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CD proteome and beyond – technologies for targeting the immune cell surfaceome

Thomas Bock [1,2,3], Damaris Bausch-Fluck [1,2], Andreas Hofmann [1,2], Bernd Wollscheid [1,2]

[1]National Center of Competence in Research (NCCR), Neuro Center for Proteomics, UZH/ ETH Zurich, Wolfgang-Pauli-Strasse 16, 8093 Zurich, Switzerland, [2]Swiss Federal Institute of Technology (ETH) Zurich, Department of Biology, Institute of Molecular Systems Biology, Wolfgang-Pauli-Strasse 16, 8093 Zurich, Switzerland, [3]ZNZ International PhD Program in Neuroscience, University of Zurich (UZH) / ETH Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

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1. ABSTRACT

Communication between cells of the immune system and the organism is dependent on information processing mediated by proteins of the cell surface. The cell surface proteome consists of a group of functionally diverse proteins, which not only enables but also limits the interaction capacities of cells within their particular microenvironment. Although these proteins represent a highly important proteome for immunological research, most routinely used technologies for their detection only allow for a fragmented view of the ensemble of cell surface located proteins. A major bottleneck is the limited availability of high quality antibodies against cell surface protein targets that altogether impedes a Systems Biology view on the cell surface proteome (surfaceome) and its concerted functions during signal processing. Recent developments in mass spectrometry-based technologies enable now complementary approaches for the qualitative and quantitative analysis of the surfaceome. Here, we highlight recent progress in the field towards the identification and quantification of the surfaceome as an important subproteome forming the information gateway of the cell.

2. INTRODUCTION

2.1. Information processing by cell surface proteins during the immune response

Immunologists have always been fascinated by the capability of the innate and adaptive immune system to successfully respond to pathogenic threats (1), regardless of their multifaceted origins. The immune system has evolved multiple defense mechanisms (2, 3) which require a complex interplay between leukocytes derived from the hematopoietic system. Leukocytes are dedicated to a variety of specific immune functions such as the recognition of pathogens and the triggering of an immune response (4) or effective pathogen neutralization (1).

The concerted response to pathogenic challenge requires a complex information exchange and efficient signal processing machinery on the tissue, cellular and protein level of the immune system. The field of Systems Immunology strives to gain an integrated overview on the interplay of these different network levels to understand immune function (5). However, a prerequisite for being able to predict and to model immune function is the qualitative and quantitative identification of all molecular

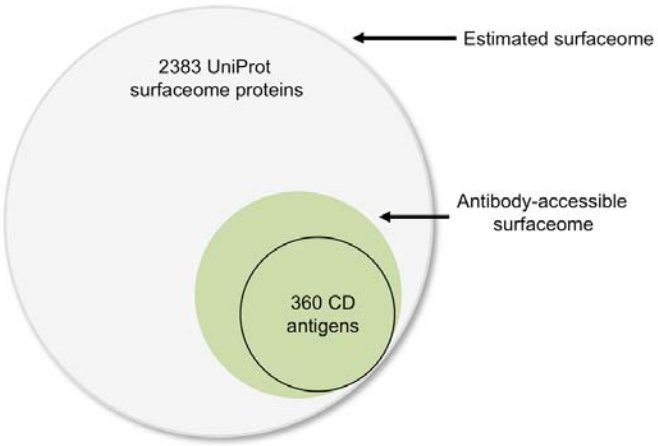

Figure 1. Current coverage of the immunological surfaceome. At least 2383 proteins are estimated from current UniProt annotations to comprise the pool of proteins that possibly constitute the surfaceome of immune cells (large grey area). Specific identification and quantification is currently restricted to a subset of cell surface proteins due to limited antibody availability (green area) against these targets. The pool of currently available antibodies consists out of antibodies from a subset of cluster of differentiation (CD) certified antibodies and other commercially available antibodies, which are often only validated for one application (FCM, IF, IHC, or WB).

and cellular players of the system, as well as an understanding of their interactions in time and space (5).

The proteins expressed on the cell surface are of particular interest to (systems) immunologists (6). Due to their prominent location they act as the main signalling mediators at the cellular interface to the microenvironment. The cell surface proteome, or surfaceome (7), plays a central role in the different major steps of immune defense such as the correct sensing of information and the instruction and guidance after triggering of immune responses (1, 2, 4, 8-10). Furthermore, many of the currently known surfaceome proteins are vital for cellular communication and for the correct functioning of immune cells within the immune system (11-13).

In order to gain a mechanistic understanding of the role of the immune cell surfaceome the identification, quantification and functional characterization of all members would be therefore highly desirable (5). Here, we will discuss the current challenges in immunological surfaceome research and highlight recent technological advancements which will eventually provide the research community with a comprehensive immune surfaceome map and additional tools to navigate this map.

2.2. Challenges in the detection of cell surface proteins by classical immunological tools

The study of the large pool of cell surface proteins still is a challenging task today. In terms of numbers, approximately a quarter (5539) of all human protein-coding genes are predicted as membrane domain containing proteins (14). While this number still includes proteins embedded within intracellular membranes, a current estimation of the number of human cell surface proteins which could be located at one point in time or another at the cell surface is at least 2383 based on a recent UniProt protein database annotation (keyword search "cell membrane", protein status: reviewed) (15, 16). This translates into the fact that approximately 10% of the UniProt described human proteome are potential members of cellular surfaceomes and can be utilized by different cell types depending on their transcriptional profile to modulate signalling capacity at the cell surface information gateway. The resulting modulations in cell surface protein expression have traditionally been used to determine different cell types, which has also been shown in a recent study that compared the transcriptional profile and the protein expression in three functional different human cell lines (17). Here, Uhlen *et al* found that most cell type specific proteins are highly enriched for proteins of the cell surface, which emphasizes the need for a more comprehensive description of the surfaceome.

