Classification of mouse B cell types using surfaceome proteotype maps

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System-wide quantification of the cell surface proteotype and identification of extracellular glycosylation sites is challenging when samples are limited. Here, we miniaturize and automate the previously described Cell Surface Capture (CSC) technology, increasing sensitivity, reproducibility and throughput. We use this technology, which we call autoCSC, to create population-specific surfaceome maps of developing mouse B cells and use targeted flow cytometry to uncover developmental cell subpopulations.
Cell types are typically identified and classified using selected cell-surface proteins as this sub-proteome reflects the maturity and functional state of a cell. A comprehensive knowledge of the surface-exposed proteome (surfaceome) is necessary for classification of cell types and to enable linkage of distinct proteotypes to functional phenotypes. Global analysis of the surfaceome is challenging for technical reasons. High-throughput protein and RNA analyses are agnostic toward spatial information, and antibody-based technologies like flow or mass cytometry are limited in their multiplexing capabilities and by the availability of high-quality antibodies. Chemical biotinylation of surface glycoproteins has been used for systematic surfaceome interrogation by affinity purification of tagged proteins or peptides prior to mass spectrometry (MS) analysis, and protein-level enrichment methods that quantify peptides adjacent to a tagged glycopeptide have provided deep coverage of the plasma membrane proteome. These methods, however, preclude a priori separation of enriched surface proteins from nonspecific background; instead, prior knowledge regarding surface localisation is used to filter for known plasma membrane proteins. In contrast, Cell Surface Capture (CSC) technology enables direct identification of extracellular N-glycopeptides. Surface biotinylated N-glycopeptides are captured and enzymatically released by peptide:N-glycosidase F (PNGase F), which catalyses cleavage of asparagine-linked glycans. This leaves a deamination within the NXS/T consensus sequence of formerly N-glycosylated peptides, indicating both surface localisation and glycosylation site. The specific site information comes at cost of sensitivity, however. Hence CSC experiments are performed with up to $1 \times 10^8$ cells per sample. The large amounts of sample required mean that it is not practical to perform CSC on primary cells.

Here, we introduce autoCSC, an automated and miniaturised CSC technology enabling surfaceome mapping of primary cell types with limited sample availability. To demonstrate the utility of autoCSC, we create population-specific surfaceome maps of developing B cells and reveal functionally distinct developmental subpopulations of immature B cells.

**Results**

**Automation and miniaturisation of cell surface capture.** Miniaturisation and automation have proven beneficial in phosphopeptide, hydrazide and antibody-based enrichment processes. We reasoned that the same principles could be applied to the biotin-streptavidin system used within the CSC workflow. Additionally, we optimised the standard CSC technology, most prominently by inclusion of a catalyst for optimal surface labelling. A schematic of the autoCSC workflow is shown in Fig. 1a. Briefly, live cells are oxidised under mild conditions with sodium-meta-periodate to generate aldehydes on cell-surface carbohydrates and are subsequently labelled with cell-impermeable biocytin-hydrazide in presence of catalyst 5-methoxyxanthorhelic acid. After cell lysis and tryptic digestion, the resulting peptides are subjected to automated processing on a tailored liquid handling robot. Repeated aspiration through filter tips containing streptavidin resin provides a confined reaction space for efficient binding of biotinylated N-glycopeptides. After extensive washing, N-glycopeptides are released by PNGase F provided in a plate heated to 37°C. Using high-resolution MS, labelled extracellular peptides are identified by deamidated asparagines within the NXS/T glycosylation consensus sequence resulting from PNGase F cleavage. Targeted feature extraction in combination with data-independent acquisition (DIA) is used to quantify surface-protein abundances across multiple conditions.

