMDB0000535	Canroic acid TMS	173.0	7 52	scyllo-Inositol	C06153	HMDB0006088	scyllo-Inositol-6TMS	318.0	16.11
Citric acid	C001565	HMDB0000094	Citric acid-4TMS	363.0	14 45	Serine	C00065	HMDB0000187	Serine-3TMS	306.0	10.58
Citrulline	C00327	HMDB0000904	Citrulline-4TMS	256.0	14.48	Stearic acid	C01530	HMDB0000827	Stearic acid-TMS	341.0	17.18
Creatinine	C00791	HMDB0000562	Creatinine-3TMS	115.0	12.58	Succinic acid	C00042	HMDB0000254	Succinic acid-21MS	247.0	10.10
Cysteine	C00097	HMDB0000574	Cysteine-3TMS	218.0	12.44	Sucrose	C00089	HMDB0000258	Sucrose-81MS	361.0	19.79
Cysteinylglycine	C01419	HMDB0000078	Cysteinylglycine-3TMS	220.0	16.14	Throitel	C16994		Z-Aminoethanesunonic acid-31WS	217.0	11.94
Cystine	C00491	HMDB0000192	Cystine-4TMS	411.0	17.67	Threonic acid	C01620	HMDB00004130	Threenic acid-4TMS	292.0	12.38
Decanoic acid	C01571	HMDB0000511	Decanoic acid-TMS	229.0	11.47	Threonine	C00188	HMDB0000167	Threonine-3TMS	291.0	10.86
Elaidic acid	C01712	HMDB0000573	Elaidic acid-TMS	93.0	17.11	Tryptophan	C00078	HMDB0000929	Tryptophan-3TMS	291.0	17.34
Erythritol	C00503	HMDB0002994	Erythritol-4TMS	217.0	11.92	Tyrosine	C00082	HMDB0000158	Tvrosine-3TMS	382.0	15.39
Fructose	C00095	HMDB0000660	Fructose-methyloxime-5TMS	307.0	14.92	Unk12*	-	-	-	196.0	14.50
Fucose	C01019	HMDB0000174	Fucose-methyloxime-5TMS	117.0	13.89	Unk3*	-	-	-	170.0	8.98
Fumaric acid	C00122	HMDB0000134	Fumaric acid-2TMS	245.0	10.37	Unk4*	-	-	-	319.0	9.56
Gluconic acid	C00257	HMDB0000625	Gluconic acid-methyloxime-5TMS	292.0	15.94	Unk6*	-	-	-	320.0	11.66
Glucose	C00031	HMDB0000122	Glucose-methyloxime-5TMS	229.0	15.32	Unk8*	-	-	-	116.0	13.96
Glucuronic acid	C00191	HMDB0000127	Glucuronic acid-methyloxime-5TMS	423.0	15.47	Unk9*	-	-	-	217.0	14.04
Glutamic acid	C00025	HMDB0000148	Glutamic acid-3TMS	246.0	12.85	Urea	C00086	HMDB0000294	Urea-2TMS	204.0	9.29
Glutamine	C00064	HMDB0000641	Glutamine-3TMS	362.0	14.11	Uric acid	C00366	HMDB0000289	Uric acid-4TMS	367.0	16.40
Glutaric acid	C00489	HMDB0000661	Glutaric acid-2TMS	261.0	10.94	Uridine	C00299	HMDB0000296	Uridine-4TMS	245.0	18.54
Glyceric acid	C00258	HMDB0000139	Glyceric acid-3TMS	292.0	10.28	Valine	C00183	HMDB0000883	Valine-2TMS	218.0	9.19
Glycerol	C00116	HMDB0000131	Glycerol-3TMS	218.0	9.69	Xanthine	C00385	HMDB0000292	Xanthine-3TMS	353.0	15.92
Glycine	C00037	HMDB0000123	Glycine-3TMS	174.0	10.20	Xylitol	C00379	HMDB0002917	Xylitol-5TMS	217.0	13.66
Glycolic acid	C00160	HMDB0000115	Glycolic acid-2TMS	205.0	7.53	Xylose	C00181	HMDB0000098	Xylose-methyloxime-4TMS	307.0	13.21
Hippuric acid	C01586	HMDB0000714	Hippuric acid-TMS	236.0	14.75						
Histidine	C00135	HMDB0000177	Histidine-3TMS	371.0	15.28						
Homocysteine	C00155	HMDB0000742	Homocysteine-3TMS	234.0	13.33						

* 'Unk' means that these metabolites were not yet identified at the time of analysis.

