**Metabolomics and Lipidomics analyses**

**Experimental procedures**

Cell samples were extracted following an in-house two-phase extraction protocol using methyl tert-butyl ether (MTBE) and methanol as a non-polar and polar extraction solvent and water as a phase separation solvent. The MTBE method was modified and optimized based on Matyash et al1. Briefly, 100 ul of methanol and 300 ul of MTBE was added to extract polar and non-polar lipid molecules, respectively. For a phase separation, 100 ul of water was used. Stable isotope labeled lipid standards (Avanti SPLASH Lipidomix) were spiked to each sample to use as a spike ins quality control. Separation of several lipid classes were maximized and validated using isotope labelled standards for each class. As a part of validation, the robustness and reproducibility of isotope labelled PS standard as well as representative lipid molecules were checked. For quality control (QC) and assurance (QA), pooled reference samples were prepared and ran at the beginning, in the middle and at the end of the biological samples.

The chromatographic separations were performed using Thermo ScientificTM TranscendTM Duo LX-2 UHPLC system interfaced with high resolution Thermo ScientificTM Orbitrap ID-XTM TribidTM mass spectrometer with a HESI ionization source, using negative ionization mode with a run time of 12 min. All samples were maintained at 4 °C in the autosampler. Data were acquired using reversed phase (RP) column in negative polarity in full scan mode with mass resolution of 60,000. A Hypersil GOLDTM RP column (3 μm, 2.1 mm x 50 mm) maintained at 45 ºC was used for chromatographic separation. 10 mM ammonium acetate in 60:40 acetonitrile: water and 10 mM ammonium acetate in 90:10 2-propanol: acetonitrile were used as mobile phase A and B respectively for RP acquisition.

Mass spectrometry data were collected with the following MS settings: mass range, 400-1000 m/z; spray voltage, 2800 V (ESI-); sheath gas, 45 Arb; auxiliary gas, 20 Arb; sweep gas, 1 Arb; ion transfer tube temperature, 325 °C; vaporizer temperature, 325 °C; full scan mass resolution, 60,000 (MS1); normalized AGC target (%), 25; maximum injection time, 100 ms. Data dependent fragmentation (dd-MS/MS) parameters for each polarity as follows: isolation window (m/z), 1.2; stepped HCD collision energy (%), 20,40,80; dd-MS/MS resolution, 30,000; normalized AGC target (%), 20; maximum injection time (ms), 54; micro scan, 1; cycle time (sec), 1.2. A full scan data-dependent MS2 (ddMS2) method was utilized to collect MS2 spectra for identification of compounds.

**Data preprocessing**

Features from the pilot metabolomics and subsequent lipidomics datasets were extracted using Asari2 (v1.10.6), and subjected to mass accuracy, chromatographic consistency, and other QC steps using customized pyOpenMS3 (v2.8.0) scripts. Blank-masking (highest intensity set to 3 times higher than blank samples), group-filtering (present in at least one group), and normalization by the mean of the top 1000 most abundant features were performed. Finally, imputation and log2-transformation were applied to the data.

**Identification of Phosphatidylserine (PS) species**

We downloaded the Glycerophosphoserines (GP03) LIPID MAPS® Structure Database (LMSD)4, computed the '[M-H+e]' adduct masses, and tentatively identified biologically relevant phosphatidylserine (PS) species in all features using JMS (0.5.1, <https://github.com/shuzhao-li/JMS>).

To compensate for the incompleteness of existing lipid databases, a computational approach was employed to generate a near-exhaustive set of chemically feasible phosphatidylserine lipids. As this list was to be used exclusively for MS1-based annotations our algorithm did not generate or consider in any way the structures of the generated lipids but instead generated only the molecular formula, their corresponding neutral masses, and the ‘[M-H+e]’ adduct masses for every computationally generated lipid. For mass calculations every atom was assumed to be the most abundant stable isotope for that element and the masses were computed using the python mpmath library. Isotope masses and electron masses were taken from NIST.

Our algorithm uses the molecular formula for the PS headgroup as a base and generates potential lipids by simulating the addition of -CH2- to the side chains as well as the removal of 2 H to form a double bond. The number of added -CH2- groups is bounded by the *m/z* range of the MS1 acquisitions assuming a charge of z=-1 (i.e., 1000 amu) while the number of double bonds possible is bounded by the number of added -CH2- groups. The headgroup formula was modified accordingly to generate LPS, PS O-, and LPS O- variants of the PS lipids. All generated formulas were compared against an in-lab target list of known PS lipids and variants to ensure the accuracy of our generated formulas.

The full feature table was annotated using the computationally generated PS lipids as follows. First, an *m/z-*only search is performed for the M-H+e adduct of every computationally generated lipid. A 5-ppm cutoff is used for this search. Second, if a match is found (hereafter referred to as the m+13C0 match) , the m+13C isotopologues of this formula are then generated in increasing order of 13C count (e.g., m+13C1 then m+13C2, etc.). These isotopologues are then searched against the feature list using their *m/z* values. Any possible matches are then further filtered to eliminate matches that did not co-elute with the m+13C0 feature. Co-elution is determined by comparing that the isotopologue retention time is bounded by the rtime\_right\_base and rtime\_left\_base of the m+13C0 feature. Isotopologues are also checked to ensure that in at least 90% of samples, the more heavily isotoped isotopologue is less intense than the previously identified isotopologue (i.e., the intensity of m+13C2 must be less than m+13C1 in at least 90% of samples). This intensity check minimizes confusion between m+13Cn isotopologues and various degrees of unsaturation between lipids when n is even. This isotopologue search continues until no such match is found. Since less abundant lipids will have fewer detectable isotopologues, using the number of observed isotopologues to measure the certainty of an assignment is not straightforward; however, this number of detected isotopologues provides subjective circumstantial evidence that an assignment is plausible.

In addition to stepwise level 4 annotations, we performed level-2 annotations to identify biologically relevant phosphatidylserine (PS) species. We selected MS/MS spectra with precursor ions exhibiting PS-specific neutral loss in their product ions and searched for spectra with precursor m/z and estimated retention times (RT) that matched tentatively annotated PS features (ppm = 5; RT window = 30 seconds). We used the resulting spectra to identify PS-specific neutral loss (m/z diff. = 87.03124, ppm = 30). We acquired the source MS/MS spectra from pooled T cell samples using regular DDA and deep scan methods. Noticeably, we annotated PS 40:0 based on MS/MS-validated PS features present in both pooled Klof1 iPSC cell and pooled T cell samples. Annotation details were stored in a JSON file. The final selected PS features were either LMSD-matched PSs with at least one C13 isotope feature identified or had MS/MS spectra with PS-specific neutral loss. This allowed us to report biologically relevant PS species with a high degree of confidence.

**Statistical analyses and visualization**

In both metabolomc and lipidomic analyses, one-way ANOVA were performed across groups and *post-hoc* Tukey tests were performed to identify pair-wise significant features (adjusted p-value < 0.05). Principal component analysis, volcano plot, heatmap and box plots were performed in customized R scripts (v4.2.3). The relevant analyses were stored in a GitHub repository (<https://github.com/gmhhope/Rafi_Ahmed_exhaustedCD8Tcell>, will make a cleaner one)