Increased sensitivity and reproducibility of automated processing. First, we evaluated the performance of the automated and miniaturised processing compared to the manual workflow. We prepared CSC-labelled peptides from $\sim 6 \times 10^8$ HeLa cells and distributed them over 30 samples containing about 5 mg peptide each. Twenty of these samples were processed in manual mode by two different researchers (Experimenters 1 and 2) and 10 were subjected to automated processing. Experimenters 1 and 2 identified 325 and 290 extracellular N-glycopeptides, respectively (median), whereas the robot identified 1811 N-glycopeptides, a more than five-fold increase over the manual process (Fig. 1b). Next to the five-fold increased sensitivity, we further asked whether autoCSC allows for more reproducible quantification of cell surface derived N-glycopeptides compared to the manual workflow. For this, deamidated N-glycopeptides were label-free quantified by integration of mass spectrometry monitored chromatographic traces. For every N-glycopeptide, quantitative values across 10 replicates were used to calculate a coefficient of variation (CV) for autoCSC, Experimenter 1 and Experimenter 2 as measure for its quantitative reproducibility. The overall distribution of obtained peptide CVs per designated workflow are visualised in Fig. 1c. Automated processing showed the lowest variation with a median CV of 28% compared to 51% and 34% for Experimenter 1 and 2, respectively (Fig. 1c). Thus, miniaturisation and automation of the biotin-streptavidin enrichment resulted in increased sensitivity and higher quantitative reproducibility for the CSC workflow.

**Surfaceome maps of 11 commonly used cancer cell lines.** To evaluate the capability for multiplexed quantification of surfaceomes, we performed autoCSC with 11 commonly used cancer cell lines. After cell labelling and digestion, samples containing approximately 1 mg peptide were processed, and N-glycoproteins were quantified with DIA-MS. We quantified 1697 unique glycosylated asparagines located within proteotypic peptides from 900 protein groups with a median of two sites per protein group (Supplementary Fig. 1, Supplementary Data 1). Of these, 192 glycosylation sites were previously unknown based on Uniprot database annotation (Supplementary Fig. 2a). However, 84% of the annotated sites in Uniprot are based on computational prediction, thus we provide experimental evidence for 1017 Uniprot-predicted glycosylation sites. The median per cell type was 602 surface proteins, two-fold higher compared to 301 reported in the largest CSC data repository, the Cell Surface Protein Atlas, despite using 5- to 33-fold lower amounts of input peptides. Hierarchical clustering and principal component analysis grouped samples originating from the same cell type in close proximity (Fig. 1d, e), demonstrating that autoCSC can reliably subclassify cell types based on surfaceome information.

**Mapping the surfaceome of developing B cells.** We hypothesised that autoCSC technology would enable phenotyping of freshly isolated primary cell populations. B cell development consists of successive cellular stages beginning in the bone marrow and continuing in peripheral lymphoid tissues. Developing B cells are particularly challenging to characterise by CSC due to limited sample availability from mice, relatively small size, and little diversity in surfaceome composition. In order to probe applicability of autoCSC, we prepared a dilution series from 30 to $0.1 \times 10^6$ cells of a B lymphoma cell line and quantified N-glycoproteins. Compared to the number of surface proteins identified from a sample of $30 \times 10^6$ cells, we recovered a median of 62% with $1 \times 10^6$, although the variance increased below $5 \times 10^6$ (Supplementary Fig. 3). With this limitation in mind, we set out to de novo map and quantitatively compare the surfaceomes of developing B cells using autoCSC. From mice, we isolated nine consecutive stages of B cell populations (Fig. 2a) with a maximum of $1 \times 10^6$ cells per sample using...
fluorescence-activated cell sorting (FACS). With autoCSC, we quantified 248 unique glycosylated asparagines located within proteotypic peptides and grouped them into 147 protein groups with a median of two unique sites per protein group (Fig. 2b and Supplementary Data 2). Besides finding 25 new glycosylation sites, we also provide experimental evidence for 139 computationally predicted sites (Supplementary Fig. 2b). Few proteins we found exclusively on a particular population, for example CD80 and CD130 on B1 cells (Supplementary Fig. 5) (Fig. 2b).
Identification of immature B cell subpopulations. Furthermore, we identified several surface proteins that were either absent or of considerably lower abundance in the bone marrow until the immature B stage, but showed stable surface abundance in later stages (Fig. 2b–f and Supplementary Fig. 5). We hypothesized that these could be used to further split the immature B stage into subpopulations with different maturities. Therefore, we performed flow cytometry analysis to identify differences in surface abundance among individual cells within each population (Supplementary Fig. 6). First, we asked whether flow cytometry reproduced the autoCSC results for the selected proteins. All showed a strong positive correlation with an average Spearman’s rho of 0.77, indicating very good agreement between the two methods (Fig. 2d). As a reference we retrieved RNA-seq data for the corresponding populations from the ImmGen database and calculated correlation coefficients with flow cytometry (Fig. 2d).
Interestingly, flow cytometry revealed a bimodal distribution of CD20 within the immature B subpopulation (Supplementary Fig. 6). For CD180, we found a broad distribution covering more than two orders of magnitude (Supplementary Fig. 6). Co-staining for CD20 and CD180 across all populations revealed that prior to CD180 upregulation, CD20 abundance increased during development (Fig. 2g). Based on our data, we were able to further divide the immature B population into three subpopulations: double negative (DN), positive for CD20 but not CD180 (SP), and positive for both CD20 and CD180 (DP) (Fig. 3a). We then asked whether the three subpopulations follow a developmental trajectory towards maturity. To test the hypothesis that the DN population differentiates to DP, we cultured CD19+ IgM+CD93+DN, SP and DP cells in vitro while monitoring CD20 and