Metabolites measured by LC-MS.

Name	Kegg ID	HMDB ID	Ion transition	Retention time(min)	Name	Kegg ID
4-Hydroxyproline	C01157	HMDB0000725	132.10>68.05	1.89	Valine	C00183
Acetylalanine	-	HMDB0000766	132.10>90.10	4.00	Xanthosine	C01762
Acetylglycine	-	HMDB0000532	118.00>76.10	3.53	Xanthurenic acid	C02470
Adenosine	C00212	HMDB0000050	268.10>136.10	8.35	asy-Dimethylarginine	C03626
Adenosylhomocysteine	C00021	HMDB0000939	385.10>136.10	8.23	sym-Dimethylarginine	-
Adenosylmethionine	C00019	HMDB0001185	399.10>250.10	2.18	trans-urocanic acid	C00785
2-Aminoadipic acid	C00956	HMDB0000510	162.10>98.20	2.35	2-Hvdroxy-3-methylvaleric acid	-
Argininosuccinic acid	C03406	HMDB0000052	291.10>70.10	2.08	2-Hydroxybutyric acid	C05984
Asparagine	C00152	HMDB0000168	133.10>74.05	1.85	2-Hydroxyglutaric acid	C01087
Aspartic acid	C00049	HMDB0000191	134.10>88.00	1.85	2-Hydroxyisovaleric acid	-
Betaine	C00719	HMDB0000043	118.10>42.05	2.07	2-Oxoqlutaric acid (α-Ketoqlutaric acid)	C00026
Creatine	C00300	HMDB0000064	132.10>44.05	2.30	3-Hvdroxybutyric acid	C01089
Creatinine	C00791	HMDB0000562	114,10>86,10	2.18	3-Hydroxyisovaleric acid	_
Cystathionine	C00542	HMDB0000099	223.10>88.00	1.84	3-Methylolutaconic acid	-
Cysteine	C00097	HMDB0000574	122.00>59.00	2.04	4-Hydroxybenzoic acid	C00156
Cystine	C00491	HMDB0000192	241.00>74.10	1.83	AMP	-
GSH	C00051	HMDB0000125	308.10>179.20	4.32	Aconitic acid	C02341
6556	C00127	HMDB0003337	613 20>231 00	8 17		-
Glutamic acid	C00025		148 10>102 05	1.08	Citric acid	C00158
Chatamic acid	000025		148.10>102.05	1.98		000100
Glutamine	C00064		76.00>20.40	1.92	Pumane acid	C00122
Giycine	000037	HMDB0000123	76.00>30.10	1.84		00469
Guanosine	000387	HMDB0000133	284.10>152.10	8.39	Hippuric acid	C01586
Histidine	000135	HMDB0000177	156.10>83.20	1.84	Indoxyl sulfate	-
Indoleacetic acid	C00954	HMDB0000197	176.10>130.10	13.67	Isocitric acid	C00311
	C02043	HMDB0000671	206.10>118.10	12.62	Lactic acid	C00186
	C00331	HMDB0060484	204.10>130.10	12.41	Malic acid	C00711
Inosine	C00294	HMDB0000195	269.10>110.10	8.40	Phenyllactic acid	C05607
Kynurenic acid	C01/1/	HMDB0000715	190.10>144.00	10.13	Pyridoxic acid	C00847
Kynurenine	C00328	HMDB0000684	209.10>192.05	9.00	Succinic acid	C00042
