A sensitive S-trap-based approach to the analysis of T cell lipid raft proteome

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Abbreviations: FASP : Filter aided sample preparation; LAT: linker for activation of T cells; S-trap : Filter aided sample preparation; TCR: T cell receptor.

ABSTRACT

The analysis of T cell lipid raft proteome is challenging due to the highly dynamic nature of rafts and the hydrophobic character of raft-resident proteins. We explored an innovative strategy for bottom-up lipid raftomics based on suspension trapping (S-trap) sample preparation. Mouse T cells were prepared from splenocytes by negative immunoselection and rafts were isolated by a detergent-free method and OptiPrep gradient ultracentrifugation. Microdomains enriched in Flotillin-1, LAT and cholesterol were subjected to proteomic analysis through an optimized protocol based on S-Trap and high pH fractionation, followed by nano-LC-MS/MS. Using this method, we identified 2680 proteins in the raft-rich fraction and established a database of 894 T cell raft proteins. We then performed a differential analysis on the raft-rich fraction from non-stimulated vs. anti-CD3/CD28 TCR-stimulated T cells. Our results revealed 42 proteins present in one condition and absent in the other. For the first time, we performed a proteomic analysis on rafts from ex-vivo T cells obtained from individual mice, before and after TCR activation. This work demonstrates that the proposed method utilizing an S-trap-based approach for sample preparation increases the specificity and sensitivity of lipid raftomics. Data are available via ProteomeXchange with identifier PXD016476.

Key words: immunology, lymphocytes, membranes, tandem mass-spectrometry, cell signaling, detergent-free, FASP, OptiPrep, raftomics

INTRODUCTION

Rafts (lipid rafts or membrane rafts) are subcellular entities defined as dynamic lateral membrane microdomains (1, 2). Their formation and dynamics have been associated, by a strong body of experimental evidence, with the regulation of cellular functions. Nevertheless, for many years their existence has been questioned owing to (i) the fact that their definition is based on experimental concepts, such as detergent resistance, and (ii) the limited sensitivity of the methods available for their study. A consensus official definition resulted from a Keystone Symposium back in 2006 (3). This definition establishes their size limits (10-200 nm) and composition (sterol- and sphingolipid-enriched domains), as well as their highly dynamic nature.

If almost any cell type can contain raft-like structures, the scientific community has widely accepted and integrated this concept in the context of lymphocytes. Compelling evidence indicates that T-cell activation encompasses membrane reorganization events involving lipid and protein rearrangement, as well as specific protein interactions that orchestrate the clustering of cholesterol and sphingolipid-rich domains (4). It is broadly acknowledged that raft dynamics plays a significant and direct role in T cell activation via TCR stimulation (5-13) and T cell migration induction via chemokine receptor stimulation (14-19).

The comprehensive characterization of the lipid raft proteome of T cells has been a challenging goal due to difficulties in the isolation of microdomains, their highly dynamic nature, and the hydrophobic character of raft-resident proteins. Nevertheless, several groups have addressed this question using state-of-the-art approaches significantly contributing to decipher some of the mechanisms involved in T cell activation (4, 9, 17, 20-27) (reviewed in (23, 28-30)). The numerous studies on rafts in any cell types published to date (reviewed in (31, 32)) have contributed to the generation of a curated growing mammalian lipid raft protein database (www.raftprot.org) (33, 34). This includes proteins identified by biochemical approaches, either positively by biochemical isolation or negatively by cyclodextrin-based raft-disruption experiments. Proteins identified in proteomic studies are also included, although it is

specified whether their presence in rafts has been validated or not by alternative methods. This underlines the issue of purity and contamination in raft preparations.

The golden standard methods for raft isolation are based on, first, cell activity arrest at 4°C followed by differential solubilization of membrane microdomains in non-ionic detergents, such as Triton X-100, Lubrol WX, and Brij 35 (35). This procedure may introduce a serious artefactual bias in that detergents are likely to alter membrane properties and, consequently, experimental resistant domains might differ significantly from those existing in physiologic conditions (36). It has been shown that detergent-based methods scramble lipid content and therefore create new artifactual detergent-resistant lipid rafts (37). Detergent-free methods have been developed to minimize this problem. They are based on membrane fragmentation by physical methods, like ultrasound treatment of cells (38), or immunoisolation targeting specific raft-resident proteins (36). These methods are considered to yield a better purity of raft fractions (38).