**Fig. 3 Molecular and functional assessment of immature B subpopulations.** a The immature B population was further divided into three subpopulations defined by CD20 and CD180 fluorescence intensity: double negative (DN), positive for CD20 (SP), and positive for both (DP). b In-vitro differentiation of the defined immature B subpopulations during three days of culturing. c Fraction of IgD+ cells in immature B subpopulations. d Mean fluorescence intensity for BAFF receptor for the three immature B subpopulations. e Modulation of in vitro survival of subpopulations with BAFF treatment. f Principal component analysis of proteotype data reflects the identified immature B subpopulations. Error bars indicate standard deviation. Statistical analysis was done with two-tailed unpaired Student’s t test. One star indicates \( P < 0.01 \); four stars indicate \( P < 0.0001 \).
CD180 surface expression. In line with our hypothesis, we found that both DN and SP populations develop into DP within 3 days of culturing (Fig. 3b). Next, we probed the status of the three subpopulations with respect to known markers of maturity. We found that the DP population had a significantly higher number of IgD+ cells (Fig. 3c) accompanied by higher surface abundance of BAFF receptor (Fig. 3d), as compared to SP and DN subpopulations. Based on these findings, we assessed the identified immature B subpopulations in their response to BAFF in vitro. Interestingly, BAFF treatment significantly increased in vitro survival of only the DP subpopulation (Fig. 3e), in agreement with the higher expression pattern observed for DP. For molecular assessment of the proteotypes underlying the observed phenotypes, we performed quantitative proteomic analysis of the identified immature B subpopulations (Supplementary Data 3). Also on the proteotype level, we found distinct clustering of DN, SP and DP subpopulations in their sensitivity towards depleting anti-CD20 antibodies. In agreement with CD20 expression levels, SP and DP subpopulations were depleted upon anti-CD20 antibody treatment in vitro, while the DN cells even slightly increased in counts (Supplementary Fig. 8a, b).

In conclusion, we identify three consecutive immature B subpopulations characterised by CD20 and CD180 surface expression and differential sensitivity towards BAFF and depleting anti-CD20 antibodies. Furthermore, we provide a comprehensive analysis of the associated proteotypes and found clustering on the proteotype-level to reflect the phenotypically defined subpopulations.

