Journal for ImmunoTherapy of Cancer

Discovery and validation of surface *N*-glycoproteins in MM cell lines and patient samples uncovers immunotherapy targets

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To cite: Oldham RAA, Faber ML, Keppel TR, *et al*. Discovery and validation of surface *N*-glycoproteins in MM cell lines and patient samples uncovers immunotherapy targets. *Journal for ImmunoTherapy of Cancer* 2020;**8**:e000915. doi:10.1136/jitc-2020-000915

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jitc-2020-000915).

Accepted 02 July 2020

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ABSTRACT

Background Multiple myeloma (MM) is characterized by clonal expansion of malignant plasma cells in the bone marrow. While recent advances in treatment for MM have improved patient outcomes, the 5-year survival rate remains ~50%. A better understanding of the MM cell surface proteome could facilitate development of new directed therapies and assist in stratification and monitoring of patient outcomes.

Methods In this study, we first used a mass spectrometry (MS)-based discovery-driven cell surface capture (CSC) approach to map the cell surface *N*-glycoproteome of MM cell lines. Next, we developed targeted MS assays, and applied these to cell lines and primary patient samples to refine the list of candidate tumor markers. Candidates of interest detected by MS on MM patient samples were further validated using flow cytometry (FCM).

Results We identified 696 MM cell surface *N*-glycoproteins by CSC, and developed 73 targeted MS detection assays. MS-based validation using primary specimens detected 30 proteins with significantly higher abundance in patient MM cells than controls. Nine of these proteins were identified as potential immunotherapeutic targets, including five that were validated by FCM, confirming their expression on the cell surface of primary MM patient cells.

Conclusions This MM surface *N*-glycoproteome will be a valuable resource in the development of biomarkers and therapeutics. Further, we anticipate that our targeted MS assays will have clinical benefit for the diagnosis, stratification, and treatment of MM patients.

BACKGROUND

The American Cancer Society estimates that there will be 32 270 new cases and 12 830 deaths from multiple myeloma (MM) in the USA in 2020. In MM patients, plasma cell neoplasia can result in bone, nervous system, renal, and hematological manifestations. Diagnosis of MM is based on morphological features, imaging studies, analysis of myeloma cell-produced proteins, and testing of the blood, urine, and bone marrow (BM).[1 2] Cell surface antigens CD38 and CD138 can be used to distinguish normal cells from clonal plasma cells, but more extensive use of immunophenotyping has been limited by a lack of universally accepted markers of MM.[3–5] Once diagnosed, the current standard-of-care for MM includes immunomodulatory drugs, proteasome inhibitors, steroids, and antibody therapies. Initial treatment with bortezomib, lenalidomide, and thalidomide have improved outcomes; however, the majority of MM patients ultimately relapse, necessitating the use of multidrug combinations.[6] The highly heterogeneous and dynamic nature of MM means that existing therapies are often unable to overcome primary refractory disease and drug-resistant relapses, resulting in a 5-year survival rate of just 50.7%.[7] A comprehensive examination of the MM cell surface is necessary to better define proteins that could be clinically useful for MM diagnosis, stratification, and minimal residual disease tracking.

Recent gene expression analyses performed on MM patient samples have defined transcriptome signatures with the potential to improve predictions of disease progression and patient survival.[8 9] These studies have also identified novel candidate therapeutic targets. However, DNA- and RNA-based approaches do not provide critical information, such as protein abundance levels and subcellular localization. Proteomic data are potentially more informative in this regard but are not as prevalent as transcriptome data for this indication to date. Present cell-surface proteomic approaches are often exclusively based on immunoassays, such as flow cytometry (FCM). FCM is a powerful tool, but requires prior knowledge of proteins of interest, and can be limited by the availability and quality

of antibodies developed for each particular protein. In contrast, mass spectrometry (MS)-based approaches for surface protein discovery, including methods such as cell surface capture (CSC), enable the semiquantitative detection of hundreds of proteins in an antibody-independent manner. CSC has recently been applied to develop cell surface protein maps for multiple cancer types.[10–15] When used together for antigen discovery and validation, MS and FCM are highly complementary techniques.

To date, efforts to define the cell surface proteome of MM have focused on individual cell lines,[16 17] have studied changes in response to one particular therapy,[18] or have used methods that are not optimal for detection of low abundance membrane proteins.[19] As a result, knowledge of clinically informative MM cell surface proteins is lacking. In this study, we first used CSC to define the cell surface *N*-glycoproteome of four MM cell lines. This discovery-based approach identified 696 MM cell surface *N*-glycoproteins. Next, targeted quantitation of 73 proteins of interest was carried out using parallel reaction monitoring (PRM) analyses of primary MM patient samples. Cell surface abundance of five proteins (CD5, CD98hc, CD147, CD66, and CD205) was further validated on MM patient samples using FCM. This combination of CSC for discovery, and PRM and FCM for validation of selected candidates, provides a detailed view of the MM cell surface, including proteins of biological and therapeutic relevance.

METHODS

Cell culture

RPMI-8226, RPMI-8226/R5, U-266, MM.1R, and RPMI-1788 cell lines were maintained in RPMI-1640 medium (Sigma), supplemented with 10% fetal bovine serum (FBS) (20% for RPMI-1788) (Gibco) and 1X Penicillin-Streptomycin-Glutamine (PSQ) (Gibco) at 37°C and 5% CO_2. An Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell line (BLCL) was maintained in RPMI-1640 medium supplemented with 10% FBS and 1× PSQ. For use in FCM assays, normal donor mononuclear cells (MNCs) were isolated by density gradient centrifugation (Lymphoprep and PBMC Isolation Tubes, StemCell Technologies) from cells derived from discarded leucocyte reduction system cones or discarded apheresis products. T and B cells were isolated according to the manufacturer's instructions using CD3+ or CD19+ selection kits, respectively (StemCell Technologies). Normal human bone marrow mononuclear cells (BMNCs) were obtained frozen from StemCell Technologies.

Patient samples

Primary human MM cells were obtained from fresh BM aspirates. BMNCs were isolated by density gradient centrifugation (Lymphoprep and PBMC Isolation Tubes, StemCell Technologies). CD138+ MM cells were isolated using the EasySep Human CD138 Positive Selection Kit (StemCell Technologies). CD138+ and CD138− cell fractions were frozen on the day of collection either as pellets or suspended in freezing media (50% X-VIVO 20 media (Lonza), 40% autologous human serum, and 10% dimethyl sulfoxide (DMSO). Lysis of the cells was required in order to remove the beads prior to MS analysis.

Flow cytometry

Following collection of patient specimens, the following subsets were analyzed by FCM: whole BM, BMNCs, CD138+ cells, and CD138− cells. For each sample, at least 200,000 cells were washed and incubated for 30 min at 4°C with CD138-PeCy7, CD38-BV421, CD45-APC, CD19-PE, and CD56-FITC, or matching isotype controls (BioLegend), at a 1:200 dilution. Stained cells were washed once and fixed with 1% paraformaldehyde (PFA). For analysis of previously frozen primary CD138+ and CD138− cells, BMNCs, T cells, and B cells, Human TruStain FcX Fc Receptor Blocking Solution (BioLegend) was used according to the manufacturer's directions. Cells were then stained with CD5-BV421, CD147-PE/Cy7, CD166-PE, CD205-APC (BioLegend), and CD98hc-FITC (ThermoFisher), or matching isotype controls (BioLegend). All samples were analyzed on a BD LSRFortessa X-20 flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (FlowJo LLC).

