Proteomics
Clinical Applications
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HIV and Proteomics: What We Have Learned from High Throughput Studies

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The accelerated development of technology over the last three decades has driven biological sciences to high-throughput profiling experiments, now broadly referred to as systems biology. The unprecedented improvement of analytical instrumentation has opened new avenues for more complex experimental designs and expands the knowledge in genomics, proteomics, and other omics fields. Despite the collective efforts of hundreds of researchers, gleaning all the expected information from omics experiments is still quite far. This paper summarizes what has been learned from high-throughput proteomics studies thus far, and what is believed should be done to reveal even more valuable information from such studies. It is drawn from the background in using proteomics to study human immunodeficiency virus 1 infection of macrophages and/or T cells, but it is believed that some conclusions will be more broadly applicable.

1. Introduction

Proteomics measures the protein repertoire of a living organism or biological system. Unless a time-course is studied, proteomics offers a snapshot of the biological system. Of note, the proteome itself is highly dynamic and changes continuously in response to internal and external stimuli. Concurrently, stimuli affecting the proteome composition act dynamically in time and strength. Proteins are undergoing constant degradation and renewal. The tempo of this process is associated with the inherent characteristics of the biological system being studied and is driven by multiple factors such as transcription and/or translation. Nevertheless, the main objective of a proteomic study is to discover unique markers of change in relevant experimental models. The question is whether or not such a marker exists in similar quantities in the proteome of every individual at the same time and whether they can be measured with satisfactory precision and accuracy. Furthermore, the time period in which these biomarkers remain detectable, for example, circulating in blood or being present in any biological material, is difficult to ascertain. For example, the proteome of humans in a healthy state will be responding to the circadian rhythm, any type of activity, and so on.[1] Aligning human responses is further complicated by comorbidities and overlapping pathologies such as infection, intoxication, cancer, and so on.

In proteomics, there are two general types of experimental approaches. One is longitudinal studies that look at a cohort of subjects over an extended period of time. The other is cross-sectional studies capturing the status of a biological system at one time point. Cross-sectional studies using large numbers of individuals can be considered as high-throughput studies per se, although they are usually driven by specific questions and/or characteristics that are present in a population affected by disease. Longitudinal studies usually try to address different questions than cross-sectional studies. For more information about cross-sectional and longitudinal studies, we refer readers to ref. [2] and https://www.verywellmind.com/what-is-a-cross-sectional-study-2794978. Regardless of whether these studies are longitudinal or cross-sectional, it has been generally expected that proteomic experiments would reveal new biomarkers in blood and/or other tissues.

Over the past twenty years, our research has focused on macrophage and/or T cell infection by human immunodeficiency virus 1 (HIV-1). Two primary targets of HIV-1 infection[3] are T cells[4] and macrophages.[5] Early proteomic profiling experiments were performed using sodium dodecyl sulfate 1D polyacrylamide gel electrophoresis- and Surface Enhanced Laser Desorption Ionization (SELDI)-based mass spectrometry. The major accomplishment during this time was protein identification and relative quantification but was limited to a very low number of proteins. Examples include orthogonally validated differential expression of superoxide dismutase Cu/ZnSOD-1 (UniProt # P00441) identified in cerebrospinal fluid (CSF)[6] and matrix metalloproteinase-9 (MMP-9), (UniProt # P14780) in the secretome of human monocyte derived macrophages