3-Hydroxykynurenine	C02794	HMDB0000732	225.10>110.15	7.49	cAMP	C00575
N'-Formylkynurenine	C02700	HMDB0001200	237.20>146.05	9.02	I hyroxine	C01829
Leucine	C00123	HMDB0000687	132.10>43.10	7.06	Pipecolinic acid	C00408
Isoleucine	C16434	HMDB0000172	132.10>69.10	6.50	3-Indolepropionic acid	-
Lysine	C00047	HMDB0000182	147.10>84.10	1.64	Quinolinic acid	C03722
Methionine	C00073	HMDB0000696	150.10>104.00	3.93	3-Methylhistidine	C01152
S-Methylmethionine	C05319	HMDB0038670	165.10>59.20	9.54	Carnitine	C00318
N-Acetyl-Asp-Glu	C12270	HMDB0001067	305.10>148.00	8.17	Acetylcarnitine	C02571
N-Acetylaspartic acid	C01042	HMDB0000812	176.10>134.00	4.08	Propionylcarnitine	C03017
N-Acetylhistidine	C02997	HMDB0032055	198.10>110.10	2.81	Isobutyrylcarnitine	-
Nicotinamide	C00153	HMDB0001406	123.10>80.05	5.13	Butyrylcarnitine	C02862
Phenylalanine	C00079	HMDB0000159	166.10>103.10	8.94	2-Methylbutyrylcarnitine	-
Phosphocholine	C00588	HMDB0001565	184.10>125.00	1.85	Isovalerylcarnitine	C20826
Proline	C00148	HMDB0000162	116.10>70.10	2.28	Hexanoylcarnitine	-
Pyroglutamic acid	C01879	HMDB0000267	130.00>56.10	5.16	Glutarylcarnitine	-
Riboflavin	C00255	HMDB0000244	377.10>243.20	10.59	Adipoylcarnitine	-
Serine	C00065	HMDB0000187	106.10>60.00	1.85	2-Aminobutyric acid	C02356
Serotonin	C00780	HMDB0000259	177.10>160.20	8.73	Choline	C00114
Putrescine	C00134	HMDB0001414	89.10>72.10	1.60	3-Aminoisobutyric acid	C05145
Spermidine	C00315	HMDB0001257	146.20>129.20	1.39	Acetylcarnosine	-
Spermine	C00750	HMDB0001256	203.20>112.20	1.33	Alanine	C00041
Taurine	C00245	HMDB0000251	126.00>44.05	1.87	Ornithine	C00077
Threonine	C00188	HMDB0000167	120.10>84.10	1.94	Arginine	C00062
Trigonelline	C01004	HMDB0000875	138.00>94.10	2.39	Citrulline	C00327
Trimethyllysine	C03793	HMDB0001325	189.20>60.25	1.82	Glucuronic acid	C00191
Tryptophan	C00078	HMDB0000929	205.10>115.20	9.84	Gluconic acid	C00257
Tyrosine	C00082	HMDB0000158	182.10>136.20	7.30	N-Acetylglucosamine	C00140
Urea	C00086	HMDB0000294	61.00>44.05	2.05	N-Acetylneuraminic acid	C00270
Uric acid	C00366	HMDB0000289	169.00>126.05	5.26	4-Guanidinobutanoic acid	C01035
Uridine	C00299	HMDB0000296	245.10>113.10	7.51	N-Acetylornithine	C00437