CSC for discovery of cell surface *N*-glycoproteins

The CSC technology[20] workflow was performed as previously described[14 21 22] with ~100 million cells per biological replicate (n=3–6). Briefly, cell surface oligosaccharides on live cells were oxidized under mild conditions and labeled with biocytin hydrazide. Following lysis under hypotonic conditions, lysates were depleted of nuclei by differential centrifugation. A membrane-enriched fraction was prepared by ultracentrifugation at 210,000 ×*g* for 18 hours. The resulting membrane pellet was digested with trypsin and biotinylated glycopeptides were captured by immobilized streptavidin resin and stringently washed to remove non-specifically bound peptides. By digestion with PNGaseF, the peptides were released from the glycan moiety and then subsequently desalted and dried under vacuum. Samples were analyzed using a Q Exactive MS (Thermo; Waltham, Massachusetts, USA). Data were analyzed using ProteomeDiscoverer 2.2 (Thermo). The exported peptide lists were manually reviewed and proteins that lacked at least one peptide with a deamidated asparagine within the *N*-linked glycosylation consensus sequence (N-X-S/T/C where X is any amino acid except proline) were discarded (online supplementary table 1).

Cell lysis, protein digestion, and peptide clean-up

For whole-cell lysate analysis of lymphocyte cell lines and patient samples, pellets of cells were lysed in 500 µL of 2X Invitrosol (40% v/v; Thermo Fisher Scientific) and 20% acetonitrile in 50 mM ammonium bicarbonate. Samples were sonicated (VialTweeter; Hielscher Ultrasonics, Teltow, Germany) by three 10-second pulses, set on ice for

1 min, and then resonicated. Beads were removed magnetically. Samples were brought to 5 mM tris(2-carboxyethyl) phosphine (TCEP) and reduced for 30 min at 37°C on a Thermomixer at 1200 RPM. Samples were then brought to 10 mM iodoacetamide (IAA) and alkylated for 30 min at 37°C on a Thermomixer at 1200 RPM in the dark. 20 µg of trypsin was added to each sample; digestion occurred overnight at 37°C on a Thermomixer at 1200 RPM. Peptides were cleaned by SP2 following a standard protocol.[23]

Targeted quantitation of proteins of interest among cell lines and primary human cells

All targeted analyses were performed using an Orbitrap Fusion Lumos Tribrid MS (Thermo; for a full description see online supplementary methods). Data were imported into Skyline[24] and chromatographic peaks were extracted from MS2 spectral data for each detected peptide from the target list. Statistical analyses were performed using Student's t-test and plots were generated in GraphPad Prism.

RESULTS

Cell surface *N*-glycoproteome of MM cell lines

Four cell lines derived from MM patients (RPMI-8226, RPMI-8226/R5, U-266, MM.1R) were analyzed. Two B cell lines (RPMI-1788, BLCL) were included for comparison. By applying CSC technology, 846 distinct cell surface *N*-glycoproteins were identified, including 171 cluster of differentiation (CD) antigens (online supplementary table 2). The list of 846 includes single-pass and multi-pass membrane proteins, glycophosphatidylinositol (GPI)-anchored proteins, and lipid-anchored proteins (figure 1A). Overall, 81% of the proteins identified are known to be membrane-associated, demonstrating a high-quality enrichment for surface-localized proteins in the dataset.

Of 696 proteins identified on the 4 MM cell lines, 104 proteins were common to all lines. Many of these 104 proteins were also found on one or both B cell lines, with 7 proteins found exclusively on all 4 MM cell lines (figure 1B). This discovery-driven screen identified hematopoietic and B cell markers (eg, human leukocyte antigen (HLA), IgM, CD80), and known MM markers, such as CD38, in addition to proteins not previously described on MM cells.

Further supporting the utility of our approach for identifying cell surface proteins with relevance to MM, we compared our results to a panel of known MM antigens. Seven proteins known to be informative for immunophenotyping and monitoring of MM (BCMA, CD28, CD33, CD38, CD44, CD45, and CD54) were detected by CSC, as expected. A further nine proteins (CD19, CD20, CD27, CD52, CD56, CD81, CD117, CD200, CD307) were not detected, consistent with a known lack of expression in MM or expression on cells from only a subset of MM patients.

PRM assay development for MM markers of interest

CSC is limited to the detection of extracellular *N*-glycopeptides and typically requires >50 million cells per experiment. This prohibits application of this approach to routine analysis of primary human cells, especially for patients with low myeloma counts. For these reasons, we applied PRM assays to whole cell lysates from individual MM patient samples, which allowed us to compare relative abundance of proteins based on the detection of multiple non-modified peptides (which can provide more reliable

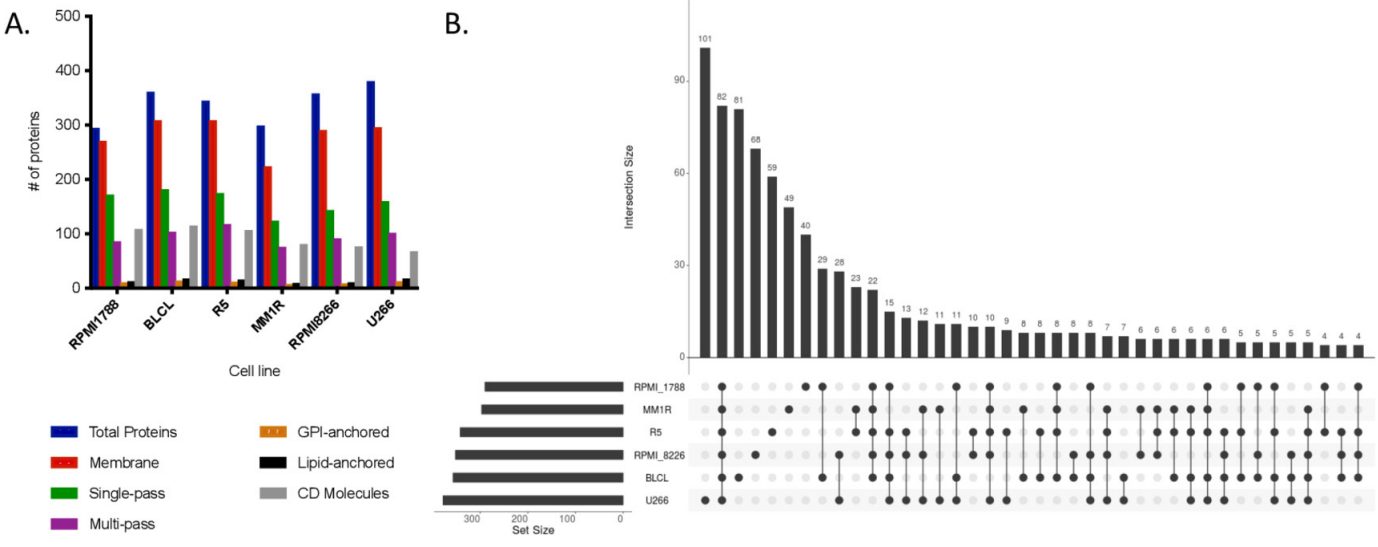

Figure 1 Overview of cell surface *N*-glycoproteins identified by cell surface capture analysis of multiple myeloma (MM) and B cell lines. (A) Distribution of protein types identified within each cell line based on UniProt annotations for cluster of differentiation antigen notations and membrane, single-pass and multi-pass, glycophosphatidylinositol (GPI)-anchored and lipid-anchored proteins. (B) Upset plot[54] showing distribution of protein observations among B and MM cell lines. BLCL, B-lymphoblastoid cell line.

quantitation than modified peptides) without having to pool patients or expand cells ex vivo. While the analysis of whole cell lysate provides a summary view of the total cell content (not only abundance at the cell surface) for a given protein, PRM assays allow for targeted, quantitative detection of pre-selected proteins with high selectivity and a lower limit of detection than CSC, and require less than 1 million cells. Thus, this approach was applied here to obtain additional evidence of cell type specificity or abundance differences for select proteins prior to subsequent analyses by FCM, which was then used to further validate their abundance at the cell surface.