Although initially helpful, transcriptional profiles do not reveal directly the identity and quantity of proteins (18, 19) or the protein location at a particular time point which is needed in order to obtain accurate information about the current status of the surfaceome, including its post-translational modifications. However, the direct detection and quantitation of the surfaceome by protein-centric methodology is a challenging task. Most modern research tools in immunology that have the sensitivity to detect cell surface proteins use antibody-based technologies. Historically, cellular antigens that currently constitute known markers of cellular classification are derived from the Cluster of Differentiation (CD) knowledgebase (http://www.hcdm.org/). The CD community built a quality controlled repertoire of affinity reagents against cell surface markers with a particular focus on cells of the immune system (20). The CD nomenclature is for example used to describe and sub-classify haematological malignancies (21-23). Although the currently 360 CD annotated molecules and characterized antibodies against these targets are a great resource, only a minor subset of the estimated 2383 cell surface proteins can be currently detected in application specific manner through antibody-based probes of varying quality (Figure 1). Furthermore, the epitopes recognized by these antibodies are for the most

part not exactly known. New technologies for epitope mapping (24) and dedicated epitope databases, such as AntiJen or BciPep might close this knowledge gap (25, 26). An effort to create quality controlled antibodies against every human protein is currently well under way in Sweden in form of the Human Protein Atlas (27, 28).

There are several reasons for the limited availability of high quality antibodies against proteins of the cell surface. Classical hybridoma technology for the generation of target specific monoclonal antibodies in mouse (29, 30) or rabbit (31) involves the immunization with the target protein and strongly depends on the availability of immunogenic protein epitopes. While epitope availability is generally difficult to predict in advance, the physico-chemical properties of cell surface proteins often challenge the production and purification even before immunization. Being (multi)-transmembrane domain containing proteins, the resulting hydrophobicity complicates the solubilization, purification and proper handling. While there are promising phage and array-based technologies (32, 33), as well as the recombinant protein epitope signature tags (PrESTs) technology (34) emerging, the measurement of the cell surface proteome of the immune interface by specific antibodies remains a challenging task today. The fact that high quality CD antibodies (20, 35, 36) for only 360 cell surface proteins and a limited number of high quality commercial antibodies for the currently estimated 500 leukocyte described cell surface molecules (6, 36) exist, highlights the need for complementary technologies for the comprehensive characterization of the surfaceome.

A second aspect towards an integrated understanding of the cell surface protein information gateway is the need for the parallel and unbiased detection of cell surface proteins. The capacity for simultaneous measurements using antibodies is mainly application dependent. Although most antibody-based technologies, such as flow cytometry (37-39), are highly sensitive and able to measure single cell level quantities of protein abundance, the number of proteins which can be analyzed simultaneously by flow cytometry in a single experiment is generally limited up to a dozen with specialized expertise. However, technological advancements in the antibody microarray field demonstrate potential towards a more extended, parallel immunophenotyping of living cells by known surfaceome classification markers. For example, live melanoma cell suspensions were profiled for general leukocyte markers and additional markers of disease development, metastasis, progression and clinical prognosis by establishing an extended antibody microarray (40). For this purpose, the melanoma cells were captured on nitrocellulose slides pre-printed with an antibody panel that was mostly derived from the CD collection of antigens. It has been proposed that the resulting melanoma binding pattern may reveal antigen signatures for different melanoma subtypes or may provide detailed insight into the prognosis of the disease (40). Although these antibody arrays are already very useful for clinical prognosis of disease, the lack of high quality affinity reagents for the majority of cell surface proteins enable only a peek preview of the complexity of the surfaceome.

Recent progress in the development of mass spectrometry (MS)-based proteomic technologies enable now the implementation of strategies for the discovery-driven identification of the surfaceome. A large toolbox of proteomic technologies for the system wide analysis and the quantitation of proteins by MS was developed over the past ten years (41-43). Without question, the progress in unraveling the proteome using MS-based approaches is striking. Recently, a proteomic study revealed previously unknown differences in viral pattern recognition pathways between subsets of mouse dendritic cells with the analysis of over 6000 cellular proteins (44). However, currently available technology is still not capable yet to reveal the complexity of expressed proteins and their dynamic range within an immune cell in a single experiment. Therefore, tailored MS-based strategies were developed which enable the in-depth analysis of the variety of cellular subproteomes such as the mitochondria, golgi, nuclei (45, 46) and cellular membranes (47, 48).

In the next section we will highlight advances made in the field of MS-based proteomics towards the comprehensive detection of the surfaceome as a dynamic information gateway of the immune cell that is required for an integrated understanding of the immune system.

