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Metabolic profiling and compound-class identification reveal alterations in serum triglyceride levels in mice immunized with human vaccine adjuvant Alum

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Abstract

Alum has been widely used as an adjuvant for human vaccines; however, the impact of Alum on host metabolism remains largely unknown. Herein, we applied mass spectrometry (LCMS)-based metabolic and lipid profiling to monitor the effects of Alum adjuvant on mouse serum at 6, 24, 72 and 168 h post-vaccination. We propose a new strategy termed *Subclass Identification and Annotation for Metabolomics (SIAM)* for class-wise identification of untargeted metabolomics data generated from high-resolution MS. Using this approach, we identified and validated the levels of several lipids in mouse serum that were significantly altered following Alum administration. These lipids showed a biphasic response even 168 h after vaccination. The majority of the lipids were triglycerides (TAGs), where TAGs with long chain unsaturated fatty acids were decreased at 24 h, and TAGs with short chain fatty acids were decreased at 168 h. To

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Author contribution

SK designed the study, performed metabolomics experiments, processed and analyzed data, prepared figures and wrote the manuscript. IN designed the study, processed and analyzed data, prepared figures, and finalized the manuscript. PS analyzed data and prepared figures. TAO designed the study, contributed to animal experiments, and sampled the serum samples. JP contributed to animal experiments and sampling the serum samples. TM contributed to lipid analysis and lipid data analysis. PA contributed to animal experiments. AMH designed the study, contributed to animal experiments and resourced the study. JN designed the study, resourced the study, and finalized the manuscript. All authors reviewed and approved the manuscript.

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Competing interests

The authors declare no competing interest.

our knowledge, this is the first report on the impact of the human vaccine adjuvant Alum on host metabolome, and may provide new insights into the mechanism of action of Alum.

Keywords

Alum adjuvant; Human vaccine; Mass spectrometry; Metabolomics

Introduction

Aluminium-containing adjuvants, commonly referred to as ‘Alum’, have been widely used in human vaccines. Like other adjuvants, Alum is administered together with subunit and inactivated vaccines with the goal to enhance, accelerate, and/or modulate immune responses to co-administered antigens^{1, 2}. In the past decade, several mechanisms of action have been proposed for Alum adjuvanticity, such as depot formation³, activation of eosinophil and macrophages, increased uptake by antigen presenting cells⁴, and activation of the Nalp3 inflammasome⁵. More recently, Alum was proposed to bind to lipids in the cell membranes inducing receptor-independent activation of dendritic cell via the Syk-PI3 kinase pathway. The activation of Syk-PI3 kinase pathway could explain the induction of Th2 rather than Th1 responses by Alum. Although Alum has been used in the clinic over the past 90 years, the effect of Alum adjuvants on the regulation of human metabolism remains largely unexplored. The advent of omics technologies along with bioinformatic tools⁶ has recently been used to identify the molecular and cellular signatures of human vaccines and adjuvants^{7–11}. Metabolomics has been applied in various biomedical applications, particularly in drug discovery and precision medicine^{12, 13}. However, the application of metabolomics in vaccine research remains largely unexplored¹⁴. In a 2008 study by Yang *et al.*¹⁵, a combination of metabolomics and proteomics (MS-based), revealed changes in lipid profiles and protein composition in the sera of mice after immunization with a DNA vaccine. Furthermore, a recent exploratory study by McClenathan *et al.* in 2017¹⁶ using NMR-based metabolomics suggested differences in the metabolite profiles of an individual before and after smallpox immunization.