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/prca.202000040

DOI: 10.1002/prca.202000040

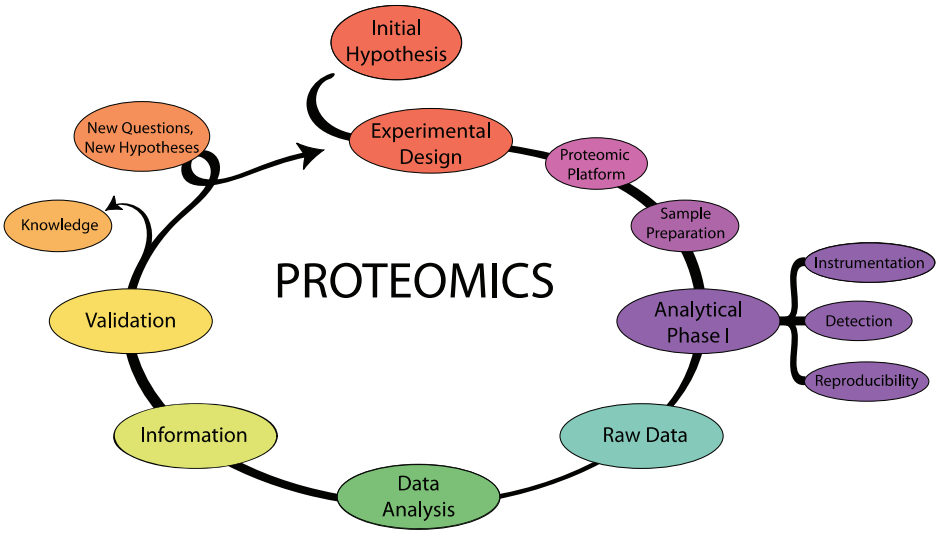

Figure 1. Complexity of a proteomic study consisting of multiple steps. Each step may have profound effect on an overall outcome of proteomic profiling. Analytical phase itself is of high complexity and includes instrumentation (choice of mass spectrometric platform), detection, reproducibility. This schematic illustration shows steps leading from initial to subsequent hypothesis in proteomic study.

(hMDM) (PMID:15579275). Additional proteomic investigations extended the list of proteins differentially expressed as a result of HIV-1 infection to include cystatins B and C, Cofilin-1, L-plastin, superoxide dismutase, and Hcgp39.[7] The role of cystatins in HIV-1 infection is still being explored.[8,9] Later proteomic studies were more focused on the effects of HIV on cellular structures and processes such as the cytoskeleton[10] and transcriptional activity.[11] These studies revealed enrichment of proteins linked to chromatin assembly or disassembly, DNA packaging, and nucleosome assembly by applying full unbiased proteomic profiling using Stable Isotope Labeling by Amino acids in cell Culture (SILAC) technology platform.[12] Intracellular interactions between viral and cellular proteins constitute a different arm of proteomic profiling applications and are described in more detail later in this review. As such, we intend to provide an overview of a small fragment in the realm of high-throughput systems biology: the proteomic dynamics between HIV-1 and the macrophage. Thus, we next discuss principles of designing and executing omics studies based on untargeted and targeted methodology, which we recognize as key to the future of proteomics studies on ex vivo human material in the context of viral infection. Because omics studies may relate to a number of variables, we will focus here on proteomics studies.

2. Quantitative Proteomics: Experimental Design to Maximize the Capabilities of Technology

Demand led to the development of several platforms for quantitative proteomics. With the exception of 2D DIGE, all are based on a LC-MS/MS analytical approach. The most commonly used techniques to study full unbiased (non-targeted) proteomics include iTRAQ, Tandem Mass Tag (TMT), SILAC, and Sequential Window Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH-MS). ICAT or spectral count methods are less commonly employed. SELDI-TOF is still occasionally used by some researchers,[13] primarily for biomarker discovery. For targeted proteomics, MRM, SRM, and PRM techniques are used. Further distinction can be made based on data dependent (DDA) or data independent acquisition (DIA). Each approach has strengths and weaknesses, and none of them is ultimately universal. Therefore, the use of a particular platform must be selected based on the hypothesis as well as the targeted subset of proteins, that is, antibodies, serum/plasma, intracellular proteins, and so on.

Proteomic experiments are multistep and complex undertakings. In **Figure 1**, we present a set of steps leading from an initial hypothesis to a new/subsequent hypothesis. In order to successfully complete a proteomic experiment, all elements have to work to their full potential because these experiments are quite vulnerable to generating false positive results at almost every step. Although omics experiments are able to measure thousands of molecules, the presence and quantity of detected molecules must be validated. The best validation method has yet to be found and agreed upon. Regardless of whether a proteomic experiment is based on full unbiased profiling or is a targeted type of study, the general steps are the same; the primary difference is in experimental design.

As stated above, the advances in high-throughput experimental technologies have led to an explosion of data, but not necessarily a cohesive knowledgebase from which definitive conclusions about a biological system in question may be drawn. Matters are complicated by the fact that different experimental designs will inevitably give some differing results. Proteomics experiments are no exception. These experiments consist of multiple steps that all must be successfully executed. This may ultimately lead to problems of comparability of different studies. Every step brings with it some degree of variability.[14] Sample preparation, instrumentation, targeted versus global approaches, and data analysis are a few of the factors that influence results, and thus the conclusions are drawn and knowledge gained. These issues can make it difficult to compare study results and to build a clearer picture of HIV infection in macrophages, and ultimately, of HIV infection in humans overall.