3. A PROTEOMIC VIEW OF THE IMMUNOLOGICAL SURFACEOME

3.1. Mass spectrometry-based proteomic technology

The term “proteome” (49, 50) is defined as the composition of the whole protein content of an organism, including the large variety of post-translationally modified proteins, functionally distinct multiprotein complexes and the different protein isoforms that exist at a given point in time (51). As translated and processed proteins in immune cells are of particular interest for signal transduction and immune function, MS-based proteomics developed into an important research tool for immunologists over the course of the last decade. Today, the field of proteomics provides biologists with a rich toolbox (52-54) of workflows that can be incorporated in the design of experiments geared towards gaining biological knowledge at the protein level.

One key advantage of MS-based proteomic studies is the ability to study the functional “end-product” of gene expression in comparison to studies on the transcriptome and genome level. Apart from the possibility of quantitative measurement of protein abundances, proteomic technologies further enable the determination of post-translational modifications, such as the glycosylation or phosphorylation status of a protein (54, 55). Combined with biochemical pre-fractionation workflows or chemical tagging strategies proteomic technologies can provide experimentalists with specific information about the location of proteins within a cell or within a protein complex, adding additional value to MS-based proteomic studies (43).

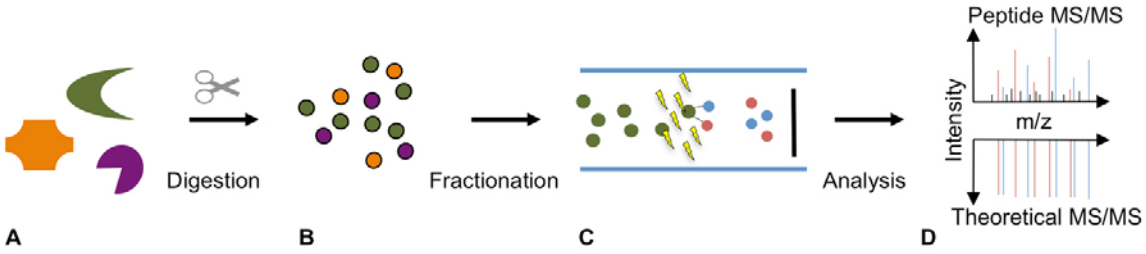

Figure 2. Common MS-based proteomics workflow. A: Proteins are processed into peptides by proteolytic digestion. B: The complexity of the peptide mixture is reduced by reversed phase C18 chromatography before MS analysis. C: Peptide masses and peptide fragment ions are analyzed by MS. D: Fragmentation spectra are computationally matched against theoretical spectra derived from protein databases for sequence identification.

MS-based proteomics experiments are typically focused on the identification of peptides (Figure 2). Upon a proteolytic protein digest by sequence specific proteases such as trypsin, peptide sequences of about 5-25 amino acids in length can be analyzed efficiently using current mass spectrometric workflows (43, 56). The digest of a full-length protein into many small peptide sequences offers two advantages for the protein analysis by MS. First, the small peptides share relatively similar physical and chemical properties compared to proteins. Therefore, the MS-based workflow becomes less prone to characteristics that make classical biochemical protein analysis a sometimes tedious and challenging task (stickiness by protein domain interaction, hydrophobicity, precipitation). Second, the protein identification by MS requires only the measurement of one proteotypic peptide in order to establish the identity of the protein group. A proteotypic peptide is defined here as a peptide, which is unique for a particular protein in comparison to all other proteins within a reference proteome based on a protein database, such as UniProt (15, 16). Further peptide identifications for the same protein ultimately lead to higher sequence coverage and to increased confidence in the specific identity of the full length protein, for example in case of a particular isoform of a protein. Therefore, the availability of a large variety of peptides after protein digestion increases the overall chances for a positive and specific protein identification (56). It is important to point out, that this protein identification scheme is based on the MS identification of several structural features, eg. peptides, of one protein in contrast to most monoclonal antibody (mAB)-based technologies, which establish the presence or absence of a protein solely based on the recognition of one short epitope. In analogy, in order to reach a similar quality of protein identification with mAB as in an MS experiment, antibody-probes for each proteotypic peptide would have to be generated.

An important ability of MS-based proteomic workflows is the capacity for identification of hundreds of proteins in parallel out of the complex sample, which enables systems immunologists to follow protein network changes in a discovery-driven fashion. However, the analysis of complex protein mixtures by MS still has certain limitations. The dynamic range of proteins expressed in a cell, which for example in yeast already covers at least six orders of magnitude (57, 58) which precludes the analysis of the complete cellular proteome in a single experiment (43, 55). Furthermore, current MS instruments are limited in their scanning speed, which enables only the identification of a subset of the peptides in a sample, but not all of them. The limited dynamic range of detection is for example a particular problem for the investigation of lower abundant protein species, such as G-protein coupled receptors within the surfaceome. More abundant cytoplasmic proteome "contaminants", such as structural proteins like tubulin, or mitochondrial proteins, often interfere with the successful detection of lower abundant cell surface proteins. A further issue, which contributes to the low detection rate of cell surface proteins within a complex mixture, is the hydrophobicity of membrane proteins, which confers poor solubility in aqueous solutions commonly used for sample preparation prior to MS analysis. And lastly, it is difficult to separate plasma membrane proteins from intracellular membrane proteins for their selective identification due to their similar biophysical protein properties. Therefore, for the identification of a particular subproteome such as the surfaceome, strategies for specific surfaceome enrichment and reduction of sample complexity have to be implemented into analytical workflows.