Detergent treatment of cell membranes is followed by ultracentrifugation on a discontinuous density gradient. Sucrose gradients are usually performed overnight, which represents a timely limitation. Shorter preparation procedures include the use of colloidal solutions, such as OptiPrep™ (36, 39, 40). The latter provides a rapid, highly reproducible and selective isolation of raft-like microdomains, where selectivity is demonstrated by raft marker enrichment. However, OptiPrep gradients have been scarcely used as a preparation step for proteomics. The few reported cases to date correspond to proteomic analyses on extracellular vesicles (41-49), exosomes (50-53), mitosomes (54), isolated insulin secretory granules (55), synaptosomes (56) and plant organelles (57-60). To date, OptiPrep isolation has been applied to raft proteomics in a study on virus-infected algae (61). Globally, OptiPrep interferes with the LC-MS/MS analysis and needs to be eliminated thoroughly from the sample. However, efficient cleanup steps, like SDS PAGE short separation, lead to protein loss and are not compatible with very low abundant samples, such as T cell rafts from one single mouse. Hence, there is a need for a simplified and sensitive method for proteomic analysis of T cell rafts.

In this work we explore an innovative strategy, based on a single device S-trap preparation technique for proteomics on raft-like (flotillin-1- LAT- and cholesterol-rich) microdomains. For the first time, a global proteomic analysis is performed on purified rafts from ex-vivo mouse T cells, before and after activation by TCR costimulation. Our results show an increased specificity and sensitivity of the proposed method. In addition, we have created a new database of T-cell raft proteins.

MATERIALS AND METHODS

Cell culture

HEK 293 cells (American Type Culture Collection), were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Mouse podocytes were cultured in RPMI 1640 medium containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and interferon-g (IFN-g, 50 U/ml) at 33°C. Differentiation was induced by maintaining stable podocyte cell lines at 37°C without IFN-g for 14 days in the presence of blasticidin and zeocin. Jurkat T-cells (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml) at 37°C.

Mouse T-cell isolation and synchronization

Balb/C mice were bred following the standards established by the national Ethics Committee (COMETH) under accreditation number 29/01/13-1. Four individual mice were used as biological replicates (n=4). After sacrifice, spleens were harvested, gently minced with a scalpel and passed through a 40 µM nylon mesh filter. T cells were isolated by negative immunoselection using the Pan T Cell isolation kit (Miltenyi Biotec GmbH, Germany). Immunoselected cells were confirmed as CD4+ and CD3+ by flow cytometry (Supplemental Figure 1). Before stimulation, T-cells were synchronized at the G1 phase of cell cycle by serum starvation in 2% fetal calf serum (FCS) for 6 hours. Synchronized T-cells were then activated for

15min with soluble 1 µg/ml anti-CD3 and anti-CD28 (eBiosciences, San Diego, CA, USA) in RPMI complete medium supplemented with 10% FCS.

Lipid raft preparation using OptiPrep™ and sucrose gradients

Lipid raft-like microdomains were obtained by a detergent-free method based on the one described by McDonald and Pike (38). Between 1 and 5 million cells per sample were washed twice in cold PBS, resuspended in 800µl of MBS/Na_2CO_3 buffer (25mM MES, 150mM NaCl, 250mM Na2CO3, pH6; supplemented with 1mM PMSF and phosphatase and protease inhibitor cocktails) and lysed by passaging 20 times through a 21G needle, followed by sonication 3 times for 60 seconds in a Vibra Cell 75022 sonicator. The homogenate was mixed with two volumes of either 60% OptiPrep™ (Axis Shield) or 60% sucrose for a final volume of 2ml of either 40% OptiPrep™ or 40% sucrose. A three-step discontinuous density gradient was made by sequentially placing 2ml of either 30% OptiPrep™ or 30% sucrose in MBS/Na_2CO_3 buffer, and 1ml of 5% OptiPrep™ or 5% sucrose sequencially on top of the homogenate. The mixture was spun in a TL-100 rotor at 268,000×g for 2h in an Optima MAX-XP ultracentrifuge (Beckman Coulter). After spinning, one fraction of 600µl followed by 5 fractions of 900µl were collected from top to bottom. Fraction 2 containing rafts was subjected to subsequent analysis.

