

Cell-specific proteome profiling reveals altered peripheral immune cell communication in early multiple sclerosis

Corresponding Author: Dr Hengameh Shams

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Overall, very abstract terms are being used, such as monocytes being 'the dominant source of altered intercellular communication', B cells functioning 'as key intermediaries linking immune subsets' and 'coordinated MS-associated immune perturbation centered on monocyte-driven output and B-cell mediated signal integration'. The results should be more specified and put into context. Also the mentioned overlap of dysregulated proteins with those reported in other disorders is too generically stated. When reading an abstract, it should be immediately clear what has been found.

The analysis of >4,000 proteins across 4 main immune subsets of completely treatment-naïve MS patients is an obvious strength of this study. The advantages and differences in results using this technology should be better explained or reflected against other studies looking at immune subset proteomes in general (e.g. Lee et al., Sci Data 2024) and in MS (e.g. Berge et al., Clin Proteomics 2019; Cappelletti et al, Clin Proteom 2022). The robustness of their used methodology and the new findings that come out of it will be strengthened by also confirming results from other studies.

Were the DEPs found in the 4 immune subsets influenced by disease activity close to sampling or disease progressiveness? If so, how exactly for each top significantly associated protein per subset? This should be tested and included.

How about subset distribution per immune subset? For B and T cells, at least differences in naïve and memory frequencies will skew data enormously and should preferably be corrected for. Were these frequencies similar between patients and controls and/or associated with the DEPs identified? How about monocytes with and without CD16, which represent functionally distinct subsets?

In the GO enrichment analysis, the 'cell killing' ontology that is claimed for CD4+ T cells would be more convincing by confirming that CAMP/CTSH/DEFA1 downregulation indeed positively correlates with cytolytic proteins such as GZMB and/or PRF. This should then also be the case for classical CD8+ CTLs.

Hugely different GO's were identified for the 4 immune subsets, but besides for confirmation of fibrinogen levels, other functional assays and/or interpretation or connections of ontologies in a sensible way would help to better interpret these data and linking these to the pathogenesis of MS (the main rationale for this study). Overall, many of the GO's are very generic and/or not biologically related to each immune subset ('blood coagulation', 'wound healing', neutrophil degranulation' etc), therefore lowering the impact of the results. When reading the manuscript including the Discussion, I kept asking myself what each finding has now learned us about the pathogenesis of MS. The conclusions or statements made are too general (e.g line 168: 'these findings suggest that both structural proteins, secreted factors, and ligand-receptor interactions are all affected in MS') and although attempts are appreciated, biological interpretations remain too far-fetched or speculative based on these types of data.

Line 156: fibrinogen levels were tested based on results in CD19+ B cells? This is not so clear. Why not testing functional components as found in the predominant monocyte compartment as claimed by the authors?

The authors looked at 551 putative MS susceptibility 'genes'. How were they defined? Most of the known risk SNPs for MS are located in non-coding regions and may act on other genes. Could it still be possible that risk SNPs themselves do affect

proteins in CD4+ T (and B) cells, which would make more sense as these are the most predicted cells to be genetically affected (e.g. Farh et al, Nature 2015)? Line 186: 'CD14+ monocytes and CD19+ B cells show the most pronounced overlap'; between each other of with risk genes (n=9 and n=6)? If the latter, how about CD8+ T cells showing n=7 DEPs that overlapped?

Similar to Fig. 3, neutrophil degranulation was found in their functional enrichment analysis of overlapping genes in monocytes (Fig. 2A). How to relate and interpret these data? Were these determined by the same genes and proteins?

In the Discussion at p17 (line 372), the authors highlight that CTSH is overexpressed in B cells, monocytes and T cells. However, this is not indicated in the Results section and CTSH is even found to be downregulated in CD4+ T cells (line 142-144). This should be revised. The same is true for other proteins that are discussed but not part of the Results, making it unclear to which exact dataset the authors refer to.

DEPs in several immune cells are enriched in several unrelated disorders, including Aicardi-Goutières syndrome, severe congenital neutropenia etc. No other autoimmune diseases were found, while being closer related to MS. How to explain and follow up on this? At least results from GWAS support the fact that there are overlapping immune cells, pathways and molecules between autoimmune diseases. Monocytes are also indicated as main source of immune related problems, while GWAS studies point towards B and CD4+ T cells as the primary immune subsets being deregulated (e.g. Farh et al, Nature 2015; Guo et al, Genome Biol 2022). The same accounts for EBV as the major environmental risk factor for MS, which primarily infects B cells. How can this be explained? This should be discussed. Could the highest outgoing strength by monocytes (line 239) be influenced by their increased size or granularity as compared to B and T cells?

Line 209-286: very large Results section with a lot of non-common terms and probably written by a bioinformatics expert. This should at least be clarified for readers with less bioinformatics background. I cannot properly judge the analysis and impact of their data.

Discussion, line 311: ..perturbations at the onset of clinical manifestations in MS. Have patients been selected at a very early stage of their disease? If yes, this should be emphasized. If not, this should be reformulated. The same accounts for 'early' proteomic alterations (line 442-443).

One of the pathways that is highlighted and discussed is the immune response to pathogens (line 358). Again, this is too general and already looked at in more detail over many years. For example, line 417: 'GO enrichment analysis revealed cell type-specific downstream programs which are consistent with altered ligand-receptor communication and highlight molecular programs engaged by each immune subsets'. I find it very difficult to make anything out of these kinds of statements.

Textual

P1, title: should be specified; 'identifies' should not be used in this context

P1, title: unclear what is meant by 'B cell-mediated network connectivity'

P4, line 67: 'expression' should not be used in this context

P6, line 108: unclear what is meant by 'loadings'

P8, line 171: subheading is too generic; 'proteins' should be 'protein'

Reviewer #2

(Remarks to the Author)

The manuscript by H Shams et al entitled: Immuno-proteome profiling identifies multiple sclerosis patients and reveals monocyte hubs and B cell-mediated network connectivity performs a proteomic study of CD4 and CD8 T cells, of B cells and monocytes from 29 treatment naïve MS patients and 25 healthy controls (paired in age and being all females). Differentially expressed proteins exhibited predominantly cell type-specific patterns. Authors described clearly different signatures in the four leukocyte subsets in MS patients compared to controls. The clearest differences were found in monocytes, followed by B cells. They also found an early involvement of fibrinogen cascade, confirmed by the finding of significantly higher levels of serum fibrinogen found in MS patients compared with controls. These data are interesting and may contribute to reveal MS pathology. However, some issues should be addressed.