Retention

time(min)

3.15

8.61

9.75

2.47

2.57

4.70

11.52

7.95

4.27

9.89

3.24

6.64

8 89

10.07

10.98

4.14

8 17

2.01

4.59

5.93

8.71

11.14

10.58

2.96

3.67

2.89

12 32

8.56

5.90

8.36

15.07

3.41

14.73

4.40

1.85

2.13

4.16

8.49

9.38

9.51

10.44

10.58

11.88

8.48

8.96

2.19

1.99

2.31

4.25

1.91

1.65

1.83

2.05

1.95

1.96

2.33

2.17

3.82

2.67

Ion transition

118.10>55.10

139.10>93.10

131.10>85.15

103.00>57.20

147.00>129.05

117.10>71.10

145.10>57.10

103.00>59.10

117 10>59 10

143.00>99.05

137.00>93.00

346.10>79.05

165.00>75.05

191.00>87.10

115.00>71.10

131.00>87.15

178.10>134.05

191.00>73.10

89.00>43.10

133.00>115.00

165.10>147.05

328.00>134.20

190.10>130.10

168.10>78.05

170.10>81.10

162.10>85.10

204.10>85.10

260.20>85.15

276.10>85.05

90.00>44.10

133.10>116.05

175.10>70.10

176.10>159.05

193.00>103.00

146.10>86.15

175.10>70.05

HMDB0000299 285.10>153.20

HMDB0000881 206.00>160.00

HMDB0001539 203.10>46.10

HMDB0003334 203.10>172.20

HMDB ID

HMDB0000883

HMDB0000301

HMDB0000317

HMDB0000008

HMDB0000606

HMDB0000407

HMDB0000208

HMDB0000357

HMDB0000754

HMDB0000522

HMDB0000500

HMDB0000045

HMDB0000539

HMDB0000094

HMDB0000134

HMDB0000661

HMDB0000714

HMDB0000193

HMDB0000190

HMDB0000156

HMDB0000748

HMDB0000058

HMDB0002302

HMDB0000232

HMDB0000479

HMDB0000062

HMDB0000201

HMDB0000705

HMDB0013130

HMDB0000161

HMDB0000214

HMDB0000517

HMDB0000904

HMDB0000127

HMDB0003464

HMDB0003357

HMDB0000958 173 00>85 15

HMDB0000682 212.00>132.20

HMDB0000017 182.00>108.05

HMDB0000254 117.00>73.05

HMDB0000248 777.70>731.50

HMDB0000070 130.10>84.30

HMDB0000824 218.10>85.10

HMDB0000736 232.20>85.15

HMDB0002013 232.20>85.05

HMDB0000378 246.20>85.10

HMDB0000688 246.20>85.15

HMDB0061677 290.20>85.00

HMDB0000452 104.10>41.05

HMDB0000097 104.10>60.00

HMDB0003911 104.10>86.10

HMDB0012881 269.10>110.10

HMDB0000625 195.10>129.00

HMDB0000215 222.10>138.00

HMDB0000230 310.10>274.10

Cellular markers measured by flow cytometry.

Cellular markers % of CCR6⁺ cells among CD4⁺ T cells % of CD25⁺ cells among CD4⁺ T cells % of CD4⁺ T cells among PBMC % of CD8⁺ T cells among PBMC % of CTLA4⁺ cells among CD4⁺ T cells % of CXCR3⁺ cells among CD4⁺ T cells % of FoxP3⁺ cells among CD4⁺ T cells % of FoxP3^{high} cells among CD4⁺ T cells % of FoxP3^{low} cells among CD4⁺ T cells % of FoxP3⁺ CD25⁺ cells among CD4⁺ T cells % of FoxP3^{low} CD45RA⁺ cells among CD4⁺ T cells % of FoxP3⁺ CTLA4⁺ cells among CD4⁺ T cells % of IFN γ^+ cells among CD4⁺ T cells % of IFN γ^+ cells among CD8⁺ T cells % of KLRG1⁺ cells among CD4⁺ T cells % of KLRG1⁺ CCR6⁺ cells among CD4⁺ T cells % of KLRG1⁺ cells among CD8⁺ T cells % of PD-1⁺ cells among CD4⁺ T cells % of PD-1⁺ CD45⁺ cells among CD4⁺ T cells % of PD-1⁺ FoxP3⁺ cells among CD4⁺ T cells % of PD-1⁺ cells among CD8⁺ T cells % of PD-1^{high} cells among CD8+ T cells % of T-bet⁺ cells among CD4⁺ T cells % of T-bethigh cells among CD4+ T cells % of T-bet⁺ KLRG1⁺ cells among CD4⁺ T cells % of T-bet⁺ cells among CD8⁺ T cells % of T-bet^{high} cells among CD8⁺ T cells % of EOMES⁺ cells among CD8⁺ T cells % of T-bet⁺ EOMES⁻ cells among CD8⁺ T cells % of T-bet⁺ EOMES⁺ cells among CD8⁺ T cells % of T-bet⁻ EOMES⁺ cells among CD8⁺ T cells % of Tnaive among CD4⁺ T cells % of Tcm among CD4⁺ T cells % of Tem among CD4⁺ T cells % of Temra among CD4⁺ T cells % of Tnaive among CD8⁺ T cells % of Tcm among CD8⁺ T cells % of Tem among CD8⁺ T cells % of Temra among CD8⁺ T cells % of Tim3⁺ cells among CD4⁺ T cells % of Tim3⁺ cells among CD8⁺ T cells Cell ROX Green (MFI) of CD4⁺ T cells Cell ROX Green (MFI) of CD8⁺ T cells Mito SOX Red (MFI) of CD4⁺ T cells Mito SOX Red (MFI) of CD8⁺ T cells Mito Tracker Deep Red (MFI) of CD4⁺ T cells Mito Tracker Deep Red (MFI) of CD8⁺ T cells Mito Tracker Green (MFI) of CD4⁺ T cells Mito Tracker Green (MFI) of CD8⁺ T cells p-Akt (MFI) of CD8⁺ T cells p-mTOR (MFI) of CD8⁺ T cells PGC-1αβ (MFI) of CD8⁺ T cells