It is surprising how few proteomics studies have been published on the macrophage response to HIV-1 infection.

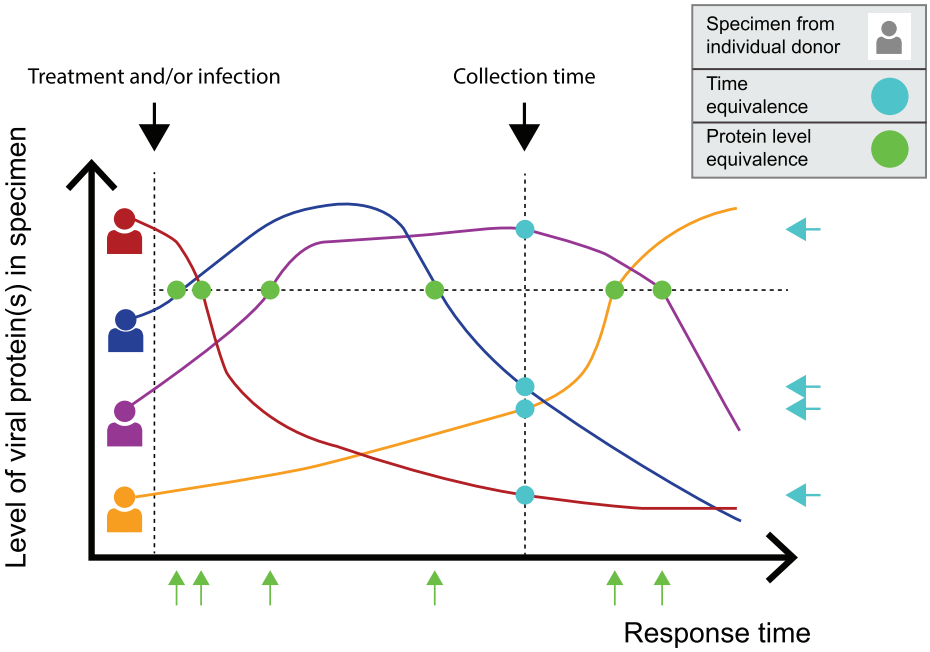

Figure 2. An illustration of a concept of dynamic response to HIV-1 infection of primary macrophages showing that proteins may be at the same level but at different time points or at different levels at one time point. This represents a challenge in experimental design, that is, time of sample collection in studying viral infection rather than specific proteins. This applies equally to intracellular, secreted, and viral proteins.[7]

Currently, there are only three publications reporting on the HIV-infected macrophage secretome.[7,15,16] These three studies revealed a surprisingly small number of differentially expressed proteins, potentially discouraging the justification of further secretome investigations. Of note, these studies were performed during the early stages of proteomics development when the sensitivity of protein identification was much less than nowadays. A few more studies of the macrophage secretome have been reported, but all of them in a different biological/disease setting.

The quest for biomarkers in blood led to slightly more proteomic profiling studies of both blood (plasma, serum) and CSF in HIV-1 infected individuals. Each of these papers contributed novel information, yet not as much as we would expect. Thus, the progress is much more incremental than the hoped-for breakthroughs upon emergence and refinement of the proteomics approach.[6,13,17,18,19,20]

3. Proteomic Approach to Viral Infection: Is There Any Specific Challenge?

Investigating a viral infection poses a specific set of challenges. One of them is that whether in vivo or in any other model system, a virus does not infect all target cells at the very same moment. Infected cells start responding to infection and sending signals to the neighboring cells to turn on defensive mechanisms. Thus, when cells are collected and analyzed, the observed changes represent a net response rather than a specific response of a subset of cells. If changes triggered by infection are manifested in changes in surface markers, respective subsets can be isolated by techniques such as flow cytometry for non-adherent cells or by laser dissection for tissue sections. If changes are reflected by the secretion of substances such as cytokines, it is much harder to identify subsets in the total pool of samples. This issue is amplified when immune cells are targeted by infections such as HIV-1. An additional challenge is the inherent variability within the population of human subjects, in contrast to model systems that employ a homogenous population of transformed cells from clonally expanded cell lines. Any response might be spread over time and the sampled time points for different subjects may differ substantially. This challenge is depicted in **Figure 2**.