1. Authors should explore the generality of their findings by exploring if their results are also found in males. This is specially relevant in MS, where clear differences associated with sex exist. In this line, the disease is more prevalent in women but male sex associates with a worse prognosis.
2. In the same line, results describing high levels of fibrinogen in serum of MS patients should be confirmed in a larger cohort of MS patients and controls.
3. Authors should also explore if serum levels of any of the proteins most clearly associated with MS are elevated in serum of patients compared with controls.
4. Associations of DEP with MS susceptibility genes seem to be modest. Due to the low numbers of patients analyzed, authors should state that these data are exploratory and should be confirmed in larger series.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors satisfactorily implemented my suggestions and took away most of my concerns, besides for the abstract terms, statements and conclusions used.

Some remaining remarks:

Line 1: the use of 'signaling' suggests a signaling cascades, which is not the case. So better to use another term

Line 25: ..proteomic changes before treatment initiation.. add early disease/MS to clarify

Line 44-46: CD14⁺ monocytes display the strongest inflammatory and adhesion-associated interaction signals, whereas CD19⁺ B cells occupy a central position within the inferred immune interaction network and are enriched for pathways related to CD19⁺ B-cell differentiation, adhesion, and immunological synapse function.

Please reformulate and specify. Both are seemingly associated with adhesion molecules (but whereas suggests a contradiction) and CD19⁺ B cell is mentioned twice.

Line 48: indicate in the conclusions that specific proteins or protein profiles have been found that associate with.. still abstract terms are used throughout the abstract

Line 368: DEGs overlap with MS genetic risk loci.. this suggests things that are not shown. What is meant with 'overlap'? Gene = protein? Better to use another term to specify this finding

Line 517-519: Our multi-cell-type analytical approach highlighted a CD14⁺ monocyte-centered remodeling of immune crosstalk in early MS and identified CD19⁺ B cells as key network connectors.

Line 520: disease-associated signaling interactions

Please specify, because such statements are uninterpretable

Line 649: not only consequences, but also the cause of peripheral immune dysregulation?

Reviewer #2

(Remarks to the Author)

The authors have answered most of my questions. However, due to the relatively low numbers of patients included, despite the careful selection of patients, it should be clearly stated in the discussion that the results should be validated in larger cohorts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have processed most of my concerns, besides for my reservation about the abstractness of the results, e.g. how changes in 'adhesion', 'differentiation' or 'communication' will move the field further even if validated in larger cohorts.

Reviewer #2

(Remarks to the Author)

The authors addressed adequately all my queries.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We are pleased to submit a revised version of our manuscript now retitled “Cell-specific proteome profiling reveals altered peripheral immune signaling in early multiple sclerosis (COMMSMED-26-0472A)”. We appreciate the constructive comments of the reviewers, based on which we have made revisions that have undoubtedly improved the manuscript. Below is a point-by-point response to the reviewer’s comments. Modifications in the revised manuscript are highlighted in yellow, and new supportive data are presented in Supplementary Figures S1, S3, and S6, Table S1, and Supplementary Data1 and Data2.

Reviewers’ comments:

Reviewer #1 (Remarks to the Author):

Overall, very abstract terms are being used, such as monocytes being ‘the dominant source of altered intercellular communication’, B cells functioning ‘as key intermediaries linking immune subsets’ and ‘coordinated MS-associated immune perturbation centered on monocyte-driven output and B-cell mediated signal integration’. The results should be more specified and put into context. Also the mentioned overlap of dysregulated proteins with those reported in other disorders is too generically stated. When reading an abstract, it should be immediately clear what has been found.

We thank the reviewer for this important comment. We agree that the original wording, particularly in the abstract was overly broad and did not sufficiently specify the nature of the observed disease enrichments. We have therefore revised the abstract and Results section to more clearly describe the biological themes underlying the identified associations. For example, we now state that the dysregulated proteins were enriched for pathways related to mitochondrial dysfunction, oxidative phosphorylation, fatty acid metabolism, and innate immune responses, with the strongest enrichment signals observed in CD14⁺ monocytes and CD19⁺ B cells. Please refer to the revised Abstract and Results section in the manuscript for the updated text. These sections have not been reproduced in full in this response letter to avoid unnecessarily lengthening the document.

The analysis of >4,000 proteins across 4 main immune subsets of completely treatment-naïve MS patients is an obvious strength of this study. The advantages and differences in results using this technology should be better explained or reflected against other studies looking at immune subset proteomes in general (e.g. Lee et al., Sci Data 2024) and in MS (e.g. Berge et al., Clin Proteomics 2019; Cappelletti et al, Clin Proteom 2022). The robustness of their used methodology and the new findings that come out of it will be strengthened by also confirming results from other studies.

We thank the reviewer for this important comment regarding proteome depth and methodological considerations. The proteomic depth in this study is broadly comparable to previous studies using similar sample preparation and data acquisition strategies. For example, a 2019 study using data-dependent acquisition (DDA) identified approximately 2,031 and 2,259 proteins in CD4⁺ and CD8⁺ T cells, respectively, values that are within the same range as those obtained in our dataset despite differences in cohort design and analytical workflow (Berge et al. 2019). Importantly, our study employed data-independent acquisition (DIA), which has been shown across numerous proteomic studies to improve proteome coverage, quantitative reproducibility, and sensitivity compared to conventional DDA approaches, particularly for low-input and complex biological samples. This likely contributed to the increased number and consistency of protein identifications observed in our dataset relative to earlier comparable studies. We also note that a more recent study reported substantially higher protein identifications using an SP3/on-bead digestion workflows with magnetic beads, a protocol specifically optimized to enhance peptide recovery and digestion efficiency for low-input samples (Brunner AD et al., 2022). However, although this newer approach can improve peptide recovery, bead-based enrichment strategies may also introduce potential biases related to protein physicochemical properties, including size and charge-dependent interactions with the beads. For this reason, we chose to apply a more established analytical workflow aimed at preserving a proteomic representation as close as possible to the in vivo cellular state. Nevertheless, given the improved analytical performance of DIA relative to DDA, future

studies combining DIA acquisition with optimized low-input workflows could further expand proteome coverage beyond that reported here. We also added the following to page 25 of the Discussion:

“The proteomic depth of our analysis is broadly comparable to previous studies using similar workflows (Berge et al. 2019 & Cappelletti et al 2022). Importantly, DIA has been shown across numerous proteomic studies to improve proteome coverage, quantitative reproducibility, and sensitivity compared with conventional DDA approaches, particularly for low-input and complex biological samples, likely contributing to the increased consistency of protein identification observed in our dataset relative to earlier studies.”

Were the DEPs found in the 4 immune subsets influenced by disease activity close to sampling or disease progressiveness? If so, how exactly for each top significantly associated protein per subset? This should be tested and included.