Metabolites showing a significant difference between responders (R) and non-responders (NR).

Metabolites	Time point	Changes in R relative to NR	Modality	p-value*
Alanine	1st	higher	GC	0.0474
4-Cresol	1st	higher	GC	0.0238
Cysteine	1st	higher	GC	0.0474
Hippuric acid	1st	higher	GC / LC	0.0062 / 0.0056
Oleic acid	1st	higher	GC	0.0281
Indoxyl sulfate	1st	higher	GC / LC	0.0313 / 0.0225
Ribose	1st	higher	GC	0.0081
Unk8	1st	lower	GC	0.0273
Indoleacetate	1st	higher	LC	0.018
Uric acid	1st	higher	LC	0.0126
Trans-urocanic acid	1st	higher	LC	0.0498
Pipecolic acid	1st	lower	LC	0.0252
N-Acetylglucosamine	1st	higher	LC	0.0281
Uric acid	2nd	higher	GC / LC	0.0114 / 0.0176
Indolelactic acid	2nd	higher	GC	0.0387
Arabinose	2nd	higher	GC	0.0186
Arabitol	2nd	higher	GC	0.0089
Hippuric acid	2nd	higher	GC	0.0323
Cystine	2nd	higher	GC	0.0043
Indoxyle sulfate	2nd	higher	GC / LC	0.0454 / 0.0348
Gluconic acid	2nd	higher	GC	0.0454
Citrulline	2nd	higher	GC / LC	0.0312 / 0.0122
Creatinine	2nd	higher	LC	0.0198
N-Acetylaspartic acid	2nd	higher	LC	0.0311
Pyroglutamic acid	2nd	higher	LC	0.0064
Trimethyyllysine	2nd	higher	LC	0.0408
Asy-Dimethylarginine	2nd	higher	LC	0.0028
Sym-Dimethylarginine	2nd	higher	LC	0.0078
Pipecolic acid	2nd	lower	LC	0.0329
Methylhistidine	2nd	higher	LC	0.0176
Butyrylcarnitine	2nd	lower	LC	0.0101
3-Aminoisobutyric acid	2nd	higher	LC	0.0122
Acethykcarnosine	2nd	higher	LC	0.0367
Alanine	2nd	higher	LC	0.0348
Arginine	2nd	higher	LC	0.0114
N-accetylorinitine	2nd	higher	LC	0.0387
4-Cresol	3rd	higher	GC	0.0023
3-Hydroxyisovaleric acid	3rd	lower	GC / LC	0.0213 / 0.0119
Pyruvic acid	3rd	lower	GC	0.0348
α-ketoglutaric acid	3rd	lower	GC	0.0238
Hippuric acid	3rd	higher	GC / LC	0.0002 / 0.0003
Cystine	3rd	higher	GC	0.0201
Indoxyl sulfate	3rd	higher	GC / LC	0.0049 / 0.0011
Unk13	3rd	higher	GC	0.0451
GSSG	3rd	higher	LC	0.0068
Uric acid	3rd	higher	LC	0.0348
2-Hydrobutyric acid	3rd	lower	LC	0.0281
Pipecolic acid	3rd	lower	LC	0.0348
Butyrylcarnitine	3rd	lower	LC	0.0015