The third challenge, although not specific to viral infection, is the wide range of proteins' concentrations within one sample, which makes it difficult to capture all changes with one analytical approach. Many pre-fractionation strategies have been proposed and used, but each additional analytical step introduces variability. Considering these three challenges, an associated fourth challenge is data normalization and processing for measuring quantitative changes within a population or between populations of human subjects used for studies. Several analytical approaches have been developed and used, such as spiked in heavy isotope labeled peptides or housekeeping proteins such as actin, but each approach has strengths and weaknesses. Therefore, the quest to identify proteins that consistently change in one direction or that remain totally unchanged and could serve as a normalization factor is ongoing. We propose to refer to these proteins as "canonical proteins". These might be a small number of proteins whose behavior (directionality of change) in HIV-1 infected macrophages allows them to be used as indicators of the status of a high-throughput experiment.

4. SILAC: A Challenge for Proteomics Studies of Non-Dividing Cells

Originally, the proteomics platform SILAC was developed with cancer cells in mind, which are actively dividing and can replace "light" with "heavy" proteins/peptides in six passages.[21] In the

case of non-dividing cells, the replacement of SILAC medium will depend on the dynamics of protein turn-over rate that can vary greatly from protein to protein. Non-dividing cells such as primary macrophages are relatively short-lived, and complete replacement of proteins might not be accomplished even in two weeks, which is the expected lifespan of these cells after differentiation. While SILAC can still be used to investigate the proteome of non-dividing cells, more limits need to be placed to reduce the number of experimental possibilities that can be studied in one setting.[22] Thus, many investigators have turned to cell models such as macrophage-like[23] transformed cell lines, Jurkat T-cells,[24] HeLa and HEK 293T[25] cells. Although SILAC remains a powerful proteomic platform, its utility to study primary and non-dividing cells is limited. Using transfected cell models is currently out of favor as it has been realized that such models may only partially reflect in vivo conditions. Thus, research has shifted to models that better reflect native cells. The recent development of organoids is just one example, yet no proteomics studies have been conducted using this model.

5. iTRAQ and TMT: The Value of Chemically Labeled Proteomic Platforms

An unquestionable advantage of the isobaric labeling method based on iTRAQ or TMT is the possibility to label up to 16 samples in a single experiment. This allows for the identification and quantification of peptides and comparison of multiple conditions simultaneously; these methods are suitable for all kinds of protein samples. Initially, iTRAQ and TMT acquired an immense interest in terms of their applications in HIV-1 pathology and the search for new biomarkers and potential drug targets. Both techniques offer insight into the complexity of this viral infection while focusing on proteins that are involved in host immune response.[26] A few examples of successful iTRAQ applications include the use to study effects of the shock-and-kill approach to latent infection,[27] to study of the common egress pathway in multiple cell types to determine the type of cells from which HIV-1 virions are derived,[28] to study comparative analysis of HIV-1 infectivity,[29] and in detecting altered protein levels that may determine susceptibility to HIV-1 infection.[30]

A further advantage of chemically labeled proteomic platforms is that they allow comparison of paired samples before and after infection as well as to a common standard.[31] For example, the study described by Linde et al.[28] used this design to differentiate HIV-1 virions derived from a T-cell line and a B-cell/T-cell hybrid cell line. Based on proteomic profiling of murine and human primary cells using iTRAQ labeling, Huang et al. postulated that Treg cells promote neuroprotection through inhibition of virus release,[31] killing of infected mononuclear phagocytes, and inducing phenotypic cell switches. The authors list a number of newly identified proteins, but further validation needs to be performed.

Another avenue being explored through proteomic profiling is the biological process of HIV infection in the presence of other comorbidities and co-infections, such as human papillomavirus,[32] Hepatitis B/C Virus,[33] *Mycobacterium tuberculosis*,[19] and *Toxoplasma gondii*.[34] One publication by Jain et al.[32] describes a very interesting experimental approach utilizing the iTRAQ platform to analyze formalin fixed paraffin embedded (FFPE) tissue sections. A relatively short list of differentially expressed proteins suggests that several protein changes may have gone undetected due to the nature of FFPE samples. Nevertheless, this study represents an example of one approach to validate proteomic profiling and histostaining.