To analyze the distribution of flotillin-1, fractions were precipitated by addition of 10% trichloroacetic acid (final concentration), incubated overnight at -20°C and washed three times in cold ethanol. The resulting dry protein pellets were solubilized in equal volumes of 1× Laemmli buffer and analyzed by western blot.

Filter aided sample preparation (FASP)

FASP was performed on OptiPrep™ raft fractions according to (62). Briefly, samples were reduced with 0.1 M dithiotreitol (DTT) at 60°C for 1 hour. Proteins were transferred to Microcon filter units (30 kDa cut-off) and washed twice with 200 µl of UA buffer (0.1M Tris, 8M urea, pH 8.9) and concentrated by centrifugation at 14,000 x g for 15 minutes. Proteins were alkylated with 100 µl of IAA buffer (0.05M

iodoacetamide, 0.1M Tris, pH 8.9) at room temperature in the dark for 20 minutes and centrifuged at 14,000 x g for 10 minutes. Proteins were then washed twice by adding 100 µl of UA buffer before centrifugation at 14,000 x g for 10 minutes, and twice with 100 µl of ABC buffer (0.05M NH_4HCO_3) before centrifugation at 14,000 x g for 10 minutes. Filter units were transferred to new collection tubes and samples were incubated with 40 µl of ABC buffer containing 1.6 µg of trypsin in a humidity chamber at 37°C for 18 hours. Tubes were centrifuged at 14,000 x g for 10 minutes, 40 µl of ABC buffer were added and tubes were centrifuged again. Peptides were finally recovered in collection tubes.

Suspension trapping (S-Trap)

S-Trap™ micro spin column digestion was performed on OptiPrep™ raft fractions according to the manufacturer's protocol. Briefly, proteins were precipitated overnight using a 10% TCA final concentration and washed four times with cold ethanol. Proteins were resuspended and solubilized in 5% SDS, 50mM triethylammonium bicarbonate (TEAB, pH 7.55), reduced with 100mM DTT solution and alkylated with the addition of iodoacetamide to a final concentration of 40mM. Aqueous phosphoric acid was added to a final concentration of 1.2%. Colloidal protein particulate was formed with the addition of 231µL S-Trap binding buffer (90% aqueous methanol, 100mM TEAB, pH7.1). The mixture was placed on S-Trap micro 1.7mL columns and centrifuged at 4,000g for 10 seconds. Columns were washed five times with 150µL S-Trap binding buffer and centrifuged at 4,000g for 10 seconds with 180-degree rotation of the columns between washes. Samples were digested with 2µg of trypsin (Promega) at 47°C for 1h. Peptides were eluted with 40µL of 50mM TEAB followed by 40µL of 0.2% aqueous formic acid and by 35µL 50% acetonitrile containing 0.2% formic acid. Peptides were finally vacuum dried.

High pH Fractionation

For library building, peptides were resuspended for high pH fractionation in 50µL of 0.1% TFA. Tips were homemade with one layer of empore disk C8 and 1mg of C18 (C18-AQ, Maisch). After washing and conditioning of C18, peptides were bound by centrifugation to C18 in acidic conditions. Peptides were

sequentially eluted in 8 fractions at basic pH (5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 50% ACN in triethylamine 0.1%). Eluted peptides were concatenated pairwise to obtain 4 final fractions (F1F5, F2F6, F3F7, F4F8). Samples were then vacuum dried.

Automated capillary immunoassay (WES)

Automated capillary immunoassay (Simple Western) was performed on a WES system (Protein Simple, San Jose, CA, USA). Akt (Cell Signaling Technologies) and Nck1/2 (Santa Cruz Biotechnology) antibodies were used at the 1:50 dilution. The analyses were performed on a 12–230 kDa Separation Module (SM-W004) according to the manufacturer's instructions.