A number of excellent workflows for protein sample pre-fractionation and enrichment have been developed over the past few years. We will discuss in the following some of the recent developments in workflows combining pre-fractionation and protein enrichment workflows with MS-based proteomic strategies towards the more comprehensive and parallel analysis of the immune cell surfaceome.

3.2. Biochemical cell surface protein enrichment strategies

The quest for the comprehensive analysis of the surfaceome by MS makes subproteome enrichment and sample pre-fractionation currently a necessity. As cell surface proteins share common characteristics with proteins from other cellular organelle membranes, purity is difficult to achieve, but necessary in order to draw biological conclusions. Although we will briefly discuss some of the most common membrane purification strategies that were used for the enrichment of the surfaceome in combination

with MS-based analysis, we further refer to two excellent reviews for a detailed review of biochemical membrane protein enrichment strategies (47, 48).

Common approaches for the enrichment of membrane proteins are density gradient and differential centrifugation strategies that separate larger membrane patches from the bulk of cellular material by shape and size (59, 60). Typically, cells are homogenized and cleared of nuclear debris with a first centrifugation step before the retrieval of membrane particles by subsequent ultracentrifugation. Membrane particles can be further resolved by the use of continuous or discontinuous density gradients (47). Furthermore, pre-enrichment of membrane particles by immune-magnetic beads before ultracentrifugation has been successfully demonstrated for the purification of plasma membrane proteins derived from dendritic cells (61). The ultracentrifugation technology is a fast and simple method for membrane particle enrichment and was therefore adapted for various subcellular fractionation strategies, although its moderate yield and limited surfaceome specificity typically requires additional steps for cell surface proteome enrichment.

Two other enrichment strategies utilize affinity purification of plasma membrane proteins for surfaceome analysis by MS. The first method enables the isolation of the target proteins through the selective labeling of specific amino acid residues within the protein backbone. Proteins residing on the cell surface are covalently labelled with biotin-containing reagent and are further affinity purified by solid phase-coupled streptavidin enrichment (62). Typically, such labeling reagents consist out of a biotin-moiety, a chemical linker und and a reactive group for specific interaction and covalent attachment to the targeted protein (63). One of the commonly used reagents for cell surface biotinylation is Biotinyl-NHS-Ester (NHS-Biotin), which specifically labels primary amines found in lysine residues or the N-terminus of proteins. Biotinyl-ester generally are water-insoluble and membrane permeable (47), which should be considered before the choice for a suitable labeling reagent. Sulfo-NHS-esters have been reported to feature better water-solubility and may be therefore preferable for cell surface proteome analysis (63). As in common with other membrane enrichment tools, the leaking of the labeling reagent into the cell and tight associations between membrane and cytoplasmic proteins (64) account for cross-contaminations and impurities during the surfaceome analysis. Certainly an advantage of cell surface biotinylation is the applicability for *in vivo* biotinylation studies as was shown for the discovery of novel therapeutic targets in tumor mouse models (65).

The second affinity-based method exploits the overall net negative charge of the plasma membrane, which allows for the attachment of cationic colloidal silica beads via electrostatic interaction to the cell surface (66). Living cells are first coated with silica beads that are further cross-linked by poly-acrylic acid, which provides membrane sheets after cell rupture (66). After separation of the membrane sheets by centrifugation, the membrane proteins are recovered by elution with detergent (67). The applicability of the silica coating particles for *in vivo* surfaceome analysis was demonstrated by the perfusion of rat lung vasculature and the successful enrichment of the endothelial cell plasma membrane proteome (67). Similar to membrane biotinylation impurities, cross-contaminations can occur while studying the surfaceome with the silica bead method, but the applicability to *in vivo* settings make both, the membrane biotinylation and the silica-bead method attractive protocols for the study of surfaceome proteins.

3.3. Carbohydrate-based affinity enrichment strategies

Glycosylated proteins are frequently used in diagnostic biomarker discovery strategies (68-70) and as potential diagnostic and therapeutic targets for clinical intervention (6). Extracellular glycosylation is a co-translational protein feature of the majority of proteins expressed on the cell surface and secreted molecules (71, 72). Protein glycosylation assists in protein folding and provides an important structure for a variety of cellular functions (73), including receptor-ligand and cell-cell interactions of the immune system.

Glycostructures can be co-tranlationally attached to three different amino acid residues within the protein backbone: serine (S) and threonine (T) in the case of O-linked glycosylation and asparagine (N) for N-linked glycosylation (74). While most N-linked carbohydrates are located within the site specific N-!P-S/T motif, where !P resembles any other amino acid than proline (P) followed either by a serine (S) or a threonine (T) (75), a general consensus sequence around the O-glycosylation attachment site is lacking. Recently, the less common N-glycosylation motives (N-x-C, N-G and N-x-V) have been verified experimentally (76). The existence of a common consensus sequence and the further availability of site-specific endoglycosidases (such as PNGaseF) for general deglycosylation of N-linked carbohydrates have so far favored the study of N-linked glycosites compared to their O-linked counterparts. Therefore, we will further focus on two N-glycosylation-based enrichment strategies that are currently applied for the analysis of the immune surfaceome.