In summary, proteomic profiling studies using chemical labeling, such as iTRAQ, have provided some additional information; however, each study has identified different dysregulated proteins. This lack of overlap may suggest that some studies fail to detect certain proteins or that some detected proteins may be false positive results. The discrepancies need to be reconciled through validation via reductionistic studies. Furthermore, iTRAQ and TMT are also applicable for analysis of post-translational modifications of various proteins, such as phosphorylation[35] and acetylation.[27] Concurrently, label-free methods based on DIA such as SWATH-MS have been developed and used to perform proteomic profiling in the context of HIV-1 infection.[36] Thus, there is experiment-based evidence demonstrating the value of chemically labeled as well as label-free proteomic platforms as a mainstay for the characterization of complex proteomes.

6. Viral Proteins

Although the presence and levels of viral proteins in cells or body fluids may tell us about the ongoing state of viral infection, these proteins do not necessarily indicate any changes in host cell metabolism. Proteins encoded by the HIV-1 genome are classified as structural, regulatory, and accessory; and the role of these viral proteins as biomarkers has been studied extensively for decades. However, as our knowledge regarding these proteins has accumulated, we have realized that their role in HIV-1 pathogenicity is far greater than their role as biomarkers. If a protein plays a role in the pathogenicity of an infection, it has to exploit interactions with cellular proteins beyond those just supporting viral replication. Thus, several high-throughput proteomic studies have explored which cellular and/or extracellular proteins, such as matrix proteins,[37] are interactors and how this information can be used to create new therapeutic targets. Several comprehensive studies of the interactome have been reported[38] in addition to those more focused on specific viral proteins such as Gag[39] and Tat.[40]

Ogishi and Yotsuyanagi recently postulated that HIV associated neurocognitive disorder (HAND) can be predicted based on three genetic features derived from the HIV *env* sequences.[41] The authors analyzed 2349 sequences from 80 specimens. If their observations were to be confirmed by further studies using a larger study population, it has the potential to be a breakthrough finding showing the link between a specific viral protein sequence and a clinical diagnosis.

The interaction of HIV and cellular proteins remain incompletely understood, but elucidating these interactions is key to understanding the pathobiology of HIV infection. Some studies initially focused on the role of host cytosolic proteins in the viral lifecycle. One such study postulates that cytosolic chaperonin TRiC (TCP-1 ring complex) is involved in transient interactions with the HIV protein Gag, ultimately leading to properly folded Gag molecules and self-assembly of the immature capsid.[42] The most comprehensive study of the interactome of HIV proteins

was performed by Krogan's group,[43] and many of the identified interactions are awaiting biological validation. For a more comprehensive discussion of viral proteins, we refer readers to our previous review.[14]

As technology develops and new tools become available, investigators have access to multi-platform, sophisticated, and complex experimental designs to investigate not only the structure of post-translationally modified proteins but also the role of viral proteins in molecular mechanisms underlying the consequences of infection. Such studies usually involved more than one model and technology platform. One such review by Seissler et al.[44] demonstrated the involvement of Vif protein in the molecular mechanism of pathogenicity of HIV infection. We envision that similar studies will be more common in the future.

7. Proteomic Investigations of Sera/Plasma and CSF from HIV-1 Infected Individuals

Proteomic profiling of body fluids such as plasma/serum, CSF, saliva, lung lavages, and cervical-vaginal fluid are still being considered as potential sources for new discoveries of diagnostic and prognostic biomarkers, as well clues related to infection susceptibility, treatment efficacy, and so on. One limiting factor restraining investigators from using body fluids in proteomic investigations is the lack of good references for normalization. However, the level of any given protein has to exceed more than ≈40% change to show statistical significance to be used for normalization. Due to the detection limits of instrumentation, it is difficult to show proteins present at much lower levels, such as those originating from damaged tissue or cell leakage.[45] In a relatively recent report presented by Rodriguez-Gallego et al.,[46] the authors were able to use TMT chemical labeling to measure statistically significant proteins following immunodepletion of seven of the most abundant proteins in human plasma. Included in the list were abundant proteins that have previously been detected in the plasma of HIV-1 infected individuals. Some of these proteins, such as a-1-antichymotrypsin, are not specific to HIV-1 infection and may represent changes in low-level persistent inflammation. The same issues apply to CSF, lung lavages,[47] saliva,[48] and cervical-vaginal fluid.[49]