Metabolites	Ratio of two time points	Changes in R relative to NR	Modality	p-value*
Creatinine	2nd/1st	lower	GC	0.0329
1,5-Anhydro-D-sorbitol	2nd/1st	higher	GC	0.0367
Cystine	2nd/1st	higher	GC	0.0419
Glutamine	2nd/1st	higher	LC	0.0114
Glycine	2nd/1st	higher	LC	0.0095
Lysine	2nd/1st	higher	LC	0.0329
Pyroglutamic acid	2nd/1st	higher	LC	0.0095
Taurine	2nd/1st	lower	LC	0.0348
asy-Dimethylarginine	2nd/1st	higher	LC	0.0176
AMP	2nd/1st	lower	LC	0.0198
Isovalerylcarnitine	2nd/1st	lower	LC	0.0454
Hexanoycarnitine	2nd/1st	lower	LC	0.0367
Acetylcarnosine	2nd/1st	higher	LC	0.0264
Arginine	2nd/1st	higher	LC	0.0329
Citrulline	2nd/1st	higher	LC	0.0348
N-accetylorinitine	2nd/1st	higher	LC	0.0037
3-Hydroxybutyric acid	3rd/1st	lower	GC / LC	0.0330 / 0.0281
2-Hydroxyisovaleric acid	3rd/1st	lower	GC / LC	0.0040 / 0.0028
Creatinine	3rd/1st	lower	GC	0.0099
Hippuric acid	3rd/1st	higher	GC	0.0016
Oleic acid	3rd/1st	lower	GC	0.0252
Acetoacetic acid	3rd/1st	lower	GC	0.0483
Ribose	3rd/1st	lower	GC	0.0008
GSSG	3rd/1st	higher	LC	0.0367
Tryptophan	3rd/1st	higher	LC	0.0367
2-Hydroxyglutaric acid	3rd/1st	lower	LC	0.0099
Malic acid	3rd/1st	lower	LC	0.0266
Quinolinic acid	3rd/1st	lower	LC	0.0407
Butyrylcarnitine	3rd/1st	lower	LC	0.0043
Caproic acid	3rd/2nd	higher	GC	0.0408
4-Cresol	3rd/2nd	higher	GC	0.0138
Isoleucine	3rd/2nd	higher	GC	0.0408
Arabinose	3rd/2nd	lower	GC	0.0114
Ribose	3rd/2nd	lower	GC	0.0028
GSH	3rd/2nd	higher	LC	0.0089
GSSG	3rd/2nd	higher	LC	0.0008
3-OH-Kynurenine	3rd/2nd	lower	LC	0.0408
Hippuric acid	3rd/2nd	higher	LC	0.0165
Isobutyrylcarnitine	3rd/2nd	higher	LC	0.0095

Cellular markers showing a significant difference between responders (R) and non-responders (NR).

Cellular markers	Time point or ratio of two time points	Changes in R relative to NR	p-value*
% of CD4 ⁺ T cells among PBMC	2nd	higher	0.0107
% of CD4 ⁺ T cells among PBMC	2nd/1st	higher	0.0001
% of CD8 ⁺ T cells among PBMC	2nd	higher	0.0478
% of CD8 ⁺ T cells among PBMC	2nd/1st	higher	0.0348
% of Tnaive among CD8⁺ T cells	2nd/1st	higher	0.0176
% of Tcm among CD4 ⁺ T cells	2nd/1st	higher	0.0095
% of Tcm among CD8 ⁺ T cells	2nd/1st	higher	0.0138
% of Tem among CD8 ⁺ T cells	3rd/1st	higher	0.0213
% of Temra among CD4 ⁺ T cells	2nd/1st	lower	0.0107
% of Temra among CD4⁺ T cells	3rd/2nd	higher	0.0081
% of Temra among CD8⁺ T cells	2nd/1st	lower	0.0009
Mito SOX CD8/CD4	1st	higher	0.0028
Mito SOX CD8/CD4	2nd	higher	0.0089
Mito SOX CD8/CD4	3rd	higher	0.018
Mito SOX CD8/CD4	3rd/1st	lower	0.019
Mito mass CD8/CD4	1st	higher	0.0451
Mito mass CD8/CD4	3rd	higher	0.0348
PGC-1 $\alpha\beta$ (MFI) of CD8 ⁺ T cells	2nd	lower	0.0176
PGC-1 $\alpha\beta$ (MFI) of CD8 ⁺ T cells	2nd/1st	lower	0.0052
PGC-1 $\alpha\beta$ (MFI) of CD8 ⁺ T cells	3rd/2nd	higher	0.0001
% of PD-1 ^{high} among CD8⁺ T cells	1st	lower	0.013
% of FoxP3 ^{low} CD45RA⁺ among CD4⁺ T cells	1st	lower	0.027
% of T-bet ^{high} among CD4⁺ T cells	3rd/1st	higher	0.03
% of T-bet ^{low} among CD4 ⁺ T cells	3rd/1st	higher	0.0214
% of T-bet ⁺ among CD8 ⁺ T cells	3rd/2nd	higher	0.0295
% of T-bet ⁺ EOMES ⁺ among CD8 ⁺ T cells	3rd/2nd	higher	0.0408