3.3.1. Lectin affinity-based glycoprotein enrichment and analysis

Lectins are belonging to the carbohydrate binding family of proteins (77) and have diverse functions in the mediation of immune cell interactions (78). Their ability to bind glycoproteins was successfully exploited in applications for binding, enrichment and purification of N-glycopeptides out of complex lysates of *Caenorhabditis elegans* (79, 80), human blood serum (81, 82) as well as human platelets (83) and subsequent identification by MS-based proteomics. Due to the selectivity of individual lectins towards specific carbohydrate structures, often combinations of several lectins, like for example the commonly used concanavalin A (specificity for mannose) and wheat germ agglutinin (specificity for N-acetylglucosamine), are applied to tag and extract a larger pool of the glycoproteome (84, 85). Such a multi-lectin affinity approach was shown more recently in a

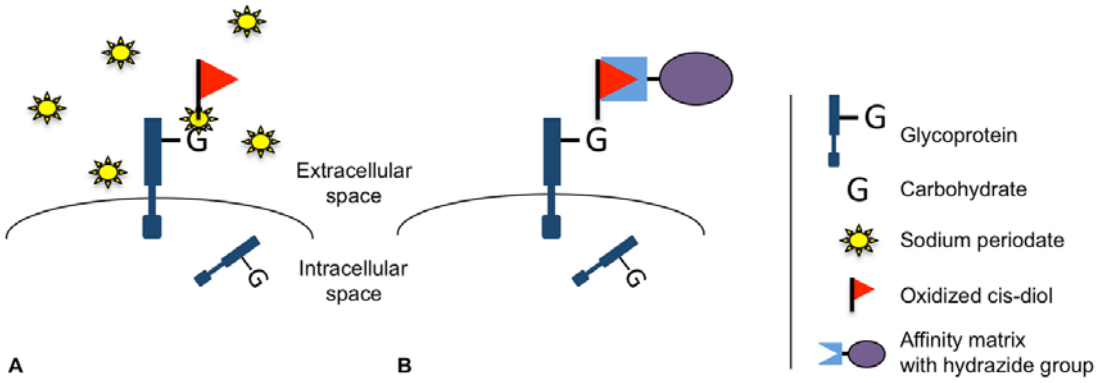

Figure 3. Location specific labeling of the cell surface exposed proteome by cell surface capturing. Temporal separation of the two-step labeling procedure preserves the specificity for currently exposed cell surface interface proteins. A: Step 1 (flagging): mild oxidation of carbohydrates (G) attached to the extracellular part of an integral membrane protein on the living leukocyte. Internal glycoprotein pools are not affected by the procedure. B: Step 2 (labeling): after removal of oxidant, only flagged carbohydrates react with the affinity reagent biocytin-hydrazide which is used for subsequent purification (Biotin-tag) after proteolytic digest. Labeling can be carried out on cellular or protein lysate level.

thorough investigation of the mouse N-glycoproteome (76). In this large mapping study on N-glycoproteins obtained from four different mouse organs and blood plasma revealed over 6000 mouse N-glycosites derived from a large variety of membrane (Golgi, endoplasmatic reticulum, lysosome and surface membrane) and secreted proteins by multi-lectin affinity.

The lectin-affinity based strategy depends on its selectivity for specific compositions and conformations of different carbohydrate groups present on the targeted glycoprotein pool (47). Therefore, combinations of different lectins need to be applied during affinity enrichment in order to cover the glycoprotein pool as comprehensively as possible. In turn, the use of selective lectins allows for the retrieval of cell surface protein pools with similar glycostructures.

3.3.2. Chemical enrichment and analysis of N-glycopeptides

Apart from lectins, selective chemical tagging of glycoproteins can be used for the enrichment of the surfaceome. Mild oxidation of cis-diols present within protein-carbohydrate structures (86) leads to the generation of new aldehyde groups that can subsequently be used for covalent tagging of these modified glycoproteins with affinity matrices. Affinity matrices include, for example, immobilized hydrazide beads, which allow for the formation of covalent hydrazone bonds to a solid support and the subsequent enrichment of N-glycopeptides, as it was first demonstrated by the targeting of N-glycoproteins in human blood serum (87). The solid phase extraction of N-linked glycopeptides (SPEG) method (88), based on hydrazide resin, has been demonstrated to yield a high specificity and sensitivity through the specific reduction of analyte complexity towards the lower abundant N-glycoproteins of interest (88, 89). The SPEG approach was further applied to the capture of whole N-glycoproteins (83, 90). Recently, the compatibility of SPEG to magnetic bead capture resins has been investigated promoting the applicability for discovery clinical proteomics (91).

In order to gain information about the pool of glycoproteins present on the cell surface at a particular time, the N-glycocapture technology was further developed and enabled the specific detection and relative quantification of cell surface proteins on neural stem cells and lymphocytes (92). The Cell Surface Capturing (CSC) technology (92) enables the tagging of glycoproteins present on the cell surface of living cells within their microenvironment in two independent steps which confer specificity. First, the exposure of living cells to mild oxidative conditions “flags” only the relevant proteome of interest, the surface exposed glycoproteome; second, the temporal separation of the ensuing labeling of previously oxidized glycosites through a bi-functional biotinylation affinity reagent (biocytin hydrazide) (93) ensures the exclusivity of tagging for cell surface exposed glycoproteins (Figure 3). During the subsequent streptavidin-based affinity purification a high degree of specificity for biotinylated glycopeptides is achieved by capturing on the peptide level after proteolytic protein digestion.