We thank the reviewer for this important point. All samples in this study were collected from newly diagnosed, treatment-naïve MS patients within the early post-diagnosis period and prior to treatment initiation window. This stringent criterion was specifically designed to minimize confounding effects from treatment, long-term disease progression, or heterogeneous disease duration. As such, the identified DEPs are expected to primarily reflect early disease-associated immune alterations rather than cumulative disease progression. While detailed longitudinal or progression-related clinical measures were not available for this cohort, we have clarified this point on page 25 of the revised manuscript:

“One important strength of this study is the highly stringent sample collection strategy, as all samples were obtained early after diagnosis and prior to treatment initiation. This design minimizes confounding effects related to therapy, prolonged disease-associated immune remodeling, and chronic inflammatory adaptation, thereby enabling more accurate characterization of immune proteomic alterations associated with early MS.”

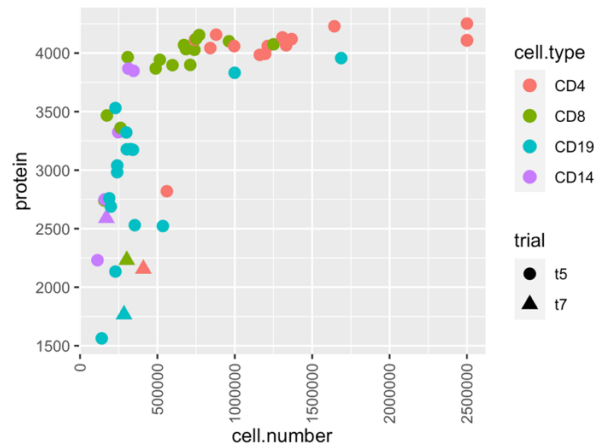
How about subset distribution per immune subset? For B and T cells, at least differences in naïve and memory frequencies will skew data enormously and should preferably be corrected for. Were these frequencies similar between patients and controls and/or associated with the DEPs identified? How about monocytes with and without CD16, which represent functionally distinct subsets?

The reviewer raises an important point. In this study, immune cell populations were sorted at the level of major subsets (CD4+ and CD8+ T cells, CD19+ B cells, and CD14+ monocytes), and we did not further separate naïve and memory T/B-cell compartments or CD16-defined monocyte subsets. We agree that differences in subset composition could, in principle, influence the observed proteomic profiles.

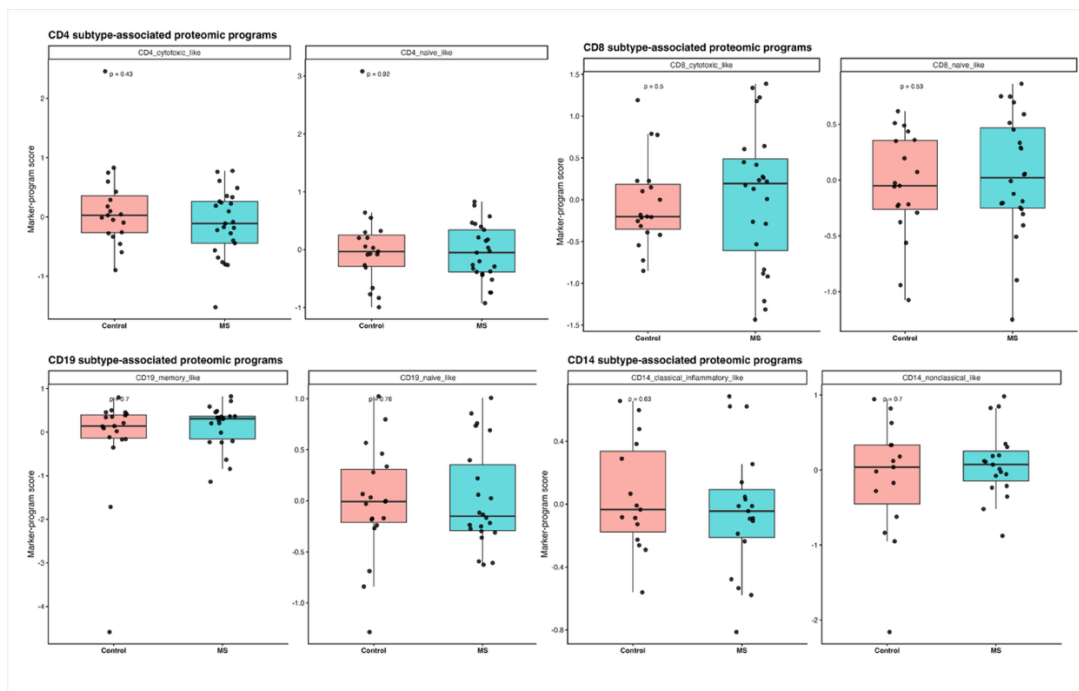
However, our study was specifically designed to capture global, cell type-level proteomic alterations in early, treatment-naïve MS rather than to resolve finer immunophenotypic heterogeneity. Importantly, all samples (patients and controls) were processed using the same sorting strategy, which reduces systematic bias between groups. In addition, as noted above, our cohort was selected to include patients at disease onset and prior to treatment initiation, thereby minimizing confounding effects related to therapy or chronic disease progression. It is noteworthy that such stringent recruitment criterion substantially limited sample availability as well as the amount of material obtained from each donor, which constrained cohort size and precluded further subpopulation sorting and additional functional validation experiments on the same samples.

We would also like to note that proteomic depth in low-input primary immune cell samples is highly dependent on the number of cells available for analysis. In our dataset, we observed a strong relationship between input cell number and the number of proteins identified, indicating that the original cohort size and sample input were not sufficient to support robust subpopulation-level proteomic analyses while maintaining adequate proteome coverage. Expanding the analysis to finer immune subsets would

therefore have required substantially larger sample numbers and/or relaxed inclusion criteria, which could in turn introduce additional biological and technical confounding effects. To illustrate this limitation, we have included an additional supplementary plot in the response letter showing the relationship between cell input and the number of proteins identified across samples from two independent runs:



To further address this point, we performed an orthogonal marker-based subtype program analysis using established subtype-associated proteins detected in our proteomic dataset. Specifically, we evaluated naïve-like, memory-like, cytotoxic/effector-like, inflammatory, and antigen-presentation-associated programs across the sorted CD4+, CD8+, CD19+, and CD14+ populations. Overall, we did not observe strong evidence for major shifts in broad subtype-associated proteomic programs between MS and control samples, including within CD19+ B cells, where naïve-like and memory-like marker-program scores were largely comparable between groups (Fig. S6, shown below). These findings suggest that the major disease-associated proteomic differences identified in this study are unlikely to be solely explained by overt shifts in broad immune subset composition.