Although high-resolution LC-MS-based metabolomics is recognized as a significant technique promoting knowledge in biology, it is challenging to utilize the majority of the chemical spectra acquired by this method. For instance, in an untargeted experiment with more than 3000 detected features (non-annotated putative metabolites), no more than 5–20% are annotated by using reference spectra and/or by *in silico* predictions¹⁷. Much of these generated spectra remain unidentified due the lack of reference spectra in mass spectrometry databases. In a typical untargeted metabolomics experiment, statistical approaches such as principal component analysis (PCA), partial least squares (PLS), and orthogonal projection to latent structures (OPLS) are often performed for group-wise classification, and thereby extract significant features which need further identification or confirmation. However, the vast majority of these detected features remain unidentified, resulting in substantial information loss. In order to maximize the use of untargeted metabolomics data generated by high-resolution LC-MS, we propose an approach called *Subclass Identification and Annotation for Metabolomics* (SIAM) to improve the prediction of untargeted metabolite

classes based on groups of metabolites that are significantly changing under certain conditions. We applied this approach to identify groups of metabolites that were altered in mouse serum within seven days following administration of Alum adjuvant. The serum samples were obtained from mice administered with clinical-grade *Mycobacterium tuberculosis* H56 (H56) antigen alone or in combination with Alum adjuvant. The metabolomics data and SIAM together showed that Alum can alter the serum lipid profiles, particularly affecting the TAGs.

Materials and methods

Ethics statement and animal sample

The experiment of mice was performed in accordance with the regulations set forth by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden and in agreement with European Community Directive 86/609. All the methods and procedures were refined to provide for maximum comfort and minimal stress to mice. Experiments performed were approved by the Ethical Committee for Animal Experimentation in Gothenburg, Sweden under license 64/2015.

Female 6–7-week-old CB6F1/OlaHsd mice (Harlan Laboratories, Netherlands) were kept in ventilated cages with free access to food and water. All animals were housed under standardized pathogen-free conditions at the experimental animal facility, University of Gothenburg, Sweden.

Adjuvant and antigen

The Alum adjuvant used was Alhydrogel 2%, which was provided by Brenntag Biosector (Denmark). The adjuvant was evaluated with 5 µg of clinical-grade *Mycobacterium tuberculosis* H56 (H56) fusion protein (Statens Serum Institute, Denmark). The adjuvant and antigen formulation were prepared according to the recommendations from manufacturers. Briefly, Alum was diluted to 3.4 mg/mL of Aluminium in saline. Thereafter, H56 in saline was added to Alum at a 1:1 ratio (v/v) and rotated at 4°C overnight for adsorption. All vaccines and reagents were prepared and used within 2 h.

Animal experiments

The first animal experiment was conducted in three groups (N=7). The first group of mice was immunized with an antigen alone (H56). The second group of mice was immunized with H56 in combination with Alum. The last group was naive (no H56 or Alum). Each mouse was injected subcutaneously on either side of the base of the tail with a total of 100 µL of the vaccine mixture. The mice from each group were sacrificed at four different time points: 6, 24, 72 and 168 h after immunization. First, mice were sedated with isoflurane and bled from the axillary plexus. The blood was left to clot on ice in a 1.5 mL Eppendorf tube for 1 h, and subsequently centrifuged at 4°C and 2500 × g for 15 min¹⁸. The serum was immediately harvested and stored at –80°C until the metabolomic analysis. The second animal experiment (for lipidomic analysis) was conducted identically as the first experiment, but with groups of N=5.

Metabolite and lipid analysis

Reagents and standards—HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Fischer Scientific (Waltham, MA, USA). SupraSolv® Chloroform (CHCl₃) was obtained from Merck (Darmstadt, Germany). HPLC-grade 2-propanol was obtained from VWR (Radnor, PA, USA). All internal standards (ISs) were analytical grade and obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

Metabolite extraction by MeOH-H₂O—The metabolite extraction protocol was adopted from previous work¹⁹. Briefly, the frozen serum was thawed on ice at room temperature for 30 min, and vortexed for 5 s. A 100 µL aliquot of serum was mixed with 900 µL of pre-cooled 90% aqueous-MeOH containing 0.09 µg/mL of internal standards (IS) (Val-Tyr-Val and Leu-Enk) in a 1.5 mL Eppendorf tube (on ice bath) and shaken in a mixer mill using QIAGEN plates for 2 min (30 Hz). Approximately 950 µL of supernatant was transferred to a new vial, of which 200 µL was transferred to an LC vial for drying and re-dissolved in 20 µL of 50% aqueous-MeOH for LC-MS analysis. The analytical blank (no serum) was performed as described above using 100 µL of water instead of serum.