*: p-value for distinction between R and NR (Wilcoxon rank sum test).

AUC value of each marker in pretreatment.

Marker name	Time point	Area under the curve (AUC)	number of patients analyzed
Mito SOX CD8/CD4	1 st	0.75	47
% of PD-1 ^{high} among CD8⁺ T cells	1 st	0.71	47
PD-L1 TPS	1 st	0.66	31

Equation of LDA for each marker combination.

Marker combination	Before the adaptation	After the adaptation
Metabolite combination I	9.92×[Cysteine 1st]−44.7×[Unk8 (1st)] +5.41×10⁻ ⁷ × [Hippuric acid (1st)] −1.50	9.92×[Cysteine 1st]−44.7×[Unk8 (1st)] +5.41×10 ⁻⁷ ×[Hippuric acid (1st)] −1.46
Metabolite combination II	$207 \times [Arabinose (2nd)] + 3.17 \times 10^{-7} \times [Arginine (2nd)] - 3.77 \times 10^{-7} \times [Butyrylcarnitine (2nd)] - 1.99$	$207 \times [Arabinose (2nd)] + 3.17 \times 10^{-7} \times [Arginine (2nd)] - 3.77 \times 10^{-7} \times [Butyrylcarnitine (2nd)] - 1.96$
Metabolite combination III	4.43×10 ⁻⁷ × [Hippuric acid (1st)]+12.1×[Cystine (2nd)] +3.55×10 ⁻⁵ × [GSSG (3rd)] -8.81×10 ⁻⁸ × [Butyrylcarnitine 3rd]-2.71	4.43 × 10 ⁻⁷ × [Hippuric acid (1st)]+12.1 × [Cystine (2nd)] +3.55 × 10 ⁻⁵ × [GSSG (3rd)] -8.81 × 10 ⁻⁸ × [Butyrylcarnitine 3rd]-2.67
Cellular combination I	-0.302×[% of PD-1high among CD8+ T cells (1st)] +2.95×[Mito SOX CD8/CD4(1st)]-1.91	-0.302×[% of PD-1high among CD8+ T cells (1st)] +2.95×[Mito SOX CD8/CD4(1st)]-1.87
Cellular combination II	-0.252×[% of PD-1high among CD8+ T cells (1st)] +3.58×[Mito SOX CD8/CD4(1st)] -1.22×[PGC-1αβ of CD8+ T cells (2nd/1st)] +1.93×[% of CD4+ T cells among PBMC (2nd/1st)]-3.35	-0.252×[% of PD-1high among CD8+ T cells (1st)] +3.58×[Mito SOX CD8/CD4(1st)] -1.22×[PGC-1αβ of CD8+ T cells (2nd/1st)] +1.93×[% of CD4+ T cells among PBMC (2nd/1st)]-3.30
Cellular combination III	-0.281×[% of PD-1high among CD8+ T cells (1st)] +3.30×[Mito SOX CD8/CD4(1st)] +2.00×[PGC-1αβ of CD8+ T cells (3rd/2nd)] +1.55×[% of CD4+ T cells among PBMC (2nd/1st)]-6.04	-0.281×[% of PD-1high among CD8+ T cells (1st)] +3.30×[Mito SOX CD8/CD4(1st)] +2.00×[PGC-1αβ of CD8+ T cells (3rd/2nd)] +1.55×[% of CD4+ T cells among PBMC (2nd/1st)]-5.97