At the same time, we acknowledge that some of the identified differences in protein abundance may still reflect, at least in part, changes in the relative proportions or activation states of specific immune subpopulations. This interpretation is consistent with our previous observation of a significant increase in plasmablast-like populations within peripheral CD19+ B cells at disease onset (Ma et al., 2020).

Nevertheless, we believe that profiling purified major immune populations at this very early clinical stage provides valuable insight into the global immune alterations associated with MS onset. We have clarified these points on page 23 of the Results section:

“Because the four major immune cytotypes included in the ligand–receptor network analysis were analyzed as bulk-sorted populations, we next assessed whether the observed proteomic alterations could reflect broad shifts in immune subset composition. Marker-based subtype program analysis was therefore performed across sorted immune populations using established subtype-associated protein signatures (Fig. S6). No strong evidence for major shifts in broad naïve-like, memory-like, or inflammatory-associated proteomic programs was observed between MS and control samples across the analyzed immune populations, suggesting that the identified disease-associated signatures are unlikely to be solely explained by large-scale differences in major immune subset composition.”

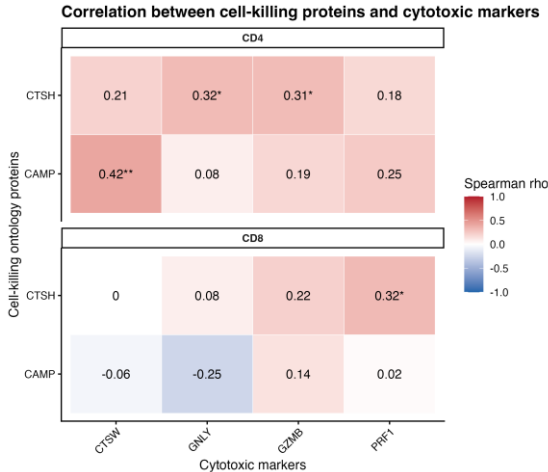
And page 30 of the Discussion:

“Several limitations still exist in our study. First, immune populations were analyzed at the level of major sorted subsets rather than finer immunophenotypic compartments. Although exploratory subtype-associated program analysis did not support major broad compositional shifts, some observed proteomic differences may still reflect disease-associated changes in the relative abundance or activation of specific subpopulations.”

In the GO enrichment analysis, the ‘cell killing’ ontology that is claimed for CD4+ T cells would be more convincing by confirming that CAMP/CTSH/DEFA1 downregulation indeed positively correlates with cytolytic proteins such as GZMB and/or PRF. This should then also be the case for classical CD8+ CTLs.

We thank the reviewer for this helpful suggestion. To further evaluate the biological relevance of the “cell killing” ontology, we examined correlations between proteins contributing to this enrichment and canonical cytotoxic markers across samples within both CD4+ and CD8+ T-cell populations. CTSH showed consistent positive correlations with cytotoxic proteins including GZMB, PRF1, and GNLY in both subsets, while CAMP showed a more selective association pattern, including correlation with CTSH in CD4+ T cells. These findings support an association between the observed enrichment and cytotoxic/effector-related immune programs rather than isolated protein changes. We have added these analyses to page 16 of the revised manuscript and Supplementary Figure S3 (copied here for convenience):

“The consistent correlation between these proteins and canonical cytotoxic proteins further support the biological association between the “cell killing” enrichment and cytotoxic/effector-associated immune programs (Fig. S3).”



Hugely different GO's were identified for the 4 immune subsets, but besides for confirmation of fibrinogen levels, other functional assays and/or interpretation or connections of ontologies in a sensible way would help to better interpret these data and linking these to the pathogenesis of MS (the main rationale for this study). Overall, many of the GO's are very generic and/or not biologically related to each immune subset ('blood coagulation', 'wound healing', 'neutrophil degranulation' etc), therefore lowering the impact of the results. When reading the manuscript including the Discussion, I kept asking myself what each finding has now learned us about the pathogenesis of MS. The conclusions or statements made are too general (e.g line 168: 'these findings suggest that both structural proteins, secreted factors, and ligand-receptor interactions are all affected in MS') and although attempts are appreciated, biological interpretations remain too far-fetched or speculative based on these types of data.

We agree that several GO terms identified across the immune subsets are broad and may not, by themselves, directly explain MS pathogenesis. In response, we have revised the Results and Discussion sections to place greater emphasis on biologically interpretable pathways and to avoid overgeneralization of the findings. Rather than focusing on isolated GO categories, we now emphasize converging functional themes across the datasets, including altered fibrinogen-associated signaling, immune cell communication, cytoskeletal remodeling, and inflammatory response programs observed at disease onset. We have also tempered several statements throughout the revised manuscript to avoid interpretations that extend beyond the scope of the data.

Line 156: fibrinogen levels were tested based on results in CD19+ B cells? This is not so clear. Why not testing functional components as found in the predominant monocyte compartment as claimed by the authors?

Regarding the fibrinogen validation experiment, the proposed model was derived from the GO enrichment analysis. Specifically, we hypothesized that increased circulating fibrinogen levels in MS may result in enhanced fibrinogen-associated interactions with peripheral immune cells. We selected the CD19⁺ B cell dataset for follow-up because "fibrin clot formation" represented the most significantly enriched ontology in this cell population. Therefore, the serum fibrinogen analysis was designed as a targeted validation of this GO-derived model rather than as a functional readout of the monocyte compartment. At the same time, we agree that the prominent signaling and interaction changes observed in CD14⁺ monocytes are highly relevant and warrant further mechanistic investigation in future studies.

The authors looked at 551 putative MS susceptibility 'genes'. How were they defined? Most of the known risk SNPs for MS are located in non-coding regions and may act on other genes. Could it still be possible that risk SNPs themselves do affect proteins in CD4+ T (and B) cells, which would make more sense as these are the most predicted cells to be genetically affected (e.g. Farh et al, Nature 2015)? Line 186: 'CD14+ monocytes and CD19+ B cells show the most pronounced overlap'; between each other of with risk genes (n=9 and n=6)? If the latter, how about CD8+ T cells showing n=7 DEPs that overlapped?

We thank the reviewer for this important comment. To clarify how the genes were defined, the list of 551 putative MS susceptibility genes was derived from GWAS-prioritized gene sets and nearest genes to risk loci, and we have now clarified this definition in the Methods section. In addition, the overlap between DEPs and HMAGMA-predicted target genes of MS risk SNPs was assessed using previously published datasets. HMAGMA integrates genome-wide association signals with 3D chromatin architecture to prioritize putative causal genes in a cell type-specific manner (Ma et al., 2023).