Discussion
In summary, miniaturised and automated CSC technology was validated and used to analyse cancer cell lines and developing primary B cells. mRNA abundance is not sufficient to infer protein levels in many scenarios,[16] and to predict genotype–phenotype relationships it is necessary to go beyond global protein levels and determine proteoform abundances within the spatial domains where activity is required for function[17]. CSC technology utilises an amino acid modification within a consensus sequence that reports on an initial cell-surface-restricted labelling event, enabling specific quantification with subcellular resolution. Direct identification of tagged sites has proven beneficial for spatial proteomic workflows[18] but requires the detection of a peptide bearing the modified amino acid. This caveat makes it particularly challenging to achieve identification of large numbers of formerly glycosylated sites when sample amounts are low. Insufficient sensitivity resulting at least partially from manual processing has limited CSC to specimens that can be produced in large quantities in the range of 50–100 Mio cells per sample. Physical confinement of the reaction space and automation of the biotin-streptavidin system within the autoCSC technology bridged this gap and reached the sensitivity and throughput required for broad application of surface proteome screening with primary cellular material.

These improvements will enable surfaceome prototype maps of primary cell samples for translational research applications and systems-scale surfaceome research.

Methods
Chemicals. All chemicals were purchased from Sigma unless stated otherwise.

Antibodies, flow cytometry and sorting. Cells were flushed from femurs and tibias of the two hind legs and from the peritoneum of the mice or single-cell suspensions of spleen cells were made. Staining was performed in PBS containing 0.1% BSA and 5 mM EDTA. The following antibodies were used for flow cytometry (from BD Biosciences, eBioscience, BioLegend, or produced in house): anti-CD117 (2B8, 1/400, 562609, BD Pharmingen), anti-CD19 (1D3, 1/400, 563333, Biolegend), anti-CD127 (SB19/9, 1/100, 25-1273-82, Invitrogen), anti-IgM (M41), anti-IgD (1.19, 1/1000, in-house), anti-CD93 (PB493, 1/1000, in-house), anti-CD11b (M170, 1/1000, in-house), anti-CD44 (IM7, 1/1000, in-house), anti-CD48 (HM48-1, 1/400, 11-0481-82, Invitrogen), anti-CD20 (M1/9, 1/100, 101823, Biolegend), anti-CD20 (SA275A11, 1/400, 150407, Biolegend), anti-CD180 (RP14/1, 1/400, 117076, Biolegend), anti-CD150 (TC15-12F12, 1/400, 115904, Biolegend), anti-CXCR5 (G2D, 1/200, 560528, BD Pharmingen), anti-PD-L1 (10 F11C2, 1/400, 124307, Biolegend), anti-CD23 (1H5B5, 1/400, 149483, Biolegend), anti-CD80 (16-10A1, 1/400, 553768, BD Pharmingen), anti-CD86 (GL1, 1/400, 533691, BD Pharmingen), an anti-mBAFF-R (9B9, 1/400, in-house). The following FACS strategy for mouse B cell populations was used: bone marrow: proB, CD19IgM+CD117'+CD127-; large preB, CD19IgM+CD117'CD127'FCSSSP; small preB, CD19IgM+CD117'CD127'FCSSSP; immature B, CD19IgM+CD3'. From the peritoneum: B1, CD19'CD127'CD23'CD11b'. From the spleen: Transitional-1, CD19'CD93'CD23'-CD21'; Transitional-2, CD19'CD93'-CD23'+CD21'; Follicular (Fo), CD19'CD93'CD23'+CD21'; and Marginal Zone (MZ), CD19+CD93'.

Flow cytometry. A BD LSRSortFortessa (BD Biosciences) was used, and data were analysed using FlowJo Software (Treestar). For cell sorting, a FACSAria IIlu (BD Biosciences) was used (~98% purity). For autoCSC, sorting was performed on four different days generating four biological replicates. Two technical replicates with 1 × 10⁶ cells per biological replicate were processed for B cell populations (SB, I, FO, MZ) with sorting yields of 2 × 10⁶ cells.