First applied for the identification on cell surface interface proteins on human Jurkat T cells, the CSC technology has been proven to simultaneously detect a wide variety of cell surface proteins with remarkable selectivity for N-glycopeptides (approximately 95%) and sensitivity down to low abundant G-protein coupled receptor molecules (92). The ability to detect all types of cell surface proteins independent of CD annotation, protein features (multi-transmembrane) or protein functions (phosphatases, kinases, receptors or adhesion molecules) was furthermore demonstrated in a variety of studies (92, 94-96). The usefulness of CSC technology for tracking cellular differentiation by monitoring cell surface molecular phenotypes was further shown in an acute myeloid leukemia model that was differentiated into mature

granulocyte-like cells using all-trans retinoic acid treatment (95). Over 500 detected proteins of the surfaceome (including 137 CD annotated proteins) were monitored for change in abundance after treatment, revealing known and new markers of granulocyte differentiation by hierarchical cluster analysis. Increased protein coverage was gained by additional capture of disulfide-bridge linked "piggyback peptides" covalently bound to the N-glycopeptides during enrichment (95). The CSC technology, similar to the other technologies described, can be combined with quantitative MS-based workflows for the relative quantification of abundance changes within the surfaceome using SILAC technology (92, 95, 97) or label free quantification workflows (94, 98). A detailed overview of different quantitative proteomic technologies can be found here (99).

A difference between lectin-affinity and hydrazide capture approach N-glycoprotein subsets has been suggested (90, 100, 101). However, the chemical method seems to offer a broader applicability compared to the selectivity dependent lectin enrichment (47) and additional further efforts will need to reveal potential differences in the glycoprotein subsets that are targeted by both strategies.

The high degree of specificity for cell surface proteins derived from a cellular state at a certain point in time (snapshot) promises to obtain a complementary, unbiased overview of the MS-detectable surfaceome and makes the CSC technology a very attractive tool for immunological cell surface proteome research. However, it is important to point out that the CSC technology, as well as other surfaceome enrichment strategies do not allow for single cell surfaceome analysis (102). Based on the sensitivity limits of currently available MS instrumentation a minimum of $1x10^7$ cells is recommended for all presented protocols in order to achieve reasonable results. However, the fast improving sensitivity of MS systems currently under development in combination with MS software improvements will drive the cell numbers required for surfaceome, and in general, proteomic analysis, further down.

3.4. Targeted verification strategy: selected reaction monitoring

Once immunological relevant cell surface proteins are defined on the MS level through their peptide MS coordinates, one can imagine the benefit of user-specific targeted proteomics workflows, allowing for the monitoring of abundance differences of specific key proteins of interest across the leukocyte differentiation tree. The immense progress in discovery-driven proteomics instrumentation and methodologies provided the community with peptide and protein databases, covering substantial amounts of the predicted human proteome (15, 16, 103, 104). Indeed, the Human Proteome Project aims to characterize the human proteome of all approximately 21 000 protein-coding genes of the known genome (http://www.hupo.org/research/hpp/). To achieve this goal, a main focus lies on targeted MS-based strategies such as the selected reaction monitoring (SRM) technology (105, 106) (also called: multiple reaction monitoring, MRM (107)) which is already widely used in the field of small molecule analytics (such as the detection of drug metabolites (108), hormones (109), pesticides (110), or protein degradation products (111)) for more than a decade with great success. The Human Proteome Project study will include the synthesis and measurement of several proteotypic reference peptides for each protein (112). This strategy will then result in mass spectrometric coordinates for each peptide, allowing for the targeted measurement of proteotypic peptides of any protein of interest by SRM based technology.

Combined with surfaceome enrichment strategies that were described above, SRM technology lends itself for the selective targeting and quantification of the respective targets. SRM offers the advantage of being highly selective due to two levels of mass selection, highly sensitive due to a non-scanning mode and was shown to have the capacity to cover a wide dynamic range of approximately 4.5 orders of magnitude (57). With spiked-in internal standard peptides, absolute protein quantities can be determined (113). The next update of SRMatlas (114) will include mass spectrometric SRM assays for public use for most proteins in yeast (97 % proteome coverage), human (95 % proteome coverage) and mouse (55 % proteome coverage) (http://www.srmatlas.org). In regard to the surfaceome, this resource will include up to five proteotypic peptides per potential cell surface expressed protein, which can be used to verify and quantify the proteins of interest. In a collaboration between the IMSB, Zürich and the ISB, Seattle, SRM assays for 7000 N-glycopeptides were generated, which enable the verification of N-glycosites in proteins located at the cell surface, but also in secreted proteins. This resource will become available later this year (R. Aebersold, personal communication) and will significantly advance basic and clinical research by supporting the repetitive and sensitive measurement of protein candidates in suitably collected cell and tissue samples as well as in biofluids such as blood, urine and CSF.