We also agree with the reviewer's interpretation. Currently available MS risk gene lists are based on in silico prioritization approaches using predefined criteria, including physical proximity to risk loci, eQTL associations, chromatin interactions, and regulatory scoring methods. While these strategies provide valuable insight into potential disease mechanisms, they remain imperfect approximations of the biological processes occurring in vivo, particularly given the complexity of long-range chromatin regulation and context-dependent gene activity. In contrast, the DEPs identified in our study are based on direct experimental measurements of protein abundance in purified immune cell populations. Since no currently

available approach can fully resolve SNP-to-gene relationships or confidently define cell type-specific regulatory effects, we aimed to avoid overinterpretation of these associations. Nevertheless, given this complexity, even modest overlaps between susceptibility genes and DEPs remain of interest and may provide biologically relevant insight into disease-associated pathways.

In line with this, we have revised the text to more carefully frame the overlap between DEPs and MS susceptibility genes, emphasizing that these associations are indirect and do not imply that the identified proteins are necessarily the direct targets of risk variants. We also acknowledge the possibility that genetic risk may preferentially act through specific immune cell types, including CD4⁺ T cells and B cells, as suggested by prior studies (e.g., Farh et al., Nature 2015), and have incorporated this context into page 28 of the Discussion as follows:

“The gene-protein associations identified in this study are indirect and should not be interpreted as evidence of direct causal regulation of the corresponding proteins by MS risk variants. In fact, currently available risk gene annotations are largely based on in silico prioritization approaches and do not necessarily identify the direct functional targets of disease-associated variants. Moreover, many MS risk variants reside in non-coding regions and may exert long-range regulatory effects, making the assignment of specific SNPs to individual genes inherently uncertain. Nevertheless, the presence of overlapping signals between experimentally measured proteomic alterations and genetically prioritized pathways remains of interest, particularly given prior evidence that MS genetic susceptibility may preferentially act through specific immune populations (Farh et al., 2015).”

Regarding the overlap counts, the reviewer is correct that the original text could be interpreted ambiguously. We have clarified that the reported numbers (e.g., n=9 for CD14⁺ monocytes and n=6 for CD19⁺ B cells) refer to overlaps between DEPs identified within each immune cell population and the MS susceptibility gene set, rather than overlaps between immune cell types themselves. We have revised the text accordingly to avoid confusion. We also note that all immune cell populations showed a broadly comparable number of overlapping DEPs, with CD14⁺ monocytes showing the highest overlap. For completeness, we have now incorporated this clarification into page 19 of the Results section as follows:

“The ‘n’ values indicate the number of overlapping proteins between DEPs identified in each immune cell population and the MS susceptibility gene set. Overall, all cell types demonstrated a comparable degree of overlap, with CD14⁺ monocytes showing the highest number of overlapping proteins.”

Similar to Fig. 3, neutrophil degranulation was found in their functional enrichment analysis of overlapping genes in monocytes (Fig. 2A). How to relate and interpret these data? Were these determined by the same genes and proteins?

We thank the reviewer for this helpful comment. We agree that the recurrence of neutrophil degranulation-related enrichment across analyses required clarification. We have now compared the underlying genes contributing to this term in Fig. S4A and Fig. 3D. The enrichment signals were not driven by identical full gene sets. In Fig. S4A, the overlap analysis was driven by CTSH, ACTR2, and RAB5C, whereas the Fig. 3D enrichment was based on a broader set of monocytes differentially expressed proteins. Thus, the shared annotation does not indicate that the two analyses are redundant, but rather that both capture components of a related myeloid lysosomal, cytoskeletal, and vesicle-trafficking program. We have revised page 19 of the Results to clarify that the “neutrophil degranulation” term should not be interpreted as evidence of neutrophil contamination, but as a pathway label reflecting proteins involved in granule biology, endolysosomal trafficking, actin remodeling, and protease-associated immune functions that are also active in monocytes:

“Although ‘neutrophil degranulation’ appeared in both all DEP- and DEP/MS susceptibility gene overlap-based enrichment analyses (Fig. 3D and Fig. S4A), comparison of the underlying proteins

showed that the signals were only partially overlapping. In the overlap-based analysis (Fig. S4A), this term was primarily driven by CTSH, ACTR2, and RAB5C, whereas the broader CD14⁺ monocyte DEP enrichment involved a larger set of proteins (Fig. 3D). We therefore interpret this recurrent annotation as reflecting a shared myeloid vesicle-trafficking and lysosomal/proteasome-related program rather than a neutrophil-specific signal.”

In the Discussion at p17 (line 372), the authors highlight that CTSH is overexpressed in B cells, monocytes and T cells. However, this is not indicated in the Results section and CTSH is even found to be downregulated in CD4⁺ T cells (line 142-144). This should be revised. The same is true for other proteins that are discussed but not part of the Results, making it unclear to which exact dataset the authors refer to.

We thank the reviewer for pointing out the imprecise wording. We revised the text to more accurately reflect the findings present in the dataset and included them on page 18 of the Results section:

“CTSH and PLEK were upregulated in both CD19⁺ B cells and CD14⁺ monocytes from MS patients. GRB2 was only upregulated in CD19⁺ B cells, while ASF1A was upregulated in CD14⁺ monocytes. In CD4⁺ T cells, CD6 and CTSH were downregulated, whereas MAPK1 and STAT3 were upregulated in CD8⁺ T cells. TNFAIP8 was downregulated in CD8⁺ T cells.”

The Discussion was also revised as follows (page 27):

“This study also identified overlaps between DEPs and known MS risk-associated genes across multiple immune cell populations, supporting the relevance of these proteomic alterations to established MS-associated pathways. GRB2 and PLEK were upregulated in CD19⁺ B cells of MS patients and are involved in intracellular signaling downstream of immune receptors and cytoskeleton remodeling^{33,34}, which is consistent with altered B-cell activation states. CTSH, which was upregulated in both CD19⁺ B cells and CD14⁺ monocytes, has previously been associated with antigen-processing pathways and immune activation³⁵. The concurrent increase of PLEK in both CD19⁺ B cells and CD14⁺ monocytes may reflect shared activation-related programs across innate and adaptive immune compartments. In T cells, CD6 and CTSH were downregulated in CD4⁺ T cells, while MAPK1 and STAT3 were upregulated and TNFAIP8 was downregulated in CD8⁺ T cells. MAPK1 and STAT3 are key regulators of inflammatory and cytokine-mediated signaling pathways^{21,36,37}, whereas CD6 and TNFAIP8 have been implicated in T cell co-stimulation and apoptosis-related processes^{38,39}. Together, these findings suggest that MS-associated proteomic alterations involve coordinated but cell type-specific immune signaling programs across peripheral immune subsets.”