NanoLC-MS/MS protein identification and quantification

Samples were resuspended in 35 µL of 1% ACN, 0.1% TFA in HPLC-grade water. For each run, 5 µL was injected in a nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000) (Thermo Scientific, Waltham MA, USA). Peptides were loaded onto a µ-precolumn (Acclaim PepMap 100 C18, cartridge, 300 µm i.d.×5 mm, 5 µm) (Thermo Scientific), and separated on a 50 cm reversed-phase liquid chromatography column (0.075 mm ID, Acclaim PepMap 100, C18, 2 µm) (Thermo Scientific). Chromatography solvents were (A) 0.1% formic acid in water, and (B) 80% acetonitrile, 0.08% formic acid. Peptides were eluted from the column with the following gradient: 5% to 40% B (120 minutes), 40% to 80% (5 minutes). At 125 minutes, the gradient returned to 5% to re-equilibrate the column for 20 minutes before the next injection. Two blanks were run between samples to prevent sample carryover. Peptides eluting from the column were analyzed by data dependent MS/MS, using a top-10 acquisition method. Peptides were fragmented using higher-energy collisional dissociation (HCD). Briefly, the instrument settings were as follows: resolution was set to 70,000 for MS scans and 17,500 for the data dependent MS/MS scans in order to increase speed. The MS AGC target was set to 3.106 counts with maximum injection time set to 200 ms, while MS/MS AGC target was set to 1.105 with maximum injection time set to 120 ms. The MS scan range was from 400 to 2000 m/z. Dynamic exclusion was set to 30 seconds duration. Three separate mass

spectrometry runs (i.e. technical replicates) were acquired for each biological replicate under the identical mass spectrometric conditions to account for instrument-related variability and to improve accuracy of the label-free quantification.

MS data processing and bioinformatic analysis

MS data processing and bioinformatics were done as previously described with some modifications (63). Briefly, raw MS files were processed with the MaxQuant software version 1.5.2.8 and searched with the Andromeda search engine against the human UniProt database (release May 2019, 20,199 entries). To search for parent mass and fragment ions, we set the mass deviation at 4.5 ppm and 20 ppm, respectively. The minimum peptide length was set to seven amino acids and strict specificity for trypsin cleavage was required, allowing up to two missed cleavage sites. Match between runs was allowed. Carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met) and protein N-terminal acetylation were set as variable modifications. The false discovery rates (FDRs) at the protein and peptide level were set to 1%. Scores were calculated in MaxQuant as described previously (63).

Statistical and bioinformatic analysis, including heatmaps were performed with Perseus software (version 1.5.5.3) freely available at www.perseus-framework.org. GO annotation was performed on Perseus software. Proteins for GO analysis were selected if annotated with the terms membrane, mitochondrion and nucleus in the Gene Ontology Slim Cellular Component database. RaftProt comparison was performed with the mouse database freely available at raftprot.org.

For the T-cell raft database, we used protein intensities to calculate a ratio between fraction 2 and the mean of the other fractions (fractions 3, 4, 5 and 6). Proteins with a ratio superior or equal to 2 were classified as “enriched in rafts” and proteins with ratio inferior to 2 were classified as “non-enriched in rafts”.

For the t-cell rafts analysis in resting vs activating conditions, we selected proteins based on the following criteria: proteins were detected in all 4 samples in one condition and completely absent in the other

condition. Proteins only identified by site, matching the reverse database and the potential contaminant database were filtered out. Selected proteins were identified with at least 2 peptides and at least 5 MS/MS to ensure robust identification of the proteins.

Proteome Discoverer ™ software (Thermo Scientific, version 1.4) was used to evaluate the number of identified proteins for the different precipitation tests.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD016476 and MS Viewer.

RESULTS

Membrane raft preparation

We optimized membrane raft preparation starting from T cells immunoselected from a single mouse. We aimed to perform biological experiments on single mice rather than on pooled mice in order to maintain higher statistical power. Membrane raft proteins were enriched by ultracentrifugation using an OptiPrep™ density gradient on a limited number of cells, (2 to 10 million) after a detergent-free cell disruption.

We optimized the method in order to collect raft proteins in a single fraction by adapting the volumes of fractions recovered after ultracentrifugation. As lipid rafts are enriched in flotillin-1, a protein that constitutes assembly sites for active signaling platforms (64), we first evaluated the distribution of flotillin-1 in the gradient fractions. As shown in Fig 1. a strong flotillin-1 signal was detected in the fraction 2, with lesser amounts in soluble protein-rich fractions 3 to 6, providing support that fraction 2 from the Optiprep™ gradient is actually enriched in the membrane rafts.