MM antigens of interest for PRM assay development were identified from the CSC dataset by comparison between the MM and B cell data, along with inclusion of data from the Cell Surface Protein Atlas (CSPA),[15] which is comprised of CSC data from over 80 human and mouse cell lines and primary cells. Publicly available expression databases, such as the human protein atlas,[25] were also used as reference.

Well-known markers of MM (eg, CD38, BCMA) were among the proteins selected for PRM assay development. This includes CD138, which was not detected by CSC. As the single predicted *N*-glycosylation site in CD138 is within a region of the protein that, after trypsin digestion, would yield a peptide that is not detectable with standard analyses, the lack of CD138 detection by CSC is not surprising. Some previously known markers for MM (eg, SLAMF7, CD305) were identified by CSC at low levels, but were not included for PRM assay development. Proteins expected to be expressed on B cells (eg, CD19, CD20), therapeutic candidates (eg, SERCA2, CD28, CD54, CD147), and diagnostic/prognostic candidates (eg, SLC3A2, CD5, CD90) were also chosen. Other selections included proteins involved in BM homing/bone disease and calcium binding/transport (MM patients often present with osteolytic lesions and bone pain), and proteins involved in cell migration, adhesion, and drug resistance. Several proteins of interest discovered during preliminary data analysis (not shown), but ultimately not identified by CSC as present in the MM cell lines (including CLPTML1, LRBA and others), were also included as candidates for PRM development. Overall, 133 proteins were chosen for further study. The CD and non-CD proteins selected for PRM assay development are listed in figure 2 and online supplementary figure 1, respectively, and in each case their previous observation among various cell types in the CSPA are indicated.

Of the 133 proteins selected, PRM assays were successfully developed for 73 candidates and applied to the MM and B cell lines for validation (figure 3; online supplementary figure 2), where all 73 antigens were detected. As expected, proteins such as CD19 and CD20 were found exclusively on the B cell lines. CD138, CD38, CD45, and CD54 were detected at varying levels across both the MM and B cell lines. Detection of BCMA (TNFRSF17) in MM cells was lower than anticipated in comparison to the B cell lines, but expression on the B cell lines was not unexpected since both the RPMI1788 and BLCL cell lines are EBV+, a factor that has been associated with BCMA expression.[26] Proteins which were detected exclusively in the MM cell lines included CD3, CD6, CD28, L1CAM, MMRN1, SORT1, PXDN, and Homer3.

Assessment of selected surfaceome proteins in primary MM samples

Primary BM was obtained from 10 MM patients (a first cohort of 6 patients, and a second cohort of 4 patients), who presented with 2%–53% (average 26.7%) plasma cells in the BM by clinically diagnostic BM differential cell count. Between 1×10^6 and 48×10^6 (average 17.9×10^6) MM cells per patient were enriched by CD138+ selection to a purity of 23.3%–98.9% (average 71.2%), as determined by FCM (online supplementary table 3). All patient samples were CD138+ and CD38+, and just one of the 10 was CD19+. Three of the 10 patient specimens were CD56+.

The 73 proteins of interest were assessed by PRM analysis of whole cell lysates of CD138+ and matched CD138– controls. In the first cohort of MM patient cells (n=6), 59 of the 73 proteins were detected in the primary cell lysates. A number of proteins typically used for immunophenotyping and/or therapy of MM were identified, though PRM assays were not developed for all known markers (online supplementary table 4). Of 59 proteins detected in human primary cells, 30 were detected at significantly higher levels in the CD138+ samples (figure 4; online supplementary figure 3, table 5). Many of these proteins are used for diagnosis and prognosis in a range of tumor types (online supplementary table 6) and are known to be expressed in malignant hematological and non-hematological cells, as well as in some non-diseased tissues (figure 5). There was no difference in the abundance of 23 proteins related to CD138 status (online supplementary table 7). Six proteins were detected at lower levels in the CD138+ than the CD138– samples (online supplementary table 8), while 14 proteins were not detected in the MM patient cell lysates (online supplementary table 9). Failure to detect these proteins in primary cells could be related to differences in proteoforms present (additional modifications or truncations not present in the cell lines), or differences in expression between cell lines and primary MM samples, among other complications.

Analysis of published literature and reported limited expression on other cell types throughout the body, narrowed our interest to nine potential therapeutic targets for MM. Five of the selected proteins (CD5, CD166 (also known as ALCAM), CD147 (also known as Basigin or EMMPRIN), CD98hc (also known as 4F2 cell-surface antigen heavy chain), and CD205), are currently under investigation as therapeutic targets in other cancer types. An additional four proteins (LRBA, CLPTM1L, Homer3, and EFNB2), have not been previously reported as therapeutic targets in any malignancy, to our knowledge. In PRM analysis of the second independent cohort of MM patient samples, six of these