SRM technology is also geared towards the quest of immunologists to gain further information about the dynamics of the immune system on the protein level. Post-transcriptional and post-translational processing often leaves multiple or differentially modified protein isoforms as the end-product of gene expression. Although their simultaneous measurement would be highly desirable, it is not trivial to distinguish these isoforms and protein modifications by currently available affinity reagents. The generation of new antibody-based probes for being able to test for all protein modifications would be theoretically desirable, but is practically impossible. Therefore, MS-based technologies might provide immunologists now with alternative strategies and a generic platform for generating protein-specific information about the glycosylation status, or the phosphorylation status of proteins within a signalling network. MS technologies, such as SRM assays, could also be employed to support results obtained from flow cytometry (FCM) experiments with antibody-based probes. A recent publication from Kemper *et al* (115) indicated the need for validation of FCM results with the antibody

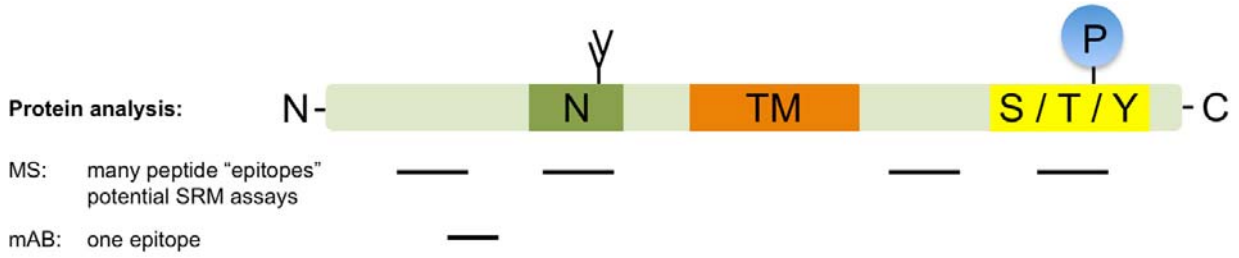

Figure 4. Mass spectrometry is a generic technology for the identification of protein features. MS-based technologies enable the identification of protein features through the identification of multiple peptides covering the protein sequence. Individual MS-identified peptide sequence length usually varies between 5-25 amino acids and can provide information about post-translational modifications (such as glycosylation, phosphorylation, acetylation, methylation, sumoylation, etc.) or protein isoforms (differential splicing, proteolytic processing, point mutation). In contrast, mAB recognize typically a linear or non-linear stretch of a short amino acid sequence (epitope). While MS technology provides a generic platform for scanning for different types of modifications, individual mAB would be required to test for these modifications in a sequence specific fashion for each protein of interest. Protein N- and C-termini are indicated. Highlighted sequence background: green: N-linked glycosylation site (N); orange: transmembrane domain (TM); yellow: phosphorylation site (S/T/Y). List of post-translational modifications is available at http://www.unimod.org.

AC133 against the cell surface protein CD133. Although the AC133 antibody revealed the expression of CD133 and its decrease upon differentiation of colon cancer stem cells by FCM analysis, it was suggested by independent experiments that the observed decrease in cell surface expression was not derived from changes of protein abundance. Furthermore, the CD133 quantities did not decrease in the cell surface location upon initial differentiation, but mere changes in the glycosylation pattern of the protein were proposed to shield the epitope recognized by the antibody AC133 and precluded identification. Here, the strength of SRM-based proteomic workflows could be exploited to specifically measure targeted peptide sequences of interest for independent validation of the location specific expression. This and other examples show how state of the art MS technologies could offer complementary strengths to currently employed antibody based technologies for surfaceome analysis which could be exploited by immunologists towards the generation of a comprehensive map of protein circuits guiding immunological responses (Figure 4).

4. SUMMARY AND PERSPECTIVE

The goal of systems immunologists to decipher the sensory and signal processing machinery during the immune response on different network levels is a challenging task. The strategies for the specific analysis of proteins that constitute the immunological surfaceome discussed here will aid immunologists in understanding and the positioning of proteins that are vital to immune response and function within immunological network maps. We discussed proteomic strategies here as complementary strategies to antibody-based workflows for the unbiased and discovery-driven analysis of cell surface proteins. Targeted SRM-based strategies were presented for the possibility of quantitative measurements of protein networks and perturbations over time. By carefully integrating these MS-based proteomic strategies for the complementary determination of the immune surfaceome into existing single-cell based approaches we expect a large benefit for the discovery of new phenomena and a better overall understanding of immune function (Figure 5).

Since we discussed proteomic tools as an alternative to the unbiased analysis of cell surface proteins, an integrated and detailed overview about downstream signal transduction and processing would be beyond the scope of this review. The proteomic analysis of protein phosphorylation and other post-translational modifications is another rapidly emerging and promising field for systems biology in general and is discussed in detail elsewhere (55). Still we would like to highlight one promising example that could further expand our view in systems immunology by combining MS with classical antibody-based approaches. Apart from the described problem in the availability of high quality affinity reagents for single cell surfaceome analysis, the possibility for multiplexed follow up measurements on all cellular levels for single cell populations would be highly desirable as discussed briefly. The primary tool for single cell analysis, fluorescence activated flow cytometry (37-39), is already capable of multiparametric detection. In principle, 12-color measurements should be manageable (although challenging) for laboratories with the necessary expertise, while up to 17-color analysis has been reported from specialized research laboratories (116). The key problem limiting the number of available simultaneous measurements is the spectral interference from cellular autofluorescence and the fluorophore conjugated antibodies used to distinguish signals derived from different proteins that were targeted during the analysis. An innovative combination of parts from a flow cytometer and a mass spectrometer, the Mass Cytometer, promises to multiply the available channels for detection. By changing from fluorescence conjugates to element-tag coded antibodies, the inductively coupled mass spectrometry (ICP-MS) technology (117) changes the method of detection from fluorescence to mass analysis (as reviewed in detail here (118)). The IPC-MS technology has already been applied to the parallel analysis of intracellular and cell surface protein markers in a human leukemia cell line (119) and has been demonstrated to work on the single cell level (120). More