Proteomic analysis of membrane rafts following different sample preparation methods

In order to identify the proteins contained in the raft-enriched fraction (F2) by LC-MS/MS analysis, we performed a classic workflow based on filter-aided sample preparation FASP, as we previously described (62). However, we encountered an unexpected challenge in analyzing this fraction. FASP digested raft proteins yielded an inconsistent number of identified proteins and flotillin-1 was not detected at all, possibly due to Optiprep™ contamination interfering with mass spectrometry analysis. Indeed, a close analysis of the base peak chromatogram of the F2 raft fraction revealed the strong presence of a doubly charged ion m/z 775.47 corresponding to the Optiprep™ compound iodixanol (Figure 2A).

To remove this persistent contamination, we combined FASP with complementary cleaning-up steps before digestion. We conducted the optimization tests on two immortalized cell lines, human embryonic kidney HEK298 cells and mouse podocytes, which constituted a non-restricted starting material. In a first attempt we performed TCA precipitation before FASP digestion, but we could still not eliminate contamination. Also, we used an additional 3-hour long ultracentrifugation step on F2 fractions, in order to wash out contaminants and spin down membrane rafts, followed by TCA precipitation and FASP digestion. Optiprep™ was still present when we associated these three steps together (Supplemental Figure 2). Other types of digestion were performed including in-solution digestion and in-gel digestion, all of them leading to similar results (data not shown).

We decided to test a more recent sample preparation method, Suspension-Trap (S-Trap), based on suspension trapping filters that potentially facilitate the washing out of contaminants, such as Optiprep™ (65). We performed S-Trap either alone or in combination with TCA precipitation and with additional SDS in the lysis step. All combinations tested led to complete and reproducible elimination of Optiprep™ contaminants, with higher protein yields observed with the combination of TCA precipitation and S-Trap (Supplemental Figure 3). To verify the suitability of the procedure on low abundant samples, we applied this method to a 2 to 10 million T cells immunoselected from a single mouse spleen and we succeeded in consistently eliminating contamination (Figure 2B). TCA precipitation allowed to concentrate the sample

and resolubilize it in 5% SDS, which contributes to solubilize membrane proteins contained in the raft fraction. Furthermore, the S-Trap protocol revealed to be faster than the other tested methods, as well as easier to perform.

Purity of raft preparation and generation of a T-cell raft protein database

To further assess the purity of the raft-enriched fraction from non-stimulated T cells, we analyzed all Optiprep^{TM} gradient fractions by LC-MS/MS. We performed TCA precipitation with S-Trap digestion, and we performed high pH fractionation of the peptides for all 6 fractions in order to gain depth in the analysis. The number of proteins identified ranged from 137 to 4533, with 2680 proteins identified in fraction 2 (Figure 3A).

In order to determine the percentage of membrane, nuclear and mitochondrial proteins, a Gene Ontology Cellular Component analysis was performed for each fraction. In fraction 2, as expected, we observed a higher percentage of membrane proteins as compared to the other fractions (Figure 3B). We could identify with a high intensity count of 8e10 a number of proteins frequently used as raft markers: linker for activation of T cells (LAT) which is a raft marker specific to T-cells, Flotillin-1 (as confirmed above by Western-Blot) and Flotillin-2. All these three proteins were abundantly identified in fraction 2 and at a much lower intensity (50-fold less) in the other fractions (Figure 4A).

To establish a raft protein database by following as stringent criteria as possible, we assumed that raft-residing proteins should present the same intensity profile in gradient fractions as LAT, Flotillin 1 and 2, *i.e.*at least two times more abundant in fraction 2 compared to other fractions. Based on our LC-MS/MS data, we could establish a raft database of 894 proteins (in blue, Figure 4B). Those proteins not falling into these criteria were classified as “non enriched in protein rafts” (1875 proteins in red, Figure 3B, Supplemental table 1). We believe that these proteins can still be associated with rafts, but they are also or mainly present in cytoplasm, hence their profile in the gradient.