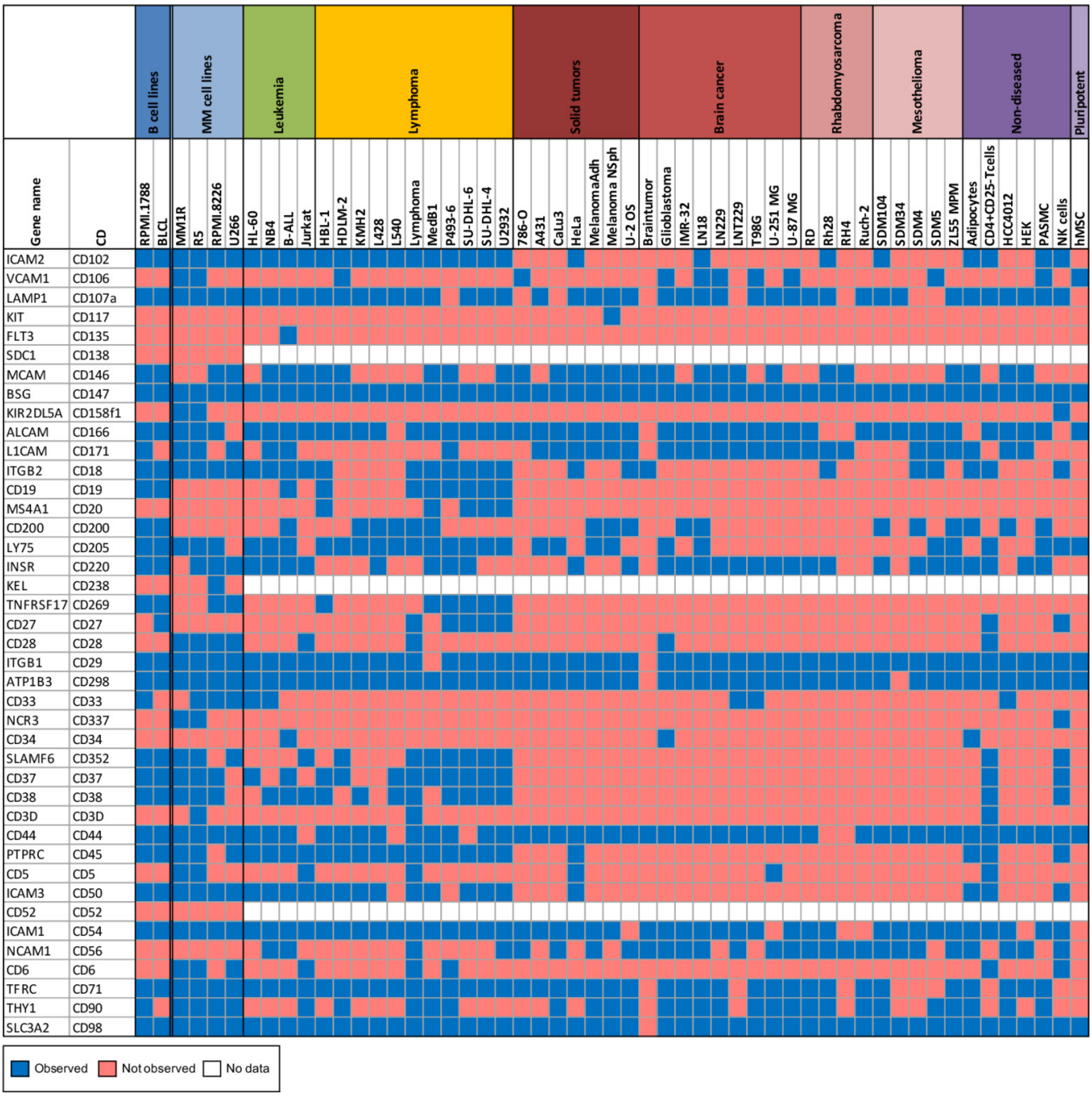

Figure 2 Matrix of cluster of differentiation (CD) molecules identified by cell surface capture (CSC) dataset that were chosen for parallel reaction monitoring assay development. For each CD molecule, its detection (observed vs not observed by CSC) among the B and multiple myeloma cell lines in the present study is indicated in the first six columns. Detection by CSC among human cell lines described in the cell surface protein atlas (CSPA)[15] is included for comparison. White squares indicate that data are not available for this protein in the CSPA.

proteins (LRBA, CLPTM1L, EFNB2, CD166, CD147, and CD98hc) were confirmed to be significantly higher on the CD138+ cell subset (figure 6). Two proteins (Homer3 and CD205) were present at higher levels but their abundance did not reach statistical significance, likely due to the small sample size (n=4). CD5 was not detected in this second analysis, thus our results for this protein are inconclusive.

Validation of therapeutic targets on live cells

Final validation regarding cell surface expression of candidate proteins was performed using FCM on CD138+ and CD138− MM patient cells. For the nine candidate proteins of interest, five corresponding monoclonal antibodies were identified that were suitable for FCM: anti-CD5, anti-CD98hc, anti-CD147, anti-CD166, and anti-CD205. Analysis of CD138+ patient samples revealed