Lipid extraction—Lipid extraction was performed based on a previously published protocol^{20, 21}. Briefly, serum samples were thawed on ice at room temperature (30 min). A 20 µL aliquot of serum was extracted with 130 µL of CHCl₃-MeOH (2:1 v/v) containing four ISs, including phosphatidylglycerol [PG (17:0/17:0); 0.923 µg], phosphatidylethanolamine [PE (17:0/17:0); 0.455 µg], monoacylglycerol [MAG (17:0/0:0/0:0); 1.833 µg], and triacylglycerol [TAG (17:0/17:0/17:0); 0.045 µg]. The mixture was shaken for 2 min (30 Hz) and allowed to phase separate at room temperature for 30 min. The mixture was then centrifuged at 4°C, (14,000 rpm) for 3 min. A 50 µL aliquot of the lower-phase was transferred to a microvial and mixed with 130 µL of ceramide [D31(Cer(d18:1/16:0)-D31); 0.152 µg] which was dissolved in CHCl₃: MeOH (2:1 v/v). The final extract was aliquoted and stored at –80 °C until LC-MS analysis.

LC-MS measurements

Metabolite profiling—Metabolite profiling was performed on a 1290 Infinity LC-system equipped with an Agilent 6550 QTOF mass spectrometer (MS), Agilent Technologies (Waldbronn) Germany. First, all samples were analyzed in positive mode (POS); thereafter, the instrument was switched to negative mode (NEG), and completed analysis of all samples. For each analysis, 2 µL of the sample was injected onto a 2.1 × 100 mm, 1.7 µm UHPLC Kinetex C18-column (Phenomenex, Torrance) CA, USA. The gradient elution buffers were A (H₂O, 0.1% formic acid) and B (75/25 Acetonitrile/2-Propanol, 0.1% formic acid) with a flow-rate of 500 µL min⁻¹. The initial condition was 0.1% B, and the proportion of this solvent was linearly increased to reach 20% B at 4 min, and 60% B at 6 min. From 6 to 9 min the proportion of B was linearly increased from 60% to 95% and it was held at 95% up to 13.5 min. From 13.5 to 14.5 min, the column was returned to its initial conditions (0.1% B), and it was equilibrated for 4.5 min before the injection. The nine pooled samples (10% combined from each sample) were distributed along the run order for each analysis. The run order was randomized using the constrained randomization method²². The metabolites were detected with an Agilent 6550 QTOFMS equipped with a jet stream

electrospray source. The capillary voltage was set at 4 kV in POS and 3.5 kV NEG. The mass range of the TOF was set to 70–1700 m/z with a scan rate of 4 scans s^{-1} . Purine (2 μ M) and HP-0921 (Hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine) (2.5 μ M), both purchased from Agilent Technologies (Santa Clara, CA, USA), were used as reference masses.