Our database containing 894 proteins was compared with the Raftprot database (3269 proteins, one-star experiment). Of note, 475 proteins from murine Raftprot overlap with our rafts T-cell database, although

the overlap increases to 669 when both human and murine Raftprot databases are used. Gene Ontology analysis revealed the presence of nearly 60 % of membrane proteins in our database, regardless of whether they are common to Raftprot or unique (Figure 4C, Supplemental Table 2). On the contrary, de group of proteins contained in F2 and classified as "non enriched in protein raft" database correspond to a ow percentage of membrane localized (28%). This Gene Ontology analysis provides support to consider the 894 proteins in F2 as true raft proteins and could be used as a reference database for non-stimulated T-cells. To determine the most significantly enriched pathways and cellular location for the 894 proteins in our raft database, we performed a Fisher's exact test using Keywords and KEGG pathways. The analysis showed a clear enrichment in membrane proteins and related membrane protein pathways as compared to all the proteins identified in all fractions (Supplemental Table 3).

T-cell rafts protein analysis in resting *vs*. activating conditions

To verify the suitability of the method on low abundant samples purified by Optiprep gradient, we used T cells from 4 single Balb/c mice. After negative immunoselection (yield between 2 and 8 million cells), T cells from each mouse were split into two groups: one group (n=4) was not stimulated (T0 minutes) and the other group (n=4) was stimulated for 15min (T15 minutes) with anti-CD3 and anti-CD28 antibodies. Next, we performed membrane raft preparation, TCA precipitation and S-Trap digestion on gradient fraction 2 and analyzed proteins by LC-MS/MS. The number of identified raft proteins significantly increased in activated T-cells compared to resting T-cells (Supplemental Figure 4A). Likewise, the abundance of FLOT1 wasalso significantly increased in activated T-cells (Supplemental Figure 4B), suggesting that the abundance of rafts is increased in these cells. However, no significant differences were obtained after label-free-based quantitation between the two conditions (t-test, FDR=0.01). We therefore proceeded with a manual selection of proteins being exclusively present in rafts either in stimulating and non-stimulating conditions. This selection was based on the detection in rafts in one condition in all 4 samples and non-detection in rafts in the other condition in at least 4 samples. A heat map showing the selected proteins is presented in Figure 5 (Supplemental Table 4). Three proteins were found present in

the raft fraction selectively at T0, while 39 proteins were selectively detected in rafts at T15. Some of these proteins are known to be involved in T-cell activation at the raft level. Among them, we found as the most relevant in the context of T cell activation and raft dynamics the following: Akt2, Nck1, TgfB1, Tbc1d10, Pdlim1, and Intesectin-2. The TGF-β ligand TgfB1 is a key molecule involved in cell signaling regulating T cell activation and polarization. Akt2 is a key player in the proximal signaling events participating in TCR activation. Nck1, is an adaptor protein involved in actin cytoskeleton remodeling. Tbc1d10 is an activator of the Rab35 GTPase. Pdlim1 has a role in the regulation of actin cytoskeleton networks. Intersectin-2 (Itsn2) is known to participate in TCR internalization.

In order to validate the mass spectrometric results, Akt2 and Nck1 were selected for capillary western immunoassay, (WES) a method that allows detection of very small amounts of proteins (few micrograms). WES analysis demonstrated the effective recruitment of Akt2 and Nck1 in rafts at T15, which is consistent with our mass spectrometric data (Figure 6).

Altogether, these data demonstrate that the described method for Optiprep^{TM} gradient raft isolation and S-Trap protein preparation is suitable for the differential analysis of low abundant raft proteins in T cells.

DISCUSSION

The present work shows, for the first time, a global analysis of the membrane raft proteome of ex-vivo T cells isolated from individual mice. Moreover, we propose a method that combines several improving features, such as the isolation capacity of OptiPrep-based density gradient, a straightforward sample preparation (S-Trap) and a label-free quantitation, globally providing a high yield of identified proteins.

The first isolation of proteins from T cells contained in triton-insoluble membranes was done back in the nineties (66). Given the early understanding that membrane rafts play a fundamental role in T cell activation (67-71) , knowing the protein composition of these microdomains has represented a key objective to understand their structure, dynamics and function. A pioneering characterization of the proteome of T cell membrane microdomains was performed on the leukemia cell line Jurkat T (20) by

LC-MS/MS, while the first study of T cell proteome in activating conditions was approached by Flotillin-1 immunoprecipitation (27) and mass spectrometry.