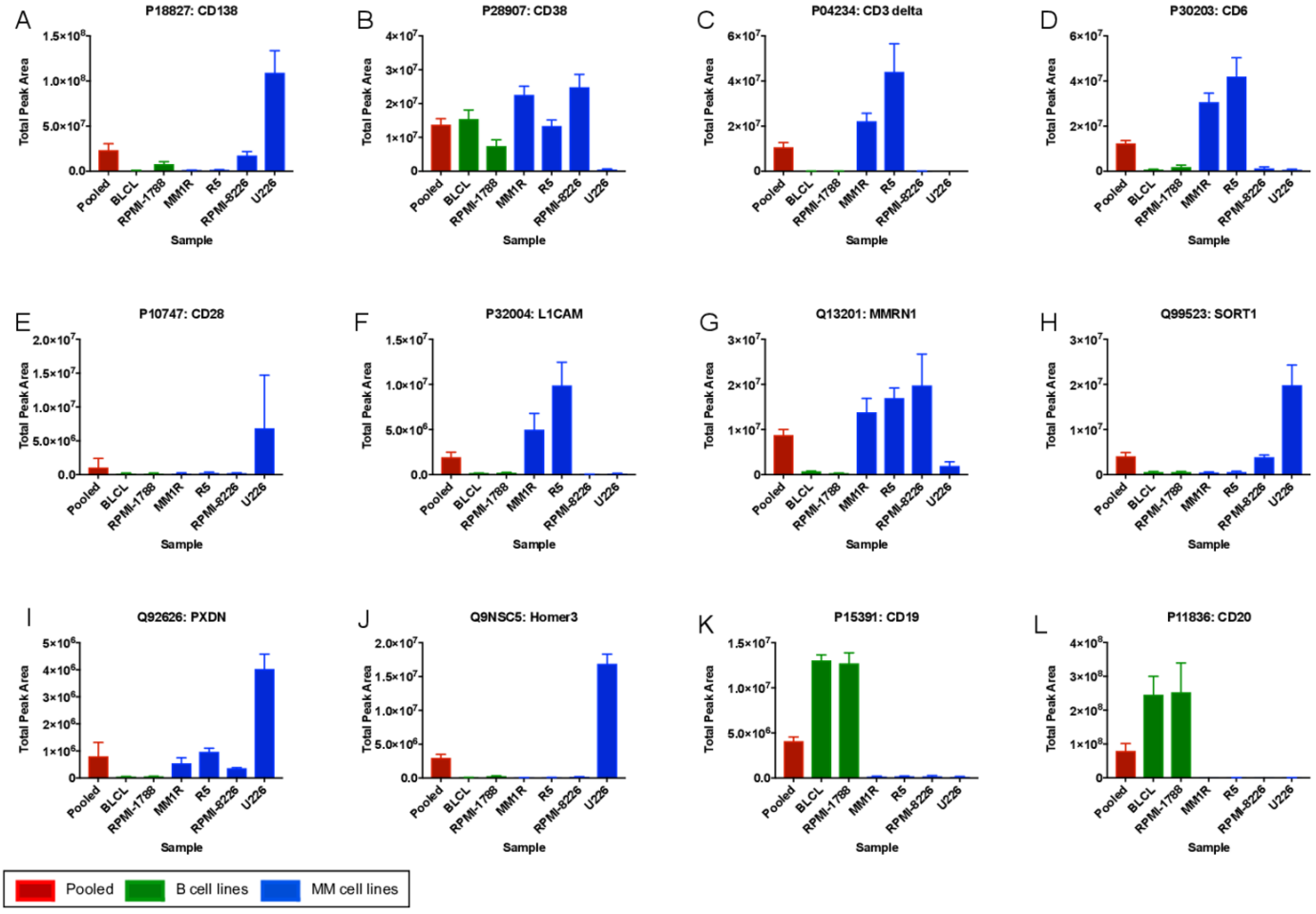

Figure 3 Relative abundance of selected proteins assessed by parallel reaction monitoring (PRM) analysis of multiple myeloma (MM) and B cell lines. (A) Syndecan-1 (CD138), (B) ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1 (CD38), (C) T-cell surface glycoprotein CD3 delta chain (CD3D), (D) T-cell differentiation antigen CD6, (E) T-cell-specific surface glycoprotein CD28, (F) neural cell adhesion molecule L1 (L1CAM, CD171), (G) multimerin-1 (MMRN1), (H) sortilin (SORT1), (I) peroxidasin homolog (PXDN), (J) Homer protein homolog 3 (Homer3), (K) B-lymphocyte antigen CD19, and (L) B-lymphocyte antigen CD20 were detectable by PRM in whole cell lysates from the MM and B cell lines. Individual B cell line peak areas are shown in green, and individual MM cell line peak areas are shown in blue. Abundance for a pooled sample control, comprising all six cell lines, is shown in red at the left of each graph.

the presence of all five antigens with some variation in expression levels (figure 7). Expression of CD5 was very low overall, present on 0.01%–5.7% of CD138+ cells, while CD147 and CD166 were expressed on nearly all CD138+ cells in most MM patient specimens examined (online supplementary table 10). MM patient-dependent variations in expression were also observed for CD98hc (1.0%–17.3% of CD38+ cells) and CD205 (25.1%–94.0% of CD138+ cells). Analysis of normal BMNCs, T cells, and B cells was included as a control; inter-donor variation in FCM assays was also observed in these cell types (online supplementary figure 4). Altogether, these FCM data provide orthogonal confirmation that the five candidate proteins of interest originally detected by MS are present on the cell surface of human primary MM cells. Monoclonal antibodies are not yet available for LRBA, CLPTM1L, EFNB2, and Homer3, precluding their validation by FCM at this time.

DISCUSSION

In this study we report a description of the MM cell surface *N*-glycoproteome based on CSC analysis of MM cell lines, followed by PRM and FCM validation of selected protein candidates in primary cells isolated from MM patient BM. While several MS-based studies of MM have been undertaken previously,[16–19] our study offers several advantages, including the use of CSC to specifically detect proteins present on the cell surface, the use of multiple cell lines to account for possible differences among patients, and the use of both PRM and FCM to determine if proteins of interest discovered on immortalized cell lines are relevant to primary human MM cells.

PRM analyses identified 30 proteins significantly higher in abundance in the CD138+ cells from MM patients compared with their CD138– subsets. Although a more informative sample for comparison would be B cells or CD138+ cells from the BM of non-MM patients,