Lipid profiling by LC-MS—The analysis followed the same strategy as the LC-MS metabolite profiling described above. The instrument was an Agilent 6540 QTOFMS equipped with an Agilent 1290 Infinity UHPLC-system, Agilent Technologies, Waldbronn, Germany. A 1 μ L aliquot of extracted sample was injected onto a reverse-phase C18 CSH (Acquity), 2.1×50 mm, 1.7 μ m column in combination with a 2.1 mm x 5 mm, 1.7 μ m VanGuard pre-column (Waters Corporation, Milford, MA, USA) at 60 °C. The gradient elution buffers were A (60:40 of ACN: H₂O, 10 mM ammonium formate, 0.1% formic acid) and B (89.1:10.5:0.4 2-propanol: ACN: H₂O, 10 mM ammonium formate, 0.1% formic acid), and the flow rate was 0.5 mL min^{-1} . The separation was achieved with a linear gradient using an initial condition 15% B, and increase to 30% B at 1.2 min, 55% at 1.5 min, isocratic to 5.0 min, increase to 72% B at 7 min, 85% at 9.5 min and 100% B at 10.0 min, and then held at 100 % for 2 minutes. An additional wash of the injection valve, with 100% B and flow rate 5.0 mL min^{-1} for 0.3 min, was performed before being decreased to the initial condition 15% B over 0.3 min; these conditions were held for 1.1 min (run time 14.3 min) to equilibrate the column before next injection. A reference interface was connected for accurate mass measurements; the reference ions purine (2 μ M) and HP-0921, both purchased from Agilent Technologies (Santa Clara, CA), USA, were infused directly into the MS at a flow rate of 0.07 mL min^{-1} for internal calibration, and the monitored ions of purine at m/z 121.05087 and m/z 119.03632; HP-0921 at m/z 922.00980 and m/z 966.000725 for POS and NEG mode respectively. The drying gas was set to 300°C, flow to 8 L min^{-1} and the nebulizer pressure 40 psig. The sheath gas temp was set to 350°C with a flow of 11 L min^{-1} . The m/z range was 70–1700, and data was collected in centroid mode with an acquisition rate of 4 scans s^{-1} (1973 transients/spectrum). The diode array detector was set to scan the interval 190–640 nm with a step length of 2 nm and a slit width of 4 nm.

Data pre-processing

Metabolites—Mass Feature Extraction (MFE) from the acquired data was performed using the MassHunter™ Qualitative Analysis software package, version B06.00 (Agilent Technologies Inc., Santa Clara, CA, USA). Extracted features were aligned and matched between samples using Mass Profiler Professional™ 12.5 (Agilent Technologies Inc., Santa Clara, CA, USA). The final output from peak picking that includes intensities, molecular mass (m/z), retention time (RT) of each feature was exported to an excel sheet.

Lipidomics—The peak areas of ISs were calculated using the Agilent MassHunter ProFinder version B.08.00 (Agilent Technologies Inc., Santa Clara, CA, USA). The targeted data processing on all samples was performed on the pooled samples using the Batch Targeted Feature Extraction algorithm with the Agilent software MassHunter ProFinder. *In-house* database with exact mass (m/z) and experimental retention (RT) times of the lipids were used for compound identification.

Multivariate analysis of metabolomics and lipidomics data

The raw data (metabolomics and lipidomics) were subjected to quality control to remove any systematic variations. The datasets (feature intensities) were log2 transformed and normalized by the Cross-contribution Compensating Multiple standard Normalization (CCMN) method²³, as deployed in the R metabolomics package. Principle component analysis (PCA)²⁴ and Orthogonal Partial Least Squares projections to latent structure - discriminant analysis (OPLS-DA)²⁵, including extraction of significant features, were performed using SIMCA (version 14.1 MSK Umetrics AB, Umeå, Sweden).