Gel electrophoresis-based approaches were among the first used to address this question. For example, 1D and 2D gels with staining and mass spectrometry have been used to resolve and identify proteins associated with TCR activation complexes in Jurkat cells (20). 2-D fluorescence difference gel electrophoresis (DIGE), based on differential fluorochrome labeling, has been applied to study lipid rafts proteins after TCR co-stimulation (22). Bini et all succeeded in studying the variation in intensity of more than 800 spots in Jurkat cells subjected to TCR costimulation for up to 15 min. using a classic 2D gel electrophoresis and MALDI-TOF setting (20). In the same experimental conditions, von Haller and colls. used a gel-free method based on LC separation (25).

Detergent solubility has been the landmark technique of raft (or DRM, for detergent resistant microdomain) isolation (20, 25, 26). In our study, a detergent-free method has been used, in which mechanical disruption of membranes is performed, with a high enrichment of raft-like fractions in raft protein markers. Any study on membrane rafts must always be taken cautiously, since cellular membranes may contain a broad range of microdomains that can differ in composition, function and dynamics (72). The experimental purification approach in all cases will determine the type of microdomain that is targeted. In our work we have chosen a procedure that allows the purification of low-density membrane fragments highly enriched in bona-fide T-cell raft markers, such as flotillins, caveolins, and LAT. The high yield of RAFTPROT-listed proteins strongly suggests that the microdomains described in this study are close to those existing in living cells. Nevertheless, alternative methods will be needed to validate the results obtained in further functional studies on T cells.

Iodixanol, the chemical component of Optiprep, was developed as an efficient and rapid way to isolate rat liver peroxisomes (73). Later, it replaced other density gradient procedures in the purification of subcellular structures, including rafts (36, 39, 40, 61, 74-86). The only report to date of a proteomic study following iodixanol-based raft isolation is that of Rose and colls. (61), in which DRM from virus-infected

plankton species *E huxleyi* were subjected in parallel to lipidomic and proteomic characterization. The authors reported the identification of 116 proteins from the infected and 86 proteins from control cells. Being fastness the main advantage of iodixanol with respect to sucrose gradients, its limiting characteristic is the spectral interference with protein identification by mass spectrometry, resulting in a limited and variable number of identified proteins. This drawback has limited proteomics on raft fractions isolated by this method.

Suspension trapping or S-trap as a preparation technique for bottom-up proteomics applied to low protein amounts was recently developed for cell lysates, membrane preparations and immunoprecipitates (65). It has been shown as more efficient than other methods such as direct FASP and in-solution digestion, and compatible with common extraction buffers and detergents (87-89). Very recently S-trap has been applied in the study of mouse brain microglial proteome (90) and in the search for oral squamous cell carcinoma biomarkers in saliva (91). Here, we have applied for the first time S-trap to T cell proteomics and to the analysis of a sub-membranous fraction (rafts). Guergues and colls. succeeded to identify and quantify nearly 4700 proteins in microglial cells from one single mouse (90), which represents a number comparable to that obtained in the current work, but from an even smaller number of cells (300,000 vs. 2 million). Even if the greater size of microglial cells as compared to lymphocytes could account for this difference, this suggests that our method could be further optimized to the analysis of rafts from T cell subpopulations. Due to the multiple advantages of this technique, it is likely to consolidate as a reference procedure in bottom-up proteomics in the years to come.

Interestingly, in our study comparing non-stimulated cells with cells stimulated with anti-CD3/CD28 antibodies for 15 min, label-free quantitation did not result in any statistically significant difference. It could be argued that this is due to the fact that 15min of stimulation might be a too long lapse to find subtle protein recruitment changes in rafts. In fact, although the immunological synapse can be assembled within seconds or a few minutes, costimulation of TCR provides a more sustained response and raft redistribution is observable after at least 20min (1). Another potential explanation is the fact that TCR

stimulation induces mostly a clustering and reorganization of existing rafts, along with their resident proteins rather than a new recruitment of proteins. Most likely, our results are due to the combination of both hypotheses. Nonetheless, several proteins were found specifically present or absent in rafts after T cell activation.