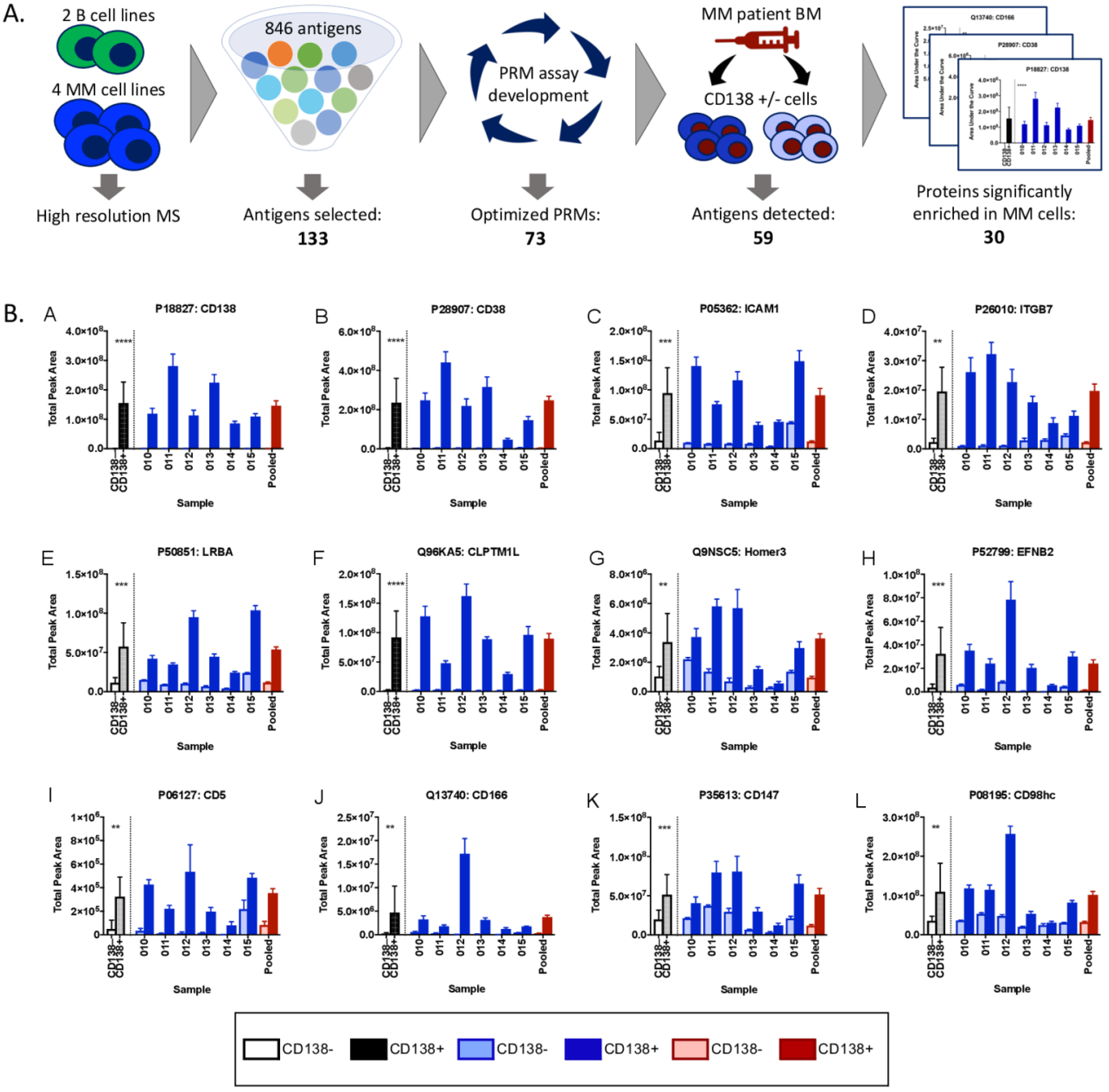

Figure 4 Relative abundance of selected proteins detected by parallel reaction monitoring (PRM) analysis of primary human multiple myeloma (MM) patient samples. (A) Target discovery and selection workflow. The workflow illustrates the steps completed and number of antigens included at each stage of analysis, from identification of cell surface proteins on MM cell lines by cell surface capture, through antigen selection, PRM development and, ultimately, detection of proteins of interest on MM patient samples using the optimized PRM assays. (B) Abundance of selected proteins detected by PRM in primary human MM patient samples (n=6). (A) Syndecan-1 (CD138), (B) ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1 (CD38), (C) CD54 (ICAM1), (D) integrin B7 (ITGB7), (E) lipopolysaccharide-responsive and beige-like anchor protein (LRBA), (F) cleft lip and palate transmembrane protein 1-like protein (CLPTM1L), (G) Homer protein homolog 3 (Homer3), (H) ephrin-B2 (Efnb2), (I) CD5, (J) activated leucocyte cell adhesion molecule (ALCAM, CD166), (K) basigin (CD147), and (L) 4F2 cell-surface antigen heavy chain (CD98hc) all have significantly higher abundance in CD138+ samples compared with CD138– samples. Individual patient peak areas are shown in blue, where dark shading represents the CD138+ fraction, and light shading represents the CD138– fraction. The average and SD of the six patients per condition is shown at the left in black (CD138+) and white (CD138–). Abundance for a pooled sample control, comprising all six cell lines, is shown in red at the right of each graph. This pooled sample was not included in statistical comparisons and is included as a control. The mean total fragment ion peak areas of the six patients' CD138+ and CD138– samples were compared using a parametric ratio paired t-test. Statistical significance is assigned by p value <0.05. On graphs, p value represented by annotations: n.s. for p>0.05, * for p<0.05, ** for p<0.01, *** for p<0.001, **** for p<0.0001. BM, bone marrow; MS, mass spectrometry.

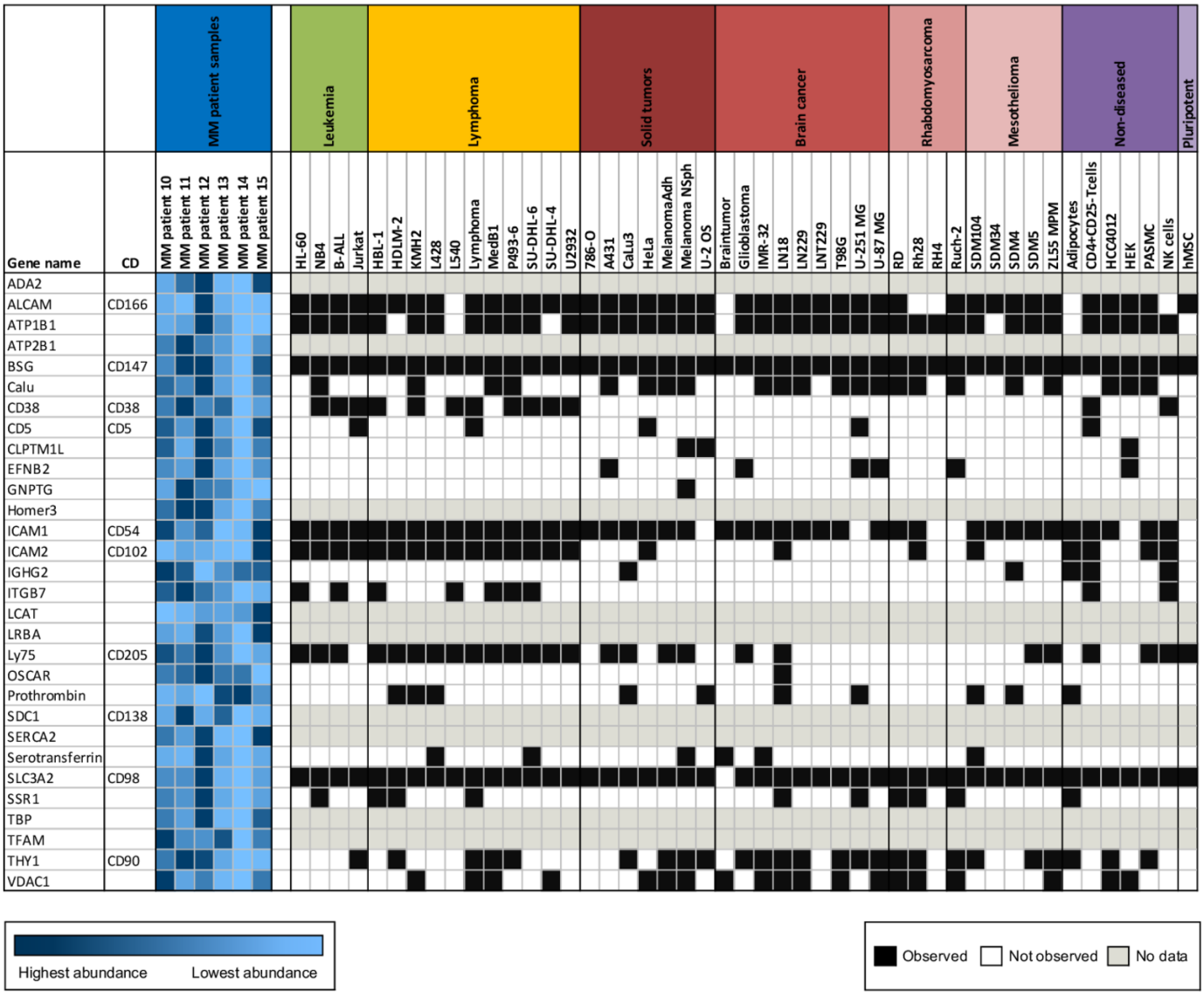

Figure 5 Matrix summarizing detection of proteins by parallel reaction monitoring (PRM) in multiple myeloma (MM) patient samples. Thirty proteins were detected at significantly higher abundance levels in the MM patient samples compared with their matched controls. For each protein, the gene name and cluster of differentiation (CD) annotation are listed. The color gradient in the first six columns indicates relative abundance by PRM within the six patient samples (dark blue=highest abundance, light blue=lowest abundance). Detection by cell surface capture among human cell lines as described in the Cell Surface Protein Atlas[15] is included for comparison (black=detected, white=not detected; gray=no data available).

BM samples from healthy individuals are challenging to obtain, and thus were not available for the present study. Our dataset of proteins with significantly higher abundance in the CD138+ subsets of cells includes known MM antigens, as well as proteins that may be involved in various MM pathologies including bone dysfunction, development and growth, metastasis and invasion, and therapy resistance.

Many of the proteins identified in this study as more abundant in MM than control cells, including: CD90, ATP1B1, CD5, CD98, ICAM-2, SERCA2, TBP, LRBA, EFNB2, Homer3, ADA2, CLPTM1L, and CD166, have been linked with poor prognoses in other cancer types. Proteins already associated with progression, poor prognosis, or drug resistance in MM include ICAM-1,[27] VDAC1,[28 29] ITGB7,[30 31] CD147,[32–34] and TFAM.[35] To our knowledge, calumenin, CD205, SSR1 (also known as translocon-associated protein (TRAP)-alpha), and GlcNAc-1-phosphotransferase subunit gamma (GNPTG) have not previously been described as diagnostic or prognostic indicators for any cancer type. Based on our results, however, they may be relevant markers for MM. Prothrombin (F2) and serotransferrin (TF) were also identified as highly abundant in our CD138+ subset, however, the universal expression of these proteins may make their development as therapeutic targets for MM unsuitable.