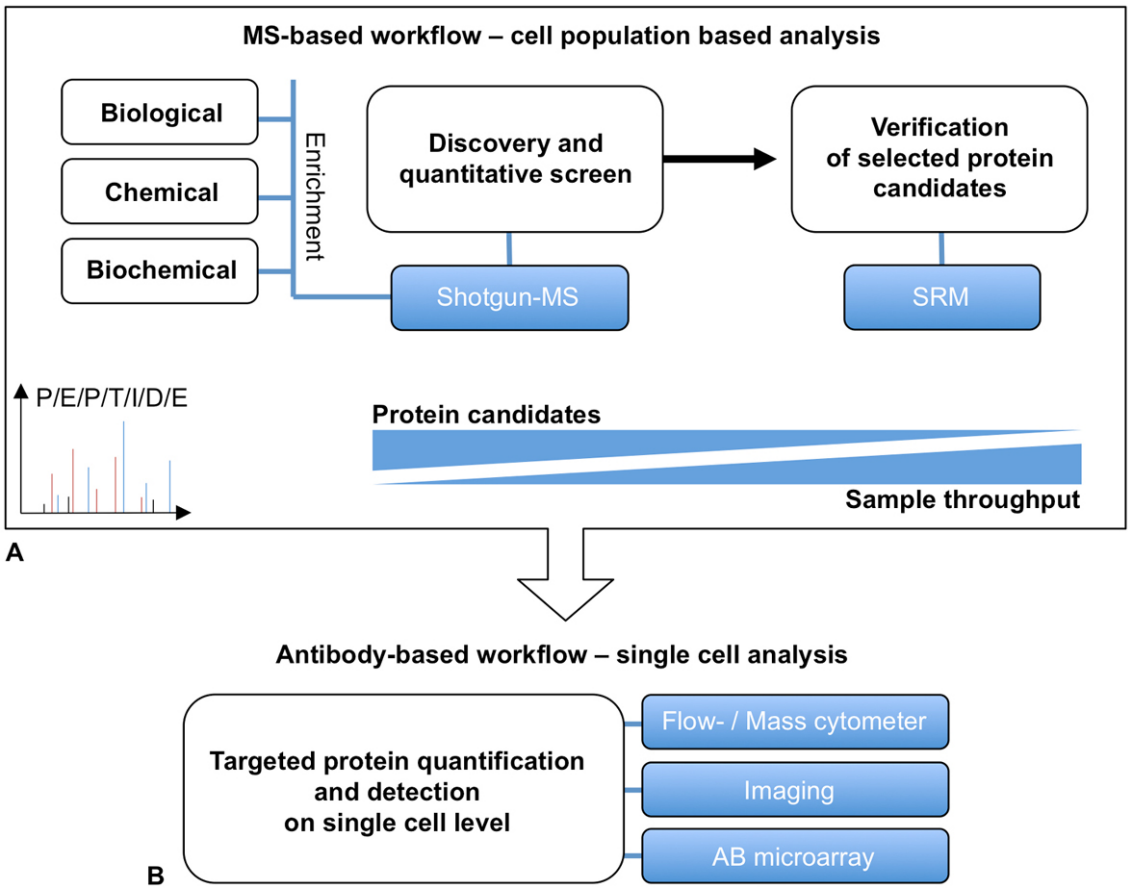

Figure 5. Mass spectrometry and antibody-based probes provide complementary technologies for the comprehensive analysis of the surfaceome. A: MS enables unbiased and multiplexed surfaceome analysis of a large number of analytes on the cell population level in discovery-driven fashion. After initial screening of quantitative changes within the surfaceome upon perturbation, selected regulated protein candidates can be further validated within larger sample sets of varying conditions (time course, dose response) using targeted SRM measurements. B: Hypothesis-driven follow-up analysis of several surfaceome proteins within cellular resolution by mAB-based technologies (if suitable antibodies are available).

recently, the mass cytometry technology was even reported to be able to increase the number of simultaneously investigated parameters up to 30 antigens (118), demonstrating the power of the technology for systems immunology.

The relation of quantitative, cell surface proteome data combined with phospho-active state of the cellular signalling network machinery would provide a more systematic view of the immune cell. The tools for the specific analysis of proteins that constitute the immunological surfaceome discussed here are one more step towards filling the current gap in knowledge about proteins vital for immune response and function.

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A.H. current address is: Novartis Pharma AG, WSJ-155.2.27.1, 4056 Basel, Switzerland.
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Abbreviations: CD: cluster of differentiation; CSC: cell surface capturing; FCM: flow cytometry; IF: immunofluorescence; IHC: immunohistochemistry; mAB: monoclonal antibody; MS: mass spectrometry; SRM: selected reaction monitoring; WB: western blot

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Send correspondence to: Bernd Wollscheid, NCCR Neuro Center for Proteomics, Institute of Molecular Systems Biology, Wolfgang-Pauli Str. 16, ETH Zurich, 8093 Zurich, Switzerland, Tel: 41-44-6333684, Fax: 41-44-6331051, E-mail: bernd.wollscheid@imsb.biol.ethz.ch

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