DEPs in several immune cells are enriched in several unrelated disorders, including Aicardi-Goutières syndrome, severe congenital neutropenia etc. No other autoimmune diseases were found, while being closer related to MS. How to explain and follow up on this?

We thank the reviewer for this important comment. The enrichment of DEPs in disorders such as Aicardi-Goutières syndrome and severe congenital neutropenia, rather than in more classically related autoimmune diseases, requires careful interpretation. We have revised the manuscript to clarify that this analysis does not imply clinical similarity between MS and these disorders. Rather, it reflects overlap in broad immune effector pathways captured at the protein level, including interferon-related inflammation, innate immune activation, myeloid biology, and granulocyte/neutrophil-associated processes during early disease development. Future studies integrating larger cohorts, longitudinal sampling, and higher-resolution immune phenotyping may help determine whether these shared inflammatory signatures represent transient early disease responses or stable features associated with specific MS phenotypes and progression trajectories. We added the following statement to page 22 of the Discussion:

“Linking immune cell-specific proteomic changes in MS to disease phenotypes revealed associations with mitochondrial, metabolic, and neurodegenerative pathways, suggesting that early MS alterations engage broader biological processes extending beyond CNS autoimmunity ⁴¹. For example, electron transfer flavoprotein subunits alpha (ETF_A) and beta (ETF_B), identified in the CD14⁺ monocyte proteome, have been associated with metabolic dysfunctions that may contribute to demyelination and neurodegeneration in MS ⁴². Although disease associations were observed across multiple immune populations, the most robust and statistically supported signals were concentrated in CD14⁺ monocytes and CD19⁺ B cells, reinforcing their central roles in immune–metabolic crosstalk during early MS. Importantly, disease-enrichment analyses should be interpreted as reflecting shared biological pathways rather than clinical similarity between disorders. In our dataset, enrichments associated with interferon-, granulocyte-, and innate immune-related conditions likely reflect broader myeloid and inflammatory immune programs engaged during early MS.”

At least results from GWAS support the fact that there are overlapping immune cells, pathways and molecules between autoimmune diseases. Monocytes are also indicated as main source of immune related problems, while GWAS studies point towards B and CD4+ T cells as the primary immune subsets being deregulated (e.g. Farh et al, Nature 2015; Guo et al, Genome Biol 2022). The same accounts for EBV as the major environmental risk factor for MS, which primarily infects B cells. How can this be explained? This should be discussed.

We would like to emphasize that this study should be distinguished from genetic susceptibility studies in MS. Unlike GWAS, our proteomic data captures dynamic immune-state changes occurring near disease onset, rather than static inherited susceptibility mechanisms. In this context, the prominent monocyte signal in our ligand–receptor analysis should not be interpreted as contradicting the genetic and EBV literature implicating B cells and CD4⁺ T cells. Instead, we now present it as evidence that monocytes may represent a major effector or amplifier compartment in early MS-associated immune activation, while B cells and T cells remain central to genetic susceptibility, antigen presentation, EBV-related biology, and adaptive immune responses. We have therefore revised the Discussion to emphasize that genetic risk and disease-associated protein expression may highlight different, complementary layers of MS biology (page 29):

“This notion expands prior genetic evidence implicating T- and B-cell biology in MS susceptibility. While GWAS studies primarily identify inherited risk architecture and immune populations associated with disease predisposition, our proteomic analysis captures dynamic immune-state alterations underlying the disease initiation process.”

Could the highest outgoing strength by monocytes (line 239) be influenced by their increased size or granularity as compared to B and T cells?

Finally, we agree that higher outgoing ligand–receptor strength in monocytes could be influenced by biological and technical features of these cells, including larger cell size, higher protein content, granularity, and innate secretory/adhesion programs. We have added this caveat and tempered our interpretation of outgoing strength accordingly on page 29 of the Discussion:

“While GWAS studies primarily identify inherited risk architecture and immune populations associated with disease predisposition, our proteomic analysis captures dynamic immune-state alterations underlying the disease initiation process. Importantly, interpretation of the prominent CD14⁺ monocyte-associated outgoing ligand–receptor signal should be considered in the context of intrinsic biological differences between lymphoid and myeloid lineages. Thus, while the strong CD14⁺ monocyte-associated LR signal observed in this study is consistent with active inflammatory and adhesion-related immune communication during early MS, outgoing interaction strength likely reflects both disease-associated immune alterations and baseline biological properties of the contributing cell populations.”

Line 209-286: very large Results section with a lot of non-common terms and probably written by a bioinformatics expert. This should at least be clarified for readers with less bioinformatics background. I cannot properly judge the analysis and impact of their data.

The Results section has been revised accordingly (pages 14-24). Please also refer to our response to the first comment above.

Discussion, line 311: ..perturbations at the onset of clinical manifestations in MS. Have patients been selected at a very early stage of their disease? If yes, this should be emphasized. If not, this should be reformulated. The same accounts for 'early' proteomic alterations (line 442-443).

Text has been revised to reflect that sample collection was during the early post-diagnosis period, prior to treatment initiation.

One of the pathways that is highlighted and discussed is the immune response to pathogens (line 358). Again, this is too general and already looked at in more detail over many years.

We thank the reviewer for this important comment. We agree that several GO terms, particularly broad immune and inflammatory programs, can appear generic when viewed in isolation and that many of these pathways have been extensively studied in the context of MS and immune biology. However, an important objective of our study was to determine which established and emerging biological programs are already perturbed at the earliest stages of clinical disease, prior to treatment initiation. In this context, we aimed not only to identify differences, but also to contextualize which biological processes remain consistently altered across studies and which appear more specific to particular immune compartments in our dataset.

For example, line 417: 'GO enrichment analysis revealed cell type-specific downstream programs which are consistent with altered ligand-receptor communication and highlight molecular programs engaged by each immune subsets'. I find it very difficult to make anything out of these kinds of statements.