In MM patients, calcium levels are often dysregulated and elevated. For instance, hypercalcemia, a result of osteoclastic bone resorption and release of calcium into

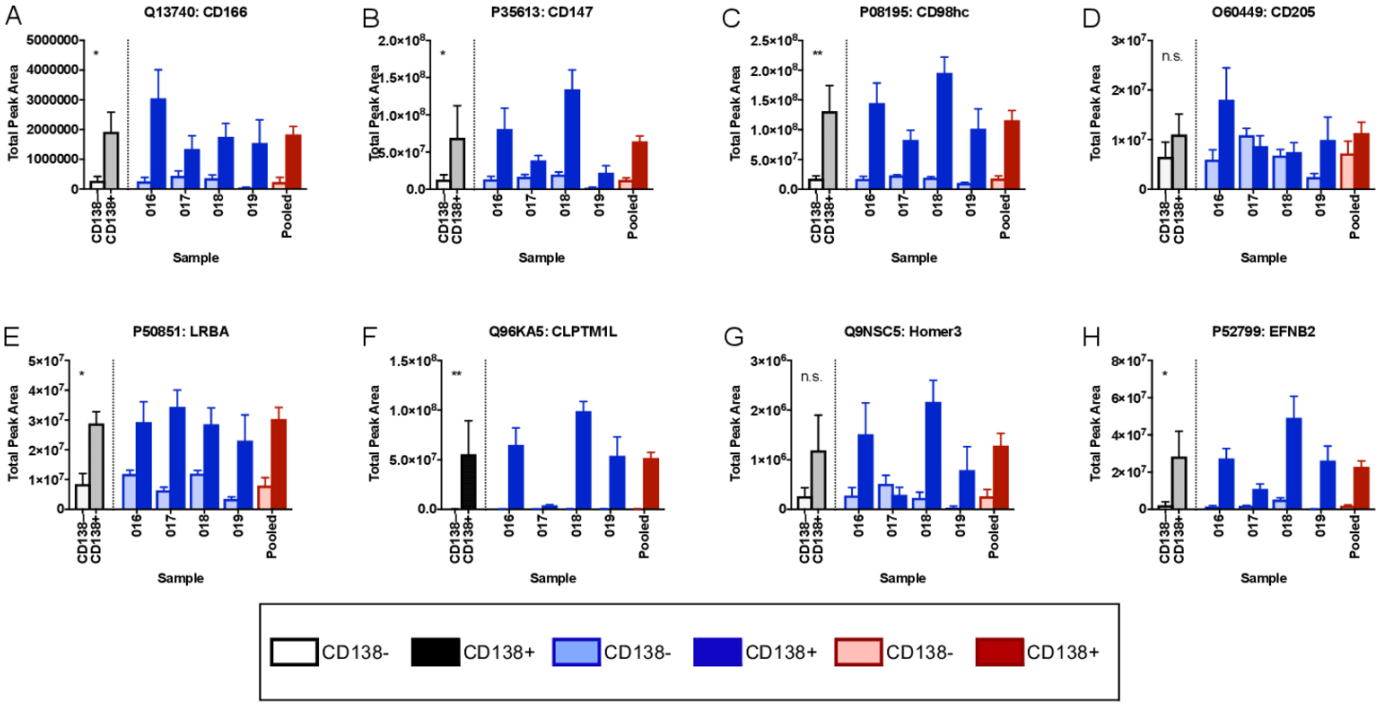

Figure 6 Validation of selected proteins detected by parallel reaction monitoring in a second cohort of primary multiple myeloma patient samples. (A) CD166, (B) CD147, (C) CD98hc, (D), CD205, (E) LRBA, (F) CLPTML1, (G) Homer 3, (H) EFNB2. Individual patient peak areas are shown in blue, where dark shading represents the CD138+ fraction, and light shading represents the CD138– fraction. The average and SD of the four patients per condition is shown at the left in black (CD138+) and white (CD138–). Abundance for a pooled sample control, comprising all six cell lines, is shown in red at the right of each graph. This pooled sample was not included in statistical comparisons and is included as a control. The mean total fragment ion peak areas of the six patients' CD138+ and CD138– samples were compared using a parametric ratio paired t-test. Statistical significance is assigned by p value <0.05. On graphs, p value represented by annotations: n.s. for p>0.05, * for p<0.05, ** for p<0.01.

the extracellular fluid, is frequently observed in MM patients with a large tumor volume. Consistent with this, various calcium-related proteins were identified in the CD138+ patient samples, including calumenin, SERCA2 (also known as ATP2A2), ATP2B1 (also known as PMCA1), and Homer3. Several proteins with known roles in BM homing and bone disease were also identified, such as TF, ITGB7, CD147, EFNB2, and CD166. Identification of known MM-linked proteins, and proteins involved in MM pathologies, supports the credibility and utility of this approach.

Unsurprisingly, differences in protein abundance levels among patient samples were observed. Several of these proteins are associated with roles in cancer migration and invasion, such as CD90 (also known as Thy-1),[36 37] CD147,[38 39] and Homer3.[40 41] Some are associated with growth/tumorigenesis, such as CD98,[42–44] SERCA2,[45] LRBA,[46] and CD166.[47 48] Proteins involved in resistance to apoptosis and therapy were also found to differ among the 10 MM patients. These variations may be related to MM stage, aggressiveness, or responsiveness to therapy, among other factors. Larger patient cohorts will be necessary in order to validate the diagnostic or prognostic impact of these findings.

We have identified nine promising MM immunotherapy targets that were originally detected in MM cell lines and subsequently validated in primary MM cells. All nine targets have been validated by PRM analysis of protein abundance at the whole cell level. Five target proteins have been further validated by FCM analysis of their abundance specifically at the surface of MM patient cells. While reported expression of some of these antigens on subsets of normal cell types does not preclude their use as immunotherapy targets, it does warrant caution in their development. Indeed, five of these nine targets, including CD5, CD147, CD205, CD98hc, and CD166, are already under investigation for other tumor types. The therapies developed in the context of those tumor types may therefore be subsequently tested for use in MM. This is important because safety information (including potential "on-target, off-tumor" effects) established in those studies may inform their application to MM. For example, a phase 1 clinical trial studying CD5 chimeric antigen receptors (CARs) for T-cell leukemia or lymphoma is currently underway (NCT03081910). A radio-immunotherapy product targeting CD147 has been evaluated in clinical trials for hepatocellular carcinoma.[49] There is also an antibody-drug conjugate against CD205 (also known as LY75) currently under phase 1 clinical trial investigation for non-Hodgkin's lymphoma (NCT03403725). A phase 1 clinical trial was recently completed for a mAb recognizing CD98 to treat