Results

Study design and implementation of SIAM

An overview of the study design, sample preparation and the implementation of SIAM is provided in Fig. 1. For the implementation of SIAM, raw datasets were first subjected to mass feature-extraction²⁶ and mass feature-alignment. Each individual feature contains information such as molecular mass (m/z), isotope distribution, adducts, retention time (RT), and intensity. In this study, we obtained 1,623 and 1,224 features from POS and NEG modes, respectively. Furthermore, the peak intensities of the two internal standards (IS) as Val-Tyr-Val and Leu-Enk, were extracted from all the samples and used for normalization. The analytical variation and systematic errors were directly visualized through the distribution of Val-Tyr-Val (<2 %RSD) and Leu-Enk (<16 %RSD) across all samples (Fig. S-1). This step helped to identify and remove any non-physiological and systematic outliers incurred by sample measurement and/or systematic variations. Thereafter, extracted features were normalized based on the intensity of Val-Tyr-Val and Leu-Enk using the CCMN²³ method (results in Fig. S-2 and full data provided in Table S-1). At this stage, unsupervised and/or supervised classification methods such as PCA, PLS or OPLS-DA²⁷ can directly be applied to the normalized datasets. To further determine the effect of Alum adjuvant, the groups of H56 and H56+Alum were matched for a group-wise comparison analysis²⁵. The aim at this stage was to identify significant features between the H56 and Alum treated groups. The selected significant features (p value 0.05 and fold change 2) were further annotated using the METLIN database²⁸, based on mass tolerance windows of 10 ppm or less ($m = 10\text{ppm}$). The putatively identified features were then defined into a subclass based on the HMDB²⁹ and Lipid Maps^{30, 31} databases (Fig. 1-C and fully detailed in Fig. S-3). At this stage, a feature may belong to one, multiple, or no subclass. This can be demonstrated by the following cases (Fig. S-3): 1) a feature at m/z 809.5887 was annotated by METLIN as phosphatidylethanolamine (PE) with $m = 1\text{ppm}$, and could be further categorized to the glycerophospholipid subclass; 2) the feature at m/z 797.5290 was identified as 34 different classes of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with the same mass accuracy ($m = 1\text{ppm}$), and we finalized these into the glycerophospholipid subclass; 3) a feature m/z at 173.0465 was identified as deisopropylatrazine ($m = 1\text{ppm}$), but no subclass information of this metabolite was available through either the HMDB or Lipid Maps databases and it was therefore marked as not available (N); 4) a feature at m/z 238.1184 was identified as different compounds and different subclasses with the same mass accuracy ($m = 1\text{ppm}$) such as N, prenol lipids, or phenols and derivatives, and was therefore defined as ambiguous (A).

Global metabolome analysis revealed a metabolic response induced by Alum adjuvant

To gain new insight into the mode of action of the Alum adjuvant, we assessed the serum metabolome longitudinally from 6h up to 1 week after single administration of H56 alone or in combination with Alum. Results from PCA (Fig. 2-A and 2-B) revealed that the changes in expression of global metabolites were subtle between H56 and H56+Alum treated mice - no clear visible separation was observed in the PCA results of the two treatment groups or among different time points. This is in line with many observations that a PCA-based classification in untargeted metabolomics often leads to a clear separation only when comparing groups that are clearly distinct; for instance, germ-free mice and conventional mice³², or transgenic plants with the wild-type³³. Hence, we applied a supervised classification method known as OPLS-DA³⁴ to our datasets. As shown in Fig. 2-C and 2-D, this resulted in an improvement in class-wise separation of groups with respect to treatments and time points. A distinct separation of naive from the other groups implied that there were changes in metabolic responses affected by H56 alone and H56 with Alum treatments. Furthermore, a clear separation of several groups at different time points suggested that there were different dynamic metabolic responses to the vaccine and adjuvant treatments.

Identification of lipid induced metabolic changes by SIAM

In order to pinpoint the metabolite changes induced exclusively by Alum, we performed matching time-point analysis (univariate) between the H56+Alum and H56 (control) groups (Fig. 1-C). We used an S-plot³³ to visualize the variable (feature) influence in the model and selected the significant features with a cut-off of $p < 0.05$ with fold changes (FC) > 2.0 . About 160 features (POS) and 102 features (NEG) were identified from four time points (Fig. S-4). Three common features m/z at 201.1001, 594.4216 and 238.1332, (POS) were found in all four time points (Fig. S5-A and S5-B). From these 262 significant features, about 50% were structurally identified by the METLIN library (accurate mass alone) with $m < 10$ ppm. By using SIAM, we categorized these tentatively identified metabolites into different subclasses based on HMDB²⁹ and Lipids Maps databases^{30, 31}. Among all 17 classes predicted, lipid was the major class that changed significantly between H56 and Alum treated groups (Fig. 3). Large changes were observed at 24 h (54% of all metabolite classes) and 168 h (43%), whereas subtle changes occurred at 6 h (19%) and 72 h (15%).