We agree that some interpretations in the original manuscript were too broad or overly abstract. In response to the reviewer's comments, we substantially revised multiple sections of the Results and Discussion to focus more directly on data-centered findings, representative proteins driving the enrichments, and biologically coherent interpretations linked to each immune subset. We also removed or rephrased statements that could not be sufficiently supported by the data or that relied too heavily on generalized pathway terminology. Specifically, to address the reviewer's concerns regarding the above statement, we revised page 20 the Discussion section describing the cell–cell communication analysis as follows:

"The integrated ligand–receptor analysis in this study suggests that early MS is associated with both intrinsic proteomic alterations within immune populations and coordinated changes in intercellular immune communication. Although many of the identified adhesion- and inflammatory-associated pathways have previously been implicated in MS and neuroinflammation, our results demonstrate that these processes are differentially distributed across peripheral immune subsets at disease onset. In particular, CD14⁺ monocytes displayed prominent outgoing disease-associated interactions and enrichment of adhesion-, stress-, and inflammatory-associated pathways, consistent with a potential role in altered extracellular immune communication during early MS. In contrast, CD19⁺ B cells occupied a central position within the inferred interaction network and showed enrichment for pathways related to B-cell differentiation, integrin-mediated adhesion, and immunological synapse-associated processes, supporting broader involvement in coordinating immune interactions across cell populations. Together, these findings highlight coordinated but cell type-specific immune alterations in early MS and suggest that altered immune communication and trafficking are already detectable within the peripheral immune compartment during the earliest stages of clinical disease."

Textual

P1, title: should be specified; 'identifies' should not be used in this context

P1, title: unclear what is meant by 'B cell-mediated network connectivity'

The title was revised to 'Cell-specific proteome profiling reveals altered peripheral immune signaling in early multiple sclerosis'.

P4, line 67: 'expression' should not be used in this context

The term on page 5 was revised to 'manifestation'.

P6, line 108: unclear what is meant by 'loadings'

We revised the sentence on page 15 to the following: "Based on these results, the contribution of individual proteins to PC1 and PC2 was visualized."

P8, line 171: subheading is too generic; 'proteins' should be 'protein'

The subheading was revised to 'Differentially expressed proteins overlap with MS genetic risk loci' for clarity and consistency.

Reviewer #2 (Remarks to the Author):

The manuscript by H Shams et al entitled: Immuno-proteome profiling identifies multiple sclerosis patients and reveals monocyte hubs and B cell-mediated network connectivity performs a proteomic study of CD4 and CD8 T cells, of B cells and monocytes from 29 treatment naïve MS patients and 25 healthy controls (paired in age and being all females). Differentially expressed proteins exhibited predominantly cell type-specific patterns. Authors described clearly different signatures in the four leukocyte subsets in MS patients compared to controls. The clearest differences were found in monocytes, followed by B cells. They also found an early involvement of fibrinogen cascade, confirmed by the finding of significantly higher levels of serum fibrinogen found in MS patients compared with controls. These data are interesting and may contribute to reveal MS pathology, However, some issues should be addressed.

1. Authors should explore the generality of their findings by exploring if their results are also found in males. This is specially relevant in MS, where clear differences associated with sex exist. In this line, the disease is more prevalent in women but male sex associates with a worse prognosis.

We thank the reviewer for raising this important point. The selection of a clinically homogeneous cohort was intended to minimize biological and treatment-related variability, thereby increasing power to detect proteomic alterations associated with early disease. We have added a statement to page 30 of the Discussion section, acknowledging that sex-specific effects are an important aspect of MS biology and that future studies with larger, sex-balanced cohorts will be necessary to systematically evaluate sex-dependent proteomic differences:

“Second, while sex differences are an important aspect of MS biology, the present cohort was intentionally restricted to female participants to achieve a highly homogeneous population at disease onset and minimize confounding influences. Future studies including both sexes will be important to further evaluate potential sex-specific differences in immune proteomic profiles in MS.”

2. In the same line, results describing high levels of fibrinogen in serum of MS patients should be confirmed in a larger cohort of MS patients and controls.

We thank the reviewer for this important point. We agree that confirmation of increased fibrinogen levels in a larger and independent cohort would strengthen this observation. In the current study, the robustness of our findings is supported by the overlap in study participants and time of sample acquisition between the unbiased cellular proteomic screen and the serum fibrinogen assessment.

Importantly, fibrinogen levels have been reported in prior MS studies (Adams et al., 2007; Davalos et al., 2012; Petersen et al., 2018), supporting the biological relevance of our results. Nonetheless, we have revised the manuscript to more clearly acknowledge the limited sample size and the need for validation in larger, independent cohorts. We have also tempered our conclusions accordingly to avoid overinterpretation.

3. Authors should also explore if serum levels of any of the proteins most clearly associated with MS are elevated in serum of patients compared with controls.

We thank the reviewer for this important suggestion. Fully addressing it and meaningfully integrating such results into the current study and our network analysis framework, would require inclusion of additional immune cell types and finer-resolution subtypes. It would also be necessary to distinguish between activated and steady-state conditions, as these functional states can substantially influence proteomic profiles and inferred ligand–receptor interactions. In addition, a substantially larger sample size would likely be required to support this level of stratification with adequate statistical power.

4. Associations of DEP with MS susceptibility genes seem to be modest. Due to the low numbers of

patients analyzed, authors should state that these data are exploratory and should be confirmed in larger series.

The reviewer raises an important limitation. It is noteworthy that we do not necessarily expect a strong concordance between DEPs and established MS susceptibility genes, as proteins reflect dynamic biological processes influenced by disease state, progression, and environmental factors, whereas genetic risk loci are static. Therefore, the modest overlap observed is not unexpected and should be interpreted within this context.

We agree that, given the relatively small sample size, these findings should be interpreted with appropriate caution. As mentioned in the previous comment, the samples in the present study were carefully selected to minimize confounding influences, which inherently constrains cohort size and limits the extent of stratified analyses. Consistent with this, we have added a paragraph to page 25 of the revised manuscript to clarify these considerations and appropriately contextualize these results as follows:

“One important strength of this study is the highly stringent sample collection strategy, as all samples were obtained early after diagnosis and prior to treatment initiation. This design minimizes confounding effects related to therapy, prolonged disease-associated immune remodeling, and chronic inflammatory adaptation, thereby enabling more accurate characterization of immune proteomic alterations associated with early MS.”

We are pleased to submit a revised version of our manuscript, now entitled “**Cell-specific proteome profiling reveals altered immune cell communication in early multiple sclerosis**” (COMMSMED-26-0472A). We appreciate the reviewers’ continued careful evaluation of our work and their constructive comments, based on which we have made additional revisions that have further improved the clarity and precision of the manuscript.