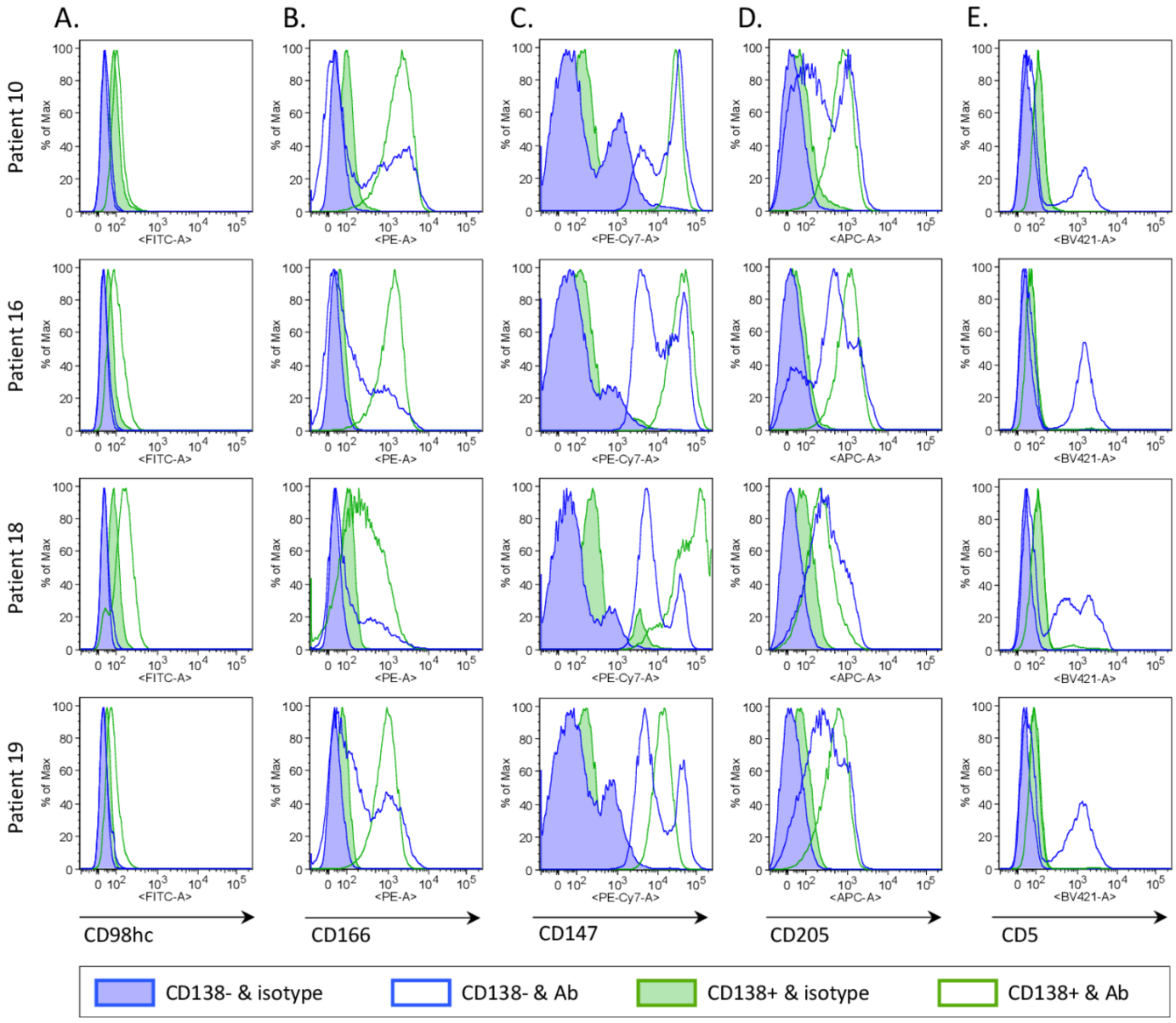

Figure 7 Flow cytometric analyses of target antigen expression on primary multiple myeloma (MM) samples. Expression of the five candidate antigens on CD138+ MM cells (blue) and CD138– BM cells (green) from matched patient samples. (A) CD98hc, (B) CD166, (C) CD147, (D) CD205, (E) CD5. CD138+ and CD138– isolated BM cells from four patients were available for flow cytometry analysis. The x-axis shows log-10 fluorescence intensities for each antibody, while the y-axis shows cell counts normalized to maximum of cells collected for each sample (20,000 cells per sample). Staining with antibody is shown as open histograms; isotype staining is shown as shaded histograms.

relapsed or refractory acute myeloid leukemia (AML) (NCT02040506). In a phase 1/2 study for selected solid tumors (NCT03149549), an antibody-drug conjugate targeting CD166 is currently being evaluated.

Beyond the well-known proteins identified in our study, additional proteins of interest have been described in MM or other cancers but, to our knowledge, have yet to be tested clinically as immunotherapy targets. This includes LRBA,[46] EFNB2[50] and CLPTM1L.[51 52] Also, Homer3 has not previously been identified as a therapeutic target; however, interestingly, anti-Homer3 antibodies have been found in MM patients with complete response to donor lymphocyte infusion,[53] suggesting that this protein may also be a promising antigen for immunotherapy. It is expected that when suitable monoclonal antibodies are available for LRBA, CLPTM1L, EFNB2 and Homer3, similar FCM-based validation efforts will be possible for these targets.

In addition to providing a detailed view of the MM cell surface and identifying new therapeutic targets, we have developed PRM assays that may be applied to patient biopsies for diagnostic or disease monitoring purposes. Currently, MM monitoring is carried out using techniques such as assessment of paraprotein levels and quantification of percentages of plasma cells in the BM; however, more informative readouts that are quick,

accurate, and sensitive would benefit the care of MM patients. This is especially relevant given the heterogeneity of MM, the continued development of new therapies, and the need for individual patient analyses when administering targeted immunotherapies. In the long-term, it is possible that MM patients could be efficiently screened using an MS-based assay to inform selection of an appropriate personalized therapy, as well as to track response or resistance to treatment over time. To support this effort, the PRM assays developed here are freely available in Panorama.

CONCLUSIONS

This study contributes to knowledge and understanding of the MM cell surface and provides a rich resource to inform future studies aimed at characterizing malignancy. We have identified nine proteins that may be relevant, novel MM immunotherapy targets, as well as multiple proteins of prognostic and/or biological interest. Further clinical validation of these novel MM targets and assays will expand the ability to diagnose, monitor, and treat this disease, with the goal of improving patient outcomes and quality of life.

Acknowledgements We would like to thank the patients involved in this study. The BLCL cell line was kindly provided by Dr Fenlu Zhu of the MCW Cell Therapy Laboratory. We thank Dr Bryon Johnson and the MCW Cell Therapy Laboratory for providing us with discarded anonymous apheresis products, and the Versiti Blood Center of Wisconsin for making available discarded anonymous LRS cones. We thank Carissa Ahrenhoerster (MCW) for technical assistance with patient sample acquisition and processing. Use of the BD LSRFortessa X-20 flow cytometer was provided by the CRI/Cancer Center Flow Cytometry Core at MCW. MS analyses were performed using instrumentation in the Center for Biomedical Mass Spectrometry Research at MCW. RAAO is a PhD candidate at the University of Toronto; this work is submitted in partial fulfillment of the requirement for the PhD degree.

Contributors RAAO, MLF, TRK, and MW performed experiments. RAAO, MLF, TRK, ARB, and RLG analyzed results and made the figures. RAAO, MLF, PH, RLG, and JAM designed the research and wrote the paper. The current affiliation for RLG is CardiOmics Program, Center for Heart and Vascular Research; Division of Cardiovascular Medicine; and Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE, 68198, USA.

Funding This work was generously supported by Midwest Athletes Against Childhood Cancer (MACC) Fund (JAM) and by National Institutes of Health awards R01-HL126785 and R01-HL134010 (RLG); F31-HL140914 (MW).

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Informed consent was obtained from all MM patients under MCW IRB approval #PRO00027134.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Datasets supporting the conclusions of this article are available in a public, open access repository. Original mass spectrometry data have been deposited to MassIVE (MSV000084858) and Panorama (https://panoramaweb.org/MedinMM.url).

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