LC-MS based lipid profiling validates lipids as a major class of metabolites affected by the Alum treatment

For the lipid analysis (Fig. S-6A), we employed a combination of CHCl_3 -MeOH (Fig. S-6B), which is highly regarded as the gold standard for extraction of lipids from serum samples. The lipid profiling yielded a total of 492 lipid features (168 identified) from POS mode and 158 lipid features (27 identified) from NEG mode (full data in Table S-2). The score plot of lipid profiling from PCA and OPLS-DA (Fig. 4-A and Fig. 4-B) revealed similar patterns of classification of different groups either treated with H56 alone or in combination with Alum.

Alum administration significantly re-modulates the serum lipidome at 168h

Next, we performed matching time-point analysis of lipid data, by comparing H56+Alum treated groups to H56 (control) at all four time points. Significantly altered lipids ($\log_2$ (fold change) ≥ 1.5 and p -value ≤ 0.05 , full results in Table S-2) were selected. In order to improve the accuracy and limit the effect of spurious variation, we assigned the precision of biological replication with RSD $\leq 10\%$. With this cut-off, we obtained 52 lipid metabolites (both identified and unidentified) that were significantly changed in mouse serum following Alum administration. These 52 lipid metabolites were obtained from only two time points: 24 h (8 identified and 18 unknowns) and 168 h (15 identified and 12 unknowns), where the unknown feature m/z 1683.4557 was found in both time points. The identity of this feature remains to be evaluated, but it undergoes the largest fold changes at 24 h (1.7-fold, $p = 0.001$) among all detected lipids. However, identifying this feature is currently not possible due to sample limitation, no availability of an authentic standard, as well as lack of reference spectra through the METLIN and other databases. To investigate the changes of all significant lipids over time, we performed hierarchical clustering (Fig. 5-A) based on abundances (Z-score) of these 52 lipids from both H56+Alum and H56 groups, which resulted in three different clusters (1–3). The profiles of the lipids categorized in cluster 1 of H56+Alum treated mice showed a significant increase in their abundance at 24 h compared to 6 h post-vaccination. Subsequently, after 24 h the lipid intensities gradually returned to base level at 168 h. Moreover, the levels of these metabolites were similar to the naive group (mice group treated with neither adjuvant nor vaccine) and at 6 h. Furthermore, we also observed the same trends in the intensities of lipids in clusters 2 and 3, but in the opposite direction. This suggests that the significant lipids detected in the Alum group could complete their cycle within seven days after vaccination (Fig. 5-B). By contrast, changes in the lipid profiles of H56 treated mice lag behind those of the H56+Alum group. As seen in cluster 1, their intensity levels were slowly increased after 6 h until 168 h without a significant decline to baseline. Among these 52 metabolites, 44% (23 lipids) were identified by our in-house library (by exact mass and experimental retention times of lipid standards). Interestingly, all of them were different forms of TAGs.

Regulation of TAGs at 24 and 168 h after immunization

Immunization with Alum had a significant effect on the levels of 23 TAGs ($1.5 \leq \log_2(\text{fold change})$ and p -value ≤ 0.05). We determined the chemical nature of these TAGs based on their acyl-chain length and degree of unsaturation (double bonds) of the FAs. The double bonds (Fig. 6A) and chain lengths (Fig. 6B) of the TAGs were associated with their relative fold changes (H56 vs. H56+Alum). It was evident that most of the long chain unsaturated TAGs were decreased at 24 h, whereas short chain TAGs decreased later (168 h following Alum administration) (Fig. 6).