We would also like to clarify our previous response regarding missing protein abundance values. While reviewing the original workflow during preparation of the code and data for deposition, we confirmed that downstream analyses were performed using the **non-imputed Spectronaut export**, with analyses based on the available protein abundance measurements. This approach was selected to avoid introducing artificial values or assumptions about unobserved protein abundances. Our previous response inadvertently stated that missing values had been imputed using Spectronaut’s background-signal approach. We have corrected this description in the revised Methods (page 9). **This clarification reflects the original analysis workflow; no analyses were repeated or modified, and the reported results remain unchanged.**

To further enhance transparency and reproducibility, we have organized the analysis code in a GitHub repository and prepared a Zenodo draft containing the code and associated data (**reserved DOI: 10.5281/zenodo.22071704**). During peer review, the Zenodo deposit will remain non-public; however, a private access link is provided at the end of this response letter so that the editors and reviewers can access the deposited materials. **The GitHub repository and Zenodo record will be made publicly accessible upon acceptance.**

We thank the reviewers again for their thoughtful comments and provide our detailed responses below. Modifications in the revised manuscript are highlighted in yellow.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors satisfactory implemented my suggestions and took away most of my concerns, besides for the abstract terms, statements and conclusions used.

Some remaining remarks:

Line 1: the use of 'signaling' suggests a signaling cascades, which is not the case. So better to use another term

We revised the title by replacing "signaling" with "immune cell communication" to more accurately represent the biological processes investigated in this study.

Line 25: proteomic changes before treatment initiation.. add early disease/MS to clarify

We revised the text to clarify that the proteomic changes were characterized during early MS, prior to treatment initiation.

Line 44-46: CD14⁺ monocytes display the strongest inflammatory and adhesion-associated interaction signals, whereas CD19⁺ B cells occupy a central position within the inferred immune interaction network and are enriched for pathways related to CD19⁺ B-cell differentiation, adhesion, and immunological synapse function.

Please reformulate and specify. Both are seemingly associated with adhesion molecules (but whereas suggests a contradiction) and CD19+ B cell is mentioned twice.

Thank you for this suggestion. We revised the text to separate the descriptions of CD14⁺ monocytes and CD19⁺ B cells, clarifying their distinct network roles while avoiding ambiguity and removing the redundant reference to CD19⁺ B cells, as follow:

“CD14⁺ monocytes exhibited the strongest predicted outgoing interaction activity and were enriched for inflammatory and adhesion-related pathways. CD19⁺ B cells occupied a central position within the inferred immune interaction network and were enriched for B-cell differentiation, adhesion, and immunological synapse pathways.”

Line 48: indicate in the conclusions that specific proteins or protein profiles have been found that associate with.. still abstract terms are used throughout the abstract

We revised the concluding statement to more explicitly summarize the identified cell type-specific proteomic alterations and their associated biological processes. Given the abstract word limit and some unavoidable overlap with the Results section, we aimed to make the Conclusion more specific while minimizing redundancy. The Conclusions section was revised as follows:

“These findings demonstrate coordinated, cell type-specific proteomic alterations at the earliest stages of MS. The identified proteomic changes highlight inflammatory and adhesion-related processes in CD14⁺ monocytes and B-cell differentiation, adhesion, and immunological synapse function in CD19⁺ B cells, providing insight into immune mechanisms that may contribute to disease initiation and early pathogenesis.”

Line 368: DEGs overlap with MS genetic risk loci.. this suggests things that are not shown. What is meant with 'overlap'? Gene = protein? Better to use another term to specify this finding

Thank you for this comment. We revised the text to clarify that "overlap" refers to the genes common to the list of genes encoding differentially expressed proteins and the curated list of MS susceptibility genes.

Line 517-519: Our multi-cell-type analytical approach highlighted a CD14⁺ monocyte-centered remodeling of immune crosstalk in early MS and identified CD19⁺ B cells as key network connectors.

Line 520: disease-associated signaling interactions

Please specify, because such statements are uninterpretable

We revised these statements to more specifically describe the findings and to avoid broad or potentially ambiguous terminology:

“Our multi-cell-type analytical approach identified coordinated, cell type-specific proteomic alterations in early MS, with prominent inflammatory and adhesion-related changes in CD14⁺ monocytes and proteomic changes in CD19⁺ B cells associated with B-cell differentiation, adhesion, and immunological synapse function. These findings underscore the value of integrated, cell type-specific proteomic profiling for identifying immune alterations associated with early MS.”

Line 649: not only consequences, but also the cause of peripheral immune dysregulation?

Thank you for this suggestion. We agree and revised the sentence to indicate that future studies should investigate both the drivers and the functional consequences of peripheral immune dysregulation in MS:

“Collectively, the present results establish a cohesive biological framework for future high-resolution, longitudinal, and mechanistic studies aimed at elucidating the temporal dynamics, drivers, and functional consequences of peripheral immune dysregulation in MS.”

Reviewer #2 (Remarks to the Author):

The authors have answered most of my questions. However, due to the relatively low numbers of patients included, despite the careful selection of patients, it should be clearly stated in the discussion that the results should be validated in larger cohorts.

Thank you for this suggestion. We have revised the Discussion to explicitly acknowledge that the findings should be validated in larger, independent cohorts. We also note that, although the cohort size is modest, it is comparable to other deep mass spectrometry-based proteomic studies of peripheral immune cells in multiple sclerosis, including studies by Berge et al. (J. Proteome Res., 2019) and Cappelletti et al. (Clinical Proteomics, 2022), which included approximately 13–20 patients with MS and a comparable number of healthy controls. The technical complexity and depth of cell type-specific proteomic profiling often limit cohort size. Nevertheless, we agree that validation in larger, independent cohorts will be important to establish the robustness and generalizability of these findings.

“Second, although this study leveraged a carefully characterized cohort of treatment-naïve patients with early MS, the relatively modest sample size is comparable to that of other deep mass spectrometry-based proteomic studies of peripheral immune cells in MS (Berge *et al.*, *Clin. Proteomic.*, 2019; Cappelletti *et al.*, *Clin. Proteomics*, 2022), reflecting the technical complexity of cell type-specific proteomic datasets. Nevertheless, validation of these findings in larger, independent cohorts will be important to establish their robustness and generalizability.”

Zenodo draft (private reviewer access):

https://zenodo.org/records/22071704?preview=1&token=eyJhbGciOiJIUzUxMiIsImIhdCI6MTc4NzUxMzA1NywiZXhwIjoxNzk4NTAyMzk5fQ.eyJpZCI6IjU4ZjNmMTZhLWlxNGUtNGFmNi05MTJjLTM0OGJlYTFmMjVIZiIsImRhdGEiOnt9LCJyYW5kb20iOiJiODY2MzdLOWUyOTQ1YjJINDAzMDE0ODE5Y2RmZjFIZSJ9.F5Vx8AB17EUsQhoh2Lb-dZjwF2c25krmiJ5ZQuOUF_-zskBVruGcEo72cPRiAJkiOhGeRC6B_LjA00UPsbq_xQ