Cell surface capture—labeling and digestion. Surface glycoproteins on live cells were gently oxidised with 2 mM NaIO₄ (20 min, 4 °C) in labelling Buffer (LB) consisting of phosphate-buffered saline, pH 6.5. Cells were washed once in LB and subsequently bionylated in LB containing 5 mM biotinyl hydrazide (Pitsch Nucleic Acids, Switzerland) and 5 mM 5-methoxysouthanilic acid for 1 h at 4 °C min. Cells were washed three times with LB and harvested, lysed in lysis buffer (100 mM Tris, 1% sodium deoxycholate, 10 mM TCEP, 15 mM 2-chloroacetamide, pH 8.5) by repeated sonication using a ViaIonTweeter (Hielser Ultrasonics), and heated to 95 °C for 5 min. Proteins were digested with trypsin overnight at 37 °C using an enzyme-to-protein ratio of 1:50. For the CSC diffusion series and experiments with B cells, LB used for washes contained 5% FBS, and digestion was done using Lysis (Wako) and sequencing-grade trypsin (Promega), both with an enzyme-to-protein ratio of 1:200. In order to inactivate trypsin and precipitate enzymes was done using LysC (Wako) and sequencing-grade trypsin (Promega), both with an enzyme-to-protein ratio of 1:50. For the CSC dilution series and experiments with B cells, LB used for washes contained 5% FBS, and digestion was done using LysC (Wako) and sequencing-grade trypsin (Promega), both with an enzyme-to-protein ratio of 1:50. In order to inactivate trypsin and precipitate enzymes was done using LysC (Wako) and sequencing-grade trypsin (Promega), both with an enzyme-to-protein ratio of 1:50. In order to inactivate trypsin and precipitate enzymes was done using LysC (Wako) and sequencing-grade trypsin (Promega), both with an enzyme-to-protein ratio of 1:50. In order to inactivate trypsin and precipitate enzymes was done using LysC (Wako) and sequencing-grade trypsin (Promega), both with an enzyme-to-protein ratio of 1:50.
membrane. Assembled tips were attached to the liquid handling robot and washed with 50 mM ammonium bicarbonate by repeated cycles of aspiration and dispensing. Biotinylated peptides were bound to streptavidin in 1% acetonitrile and 0.1% formic acid containing iRT peptides (New Objective) packed in-house with a 1-cm stationary phase ReproSil-Pur 120 A C18 1.9 µm (Dr. Maisch GmbH) and connected to an Easy-nLC 1000 instrument equipped with an autosampler (Thermo Fisher Scientific). The HPLC was coupled to a Fusion Lumos mass spectrometer equipped with a nano-electrospray ion source (Thermo Fisher Scientific). Raw data were converted to mzML using MSconvert. Fragment ion spectra were searched against UniprotKB (Swiss-Prot, Homo sapiens, retrieved April 2018) containing common contaminants and MS standards. The precursor mass tolerance was set to 20 ppm. Carbamidomethylation was set as a fixed modification for cysteine, oxidation of methionine and deamidation of arginine were set as variable modifications. Probability scoring was done with PeptideProphet of the Trans-Proteomic Pipeline (v4.6.2). Peptide identifications were filtered for an FDR of ≤1% and presence of consensus NXS/T sequence and deamidation (+0.98 Da) at asparagines. Non-conflicting peptide feature intensities were extracted with Progenesis QI (Nonlinear Dynamics) for label-free quantification and determination of CVs.