Discussion

In our previous study³⁵, we carried out genome-wide transcriptomics analysis to unravel the transcript signatures of four clinically tested vaccine-adjuvants in mice. We reported shared or mutually exclusive gene modules, networks and pathways perturbed in whole blood and the draining lymph nodes by the clinical tested vaccine-adjuvants. While large changes in

transcriptomic profiles were observed for CAF01, IC31, and GLA-SE in both whole blood and the draining lymph nodes, Alum was found to be transcriptionally quiescent in both organs. To further investigate the underlying mechanism of action of Alum at the molecular level, we herein applied an untargeted metabolomics approach based on high-resolution MS to serum samples retrieved longitudinally from mice after administration of Alum in combination with H56 antigen or H56 antigen alone. Groups of mice were administered subcutaneously with H56 alone (control) or in combination with Alum (2% Alhydrogel) (Fig. 1-A). To explore the global changes in serum metabolites, we first analyzed the polar metabolites derived from the MeOH fraction after MeOH-H₂O¹⁹ extraction (Fig. 1-B). The analysis was performed on a high-resolution MS (LC-QTOFMS) in both POS and NEG mode. To increase information of untargeted metabolomics data, we developed SIAM (Fig. 1-C), which consists of two different modules for data processing and data analysis. The metabolome data analysis by SIAM revealed that time is the most confounding factor for the changes in the metabolome signal affected Alum. Likewise, the SIAM platform allowed identifying that lipid was the major class of metabolites significantly altered following Alum administration. From this prediction, we hypothesized that lipid metabolism is presumably involved in adjuvanticity of Alum. However, we were aware that the metabolome datasets might not reflect the changes induced by vaccine and Alum on the entire lipidome owing to the fact that our analysis was based on the MeOH fraction after MeOH-H₂O extraction, which is known to be a less sensitive method for lipid extraction as compared to the mixture of chloroform (CHCl₃) and MeOH. To validate our prediction from SIAM, we subsequently carried out an additional mouse experiment (N=5 in each group), under the same experimental conditions as the metabolomics experiment. The lipid analysis results confirmed that lipid metabolism was significantly changed following Alum administration. This is demonstrated by a similar separation pattern obtained from both OPLS-DA and unsupervised PCA analysis. Furthermore, these results indicated that analyzing only one class of metabolites that were significantly impacted by Alum provided better resolution than analyzing the entire metabolome. In addition, we observed a dramatic separation between H56+Alum and H56 groups at 24 h and 168 h of vaccination, from both PCA and OPLS-DA methods. This is in contrast to the small changes induced by Alum on the transcriptome level (only a few genes at 72 h) and GO enrichment terms in the whole blood and draining lymph nodes of mice³⁵.

Moreover, the majority of these significant lipids (23 identified species out of 53) were non-essential TAGs and endogenously produced by *de novo* biosynthesis³⁶. Recently, evidence of the importance of fatty acid synthesis and oxidation in directing immune cell differentiation and function, especially T cells, have been reported³⁷. Future studies are needed to evaluate whether the lipid profile induced upon Alum administration is representative of its characteristic Th2 type response tilting propensity and how it compares with other immunologic adjuvants. Likewise, significant changes in the endogenous TAG profiles post-immunization may substantiate the fact that Alum induces the immune mediators and metabolites, which in turn might regulate the *de novo* biosynthesis of the TAGs in the liver. Thus, immunization with Alum might have a direct or indirect effect on the host immunometabolism. However, a detailed mechanistic study is required to elucidate

the underlying regulation of host *de novo* lipogenesis, and its causal effect on Alum treatment.