Among the 39 proteins we identified selectively recruited in rafts after 15min of TCR stimulation, we found some that are known to play a role in T cell function and/or in raft dynamics: Akt2, Nck1, TgfB1, Tbc1d10, Pdlim1, Intesectin-2 and Cherp. Akt2, a member of the PKB/Akt family, plays a key role in in the signaling events leading to activation, differentiation and survival of Jurkat and human primary T cells after TCR activation (92-96). Akt is recruited at the plasma membrane upon TCR activation via the interaction between its pleckstrin homology domain and membrane phosphoinositides and phosphorylated by PKC at Ser473 (97). The presence of Akt2 isoform in rafts has been specifically reported in intestinal cells (98) and platelets (99).

The adaptor protein Nck1 is also known to interact with the TCR via its SH3.1 and SH2 domains and thereby participates in T cell activation via Erk and MEK phosphorylation (100-102). Its presence in T cell rafts has not been reported to date. Conversely, it has been found in non-raft compartments in human neuroblastoma cells and embryonic cortical neurons (103).

The increased presence of the TgfB1 ligand in raft-like domains strongly suggests the presence of the cognate receptor. The TGF-β receptor has been associated with lipid rafts in previous reports (2). Most interestingly, it has been shown that TCR activation induces the recruitment of TGF-β receptor in rafts, subsequently inhibiting SMAD signaling and ultimately resulting in Th1 and Th2 differentiation (3). Intriguingly Strap (Serine-threonine kinase receptor-associated protein), known to interact with both TGF-β receptor and PDK1, and to inhibit TGF-β-dependent signaling (4), is also present within the proteins found increased in rafts in the current study. Strap has never been reported as associated with rafts or TCR activation, which evokes a complex subjacent regulatory mechanism.

Other proteins of interest include Tbc1d10, an activating protein of the GTPase Rab35. Rab35 participates in the formation of the immunological synapse, by regulating TCR transport. Tbc1d10 and Rab35 colocalize with TCR in Jurkat T cells, and knockdown of the former inhibits immunological synapse relocalization of the latter (5). Pdlim1 is involved in the formation of actin networks and could play a role in the structuration of lipid raft clusters characteristic of immunological synapse. Pdlim1 expression is increased along with that of caveolin in cells treated with dexamethasone (6), while its role in T cell activation is currently unknown. Intersectin-2 is a recently described protein participating in TCR internalization, via association with Wiskott Aldrich Syndrome protein (WASp) and Cdc42, with maximum interaction with the former at 10min post TCR activation (7). Internalization of TCR is a key step in the regulation of T cell activation. It involves actin cytoskeleton rearrangement and clathrin-dependent endocytosis. Intriguingly, Intersectin-2 is found here recruited in lipid rafts, whereas it has been reported to be involved in non-raft-dependent endocytosis (7). Finally, the calcium homeostasis endoplasmic reticulum protein Cherp is likely to play a role in the activation and proliferation of T cells, as its knocking down has been found to impair those processes in Jurkat cells (8). Although its localization to rafts has never been reported before, this finding is intriguing as Ca2+ levels are known to rise rapidly following TCR engagement.

In line with the calcium homeostasis events characteristic of TCR stimulation, one of the 3 proteins excluded from rafts at 15min post-activation, according to our results, was the potassium calcium-activated channel 4 (KCa3.1 or Kcnn4). This transporter, shown to be associated with rafts upon cell swelling (9), is critical for Ca2+ influx associated with T cell activation, and so its inhibition blocks T cell activation (10). Another raft-excluded protein, the tyrosine kinase Irak4, has been shown to participate in TCR activation. In Jurkat cells Irak4 was found to relocalize to rafts after stimulation with anti-CD3 antibodies, and to induce downstream signals including PKC and NF-κB activation (11). Irak4 was found in rafts in our unstimulating conditions, like in the previous report by Suzuki and colls., but to our surprise, it would disappear from those fractions after stimulation instead of being increased. These

unexpected and somehow contradictory results could be due either to the different cell models (mouse T cells vs. Jurkat) or to the differences in raft isolation procedures and, subsequently, in the kind of microdomains obtained. The exclusion of these two proteins from rafts 15min after TCR stimulation warrants a closer study of their raft localization dynamics and functional implications.

In conclusion, our work shows the relevance of an S-Trap-based methodological strategy to deepen into the molecular mechanisms that govern T cell activation. We also provide a new database of T cell raft proteins. Our approach can be applied to other aspects of the biology of T cells and other cell types involving membrane rafts.

Data availability statement

All data are contained either within the manuscript or as supporting information. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016476.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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