Data analysis DIA LC-MS/MS. LC-MS/MS DIA runs were analysed with Spectronaut Pulsar X version 12 (Biognosys) using default settings. Briefly, a spectral library was generated from pooled samples measured in DDA (details above). The collected DDA spectra were searched against UniprotKB (Swiss-Prot, Homo sapiens or Mus musculus, retrieved April 2018) using the Sequest IFT search engine within Thermo Proteome Discoverer version 2.1 (Thermo Scientific). Peptides were loaded onto the column with 100% buffer A (99% H2O, 0.1% formic acid) and eluted at a flow rate of 250 nL/min with a segmented gradient from 1 to 38% buffer B (80% acetonitrile, 0.15% formic acid). DDA LC-MS/MS DIA runs were analysed with Spectronaut Pulsar X version 12 (Biognosys) with the same parameters as described above for the DIA runs. Raw data were converted to mzML using MSConvert. Fragment ion spectra were searched against UniprotKB (Swiss-Prot, Homo sapiens, retrieved April 2018) containing common contaminants and MS standards. The precursor mass tolerance was set to 20 ppm. Carbamidomethylation was set as a fixed modification for cysteine, oxidation of methionine and deamidation of arginine were set as variable modifications. Probability scoring was done with PeptideProphet of the Trans-Proteomic Pipeline (v4.6.2). Peptide identifications were filtered for an FDR of ≤1% and presence of consensus NXS/T sequence and deamidation (+0.98 Da) at asparagines. Non-conflicting peptide feature intensities were extracted with Progenesis QI (Nonlinear Dynamics) for label-free quantification and determination of CVs.
Benjamini-Hochberg method was used to account for multiple testing. Protein abundance per sample or condition was used for further analysis and plotting. autoQC experiments comparing 11 cell lines and developing B cells were performed in multiple replicates (quadruplicate biological replicates and where possible additional technical replicates). Technical replicates per biological replicate were consolidated in MSstats. Outliers were removed to retain minimally three biological replicates, generating 31 samples for B cell populations and 33 samples for the 11-cell lines comparison for final quantification.

Correlation analysis. Proteins with abundance values (non-NA) for flow cytometry and CSC for at least four populations were considered for analysis (CD20, CD24, PD-L1, CD180, CD44, CD48, CD150, CD19, CXCR5). If one condition only was not applicable it was set as zero assuming an abundance value below limit of detection. Spearman correlation coefficients were calculated and plotted using R. RNA-seq data were retrieved for the same proteins from the ImmGen database (www.immgen.org). In the retrieved dataset the following populations were sorted based on the same markers as our flow cytometry analysis: Transitional 1, Transitional 2, Follicular and Marginal Zone. The following populations were considered equivalent between ImmGen and our flow cytometry analysis: Pro-B to FrB/C, immature B to FrE, and B1 to B1b. Large and small pre-B are not included in ImmGen database and were therefore not included in the correlation analysis between flow cytometry and RNA.

Glycosylation site analysis. For glycosylation site counting the following rules were followed: (i) only glycosylated peptides conforming to the NX(STC) consensus sequence were considered; (ii) to avoid inflating the count, non-protootypic peptides were arbitrarily assigned to a single protein in the protein group; (iii) if a glycosylated peptide could be mapped to multiple positions within the same protein, both positions were kept, unless one of the mappings resulted in a higher number of sites matching the consensus NX[STC] motif, in which case only this one was kept. When comparing the glycosylation sites identified in this study to the ones annotated in UniProt, only proteins identified in this study as having at least one glycosylation site were considered in UniProt.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All mass spectrometric data and acquisition information were deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository (data set identifier: PXD013627).

Code availability
All in-house developed computer code supporting the findings of this study are available from the corresponding author upon request.

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Author contributions
M.v.O. and M.M. performed all experiments except those noted below. F.K. and P.T. performed flow cytometry and FACS. M.v.O., M.M., R.B. and L.R. and B.W. optimised and performed LC-MS/MS acquisition. M.v.O., M.M., F.K., P.T., and P.G.A.P. analysed data. P.G.A.P. performed glycosylation site analysis. H.Z. contributed new analytical tools. M.v.O., M.M., F.K., P.T. and A.R. and B.W. designed research. M.v.O., M.M. and B.W. conceived the project and wrote and revised the paper.

Competing interests
R.B. and L.R. are employees of Biognosys AG. Spectronaut is a trademark of Biognosys AG. All other authors declare no competing interests.

Additional information
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