Conclusion

In summary, we have developed the SIAM strategy for the analysis of untargeted metabolomics data, and demonstrated its ability to enhance the accuracy, precision, sensitivity and specificity of metabolomics data analysis. This strategy is particularly useful when dealing with clinical metabolomics data, as they often suffer from low signal to noise ratios⁶. Hence, this will greatly help to extract information from metabolomics data. Furthermore, most analytical methods used for targeted metabolite analysis are designed to quantify a particular class of metabolites, such as fatty acids³⁸, lipids³⁹, or amino acids⁴⁰. By applying this method as a screening strategy, we could determine that lipids were the metabolites most affected by Alum administration. The majority of these lipids were TAGs, as well as several unidentified species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Experimental design and development of the *Subclass Identification and Annotation for Metabolomics (SIAM)* platform. (A) Schematic view of the experimental design. (B) Schematic view of untargeted metabolomics of serum sample extracted by MeOH-H₂O and analyzed by UPLC-QTOFMS. (C) Schematic view of the SIAM platform. SIAM consists of two parts: data processing, and data analysis. The raw files obtained from MS were subjected to Mass Hunter software (Agilent Technologies Inc.) to extract a feature containing information such as molecular ion (m/z), intensity and RT. The data was then normalized with ISs using a normalization known as CCMN method (performed in R). At this state, the normalized data can be directly subjected to PCA or OPLS-DA for a global view of metabolite profiling. Annotation of significant features between H56+Alum and H56 with cut-off p corr 0.5, Log2(fold changes) 0 was performed using SIMCA software (MKS Umetrics). Significant features selected from the previous step were putatively identified by METLIN library with m 10ppm. The putatively identified metabolites were redefined into different subclasses based on HMDB and Lipid Maps systems.

Fig. 2.

PCA and OPLS-DA

(A) PCA: score plot (POS); (N = 8), $R^2X[1] = 0.141$, $R^2X[2] = 0.10$, $Q^2 = 0.194$.

(B) PCA: score plot (NEG); (N = 8), $R^2X[1] = 0.115$, $R^2X[2] = 0.097$, $Q^2 = 0.061$.

(C) OPLS-DA: score plot (POS); (5+2+0) $R^2X[1] = 0.0726$, $R^2X[2] = 0.0503$, $R^2Y = 0.498$, $Q^2 = 0.11$.

(D) OPLS-DA: score plot (NEG); (8+4+0) $R^2X[1] = 0.0571$, $R^2X[2] = 0.0382$, $R^2Y = 0.878$, $Q^2 = 0.218$.

Note that N= number of components, R^2X represents the explained variance of the component. The R^2Y value represents the modelled class discrimination, ranging from 0 (no discrimination) to 1 (perfect discrimination). The Q^2 represents the predicted class discrimination ranging from 0 to 1.

Fig. 3.

Pie charts showing the different subclasses of metabolites at 6, 24, 72 and 168 h after treatment.

(A) Subclass metabolites at 6 h [POS: 32 features and NEG:27 features]

(B) Subclass metabolites at 24 h [POS: 48 features and NEG: 14 features]

(C) Subclass metabolites at 72 h [POS: 48 features and NEG: 38 features]

(D) Subclass metabolites at 168 h [POS: 32 features and NEG: 23 features]

*Total significant ($p < 0.5$ with fold changes (FC) ≥ 2.0) number of features from POS and NEG.

**Total number of features that were tentatively identified by METLIN.

Fig. 4.
PCA and OPLS-DA of lipid metabolites (Log2 peak intensities).
(A)PCA: score plot of lipid metabolites (POS + NEG)
(B)OPLSDA: score plot of lipid metabolites (POS + NEG)

Fig. 5. Change of 52 significant lipid-metabolites over time. (A) Hierarchical clustering of 52 significant lipid metabolites based on the phase of their maximal expression. Each row corresponds to a lipid metabolite (identified or unidentified), and each column indicates the intensities for all of the biological replicates (N=5) at each time point. The color scale of the intensity values (Z-scored normalized log2 intensities) is shown in the top left bar [high: red, low: blue]. (B) Summary cycle changes of an individual lipid metabolite from 6 to 168 h. (Ah56+ Alum groups)

Fig. 6. Types of triglycerides (TAGs) significantly affected by Alum administration. (A) A scatter plot of the log2 fold changes (FCs) of TAGs significantly altered ($p < 0.05$) between H56 and H56+Alum as a function of their number of acyl chain double bonds. (B) A scatter plot of the FCs of these TAGs as a function of their number of acyl chain carbon atoms (chain length). The blue and magenta points denote 24 and 168 h of immunization, respectively.