The spike-in approach can serve as good control of sample preparation and analysis, but it cannot offset the inherent variability of proteins over time and between the individuals from which samples are taken. Highly abundant proteins with widely ranging concentrations can fluctuate hourly, daily, weekly, or monthly contributing to the already existing large donor-to-donor variability. For example, serum albumin ranges from 34 to 54 g L^{-1} (reference serum values) making the difference between the minimum and maximum greater than 40%. Averaging values from large cohorts of samples will result in a high standard deviation that potentially exceeds the changes due to the pathological process. Thus, small changes (statistical significance) will not be detected in proteins present in very low concentrations.[50] Despite these obstacles, proteomic profiling, more thoroughly discussed in our review paper from 2016,[14] is progressing.

One major challenge in plasma/serum/CSF proteomics is the extremely wide dynamic range of concentrations of proteins that varies at any given time. Based on reports from the Human Plasma Proteome Project, the Core Dataset for human blood contains 9504 IPI proteins identified with one or more peptides and 3020 proteins identified with two or more peptides. The twelve most abundant proteins constitute ≈96% of the protein mass, creating an incredibly difficult challenge for sample preparation and analytics. One approach to offset the difficulty associated with this range of protein concentration is removing the most abundant proteins. Among the immunodepletion methods, the use of IgY columns seems to be the most efficient[17] in general enrichment protocols. More targeted approaches include enrichment of glycopeptides and performing a search with PNGase F-catalyzed conversion of Asn to Asp. This method has allowed for the identification of 829 unique glycopeptides from 411 unique glycoproteins with N- deamidation within the consensus N-linked glycosylation motif.[50]

A recent proteomic profiling study of CSF performed by Landi et al. confirmed previously reported changes in proteins altered by inflammation but did not reveal information specific to advancing our understanding of HIV-1 infection pathobiology.[51] The authors postulate that oral bacteriotherapy might be beneficial as a supplement to combined antiretroviral therapy (cART) with the goal of alleviating the level of inflammation at peripheral and CNS levels.

In recent years, proteomics research aimed at identifying novel biomarkers associated with HIV-1 infection has moved to more focused studies with the intention of finding possible markers associated with a specific co-pathology. This comprises efforts to validate earlier findings using new methodologies[52] to include better characterized cohorts of infected individuals, new platforms,[20] and so on. One such platform developed by Olink Proteomics (https://www.olink.com/) provides panels to monitor specific changes related to immunity and other cellular processes. While such panels have benefits, the pre-determined factors have lower utility in discovering new biomarkers. In addition, Olink Proteomics has proposed to use a number of cytokines and chemokines that are investigated by methods other than mass spectrometry. Another method to search for new biomarkers is presented by Colon et al.[16] The authors investigated the changes of matrix metalloproteinase-9 (MMP-9) levels in culture supernatants of macrophages isolated from patients with various levels of neurocognitive impairment due to HIV infection. Their work confirmed our earlier finding that secretion of MMP-9 is diminished in the secretome of HIV-1 infected macrophages.[53]

As bioinformatic tools and methods advance, more reliable information can be extracted from large datasets not only from proteomics profiling experiments but also from other high-throughput studies. Yet, the integration of data from various platforms remains a challenge and needs to be investigated in a separate review.

For more information regarding proteomic platforms used and experimental outcomes, we direct readers to our previously published review from 2016.[14] Over the last five years, a limited number of proteomic profiling studies of plasma/serum samples from HIV-1 patients have been reported; and no break-through information has been presented. Finding a way to normalize protein levels in this specific sample type may produce different results.

8. Biological and Technical Validation

Validation of high-throughput data such as proteomic profiling data is meant to weed out false positive results. One goal of cross-validation is to test the experimental model's ability to predict new data. Thus, cross-validation[54] (rotation estimation, out-of-sample testing) is a technique used to assess how the results of a statistical analysis will generalize to an independent data set and to estimate how accurately a predictive model will perform in practice. On the other hand, the orthogonal method of validation can be used to evaluate the primary method. For example, immunohistostaining of cells would be orthogonal validation of proteomic profiling performed using these same cells. Another aspect of proteomic validation is technical versus biological validation. Technical validation is more straightforward and incorporates reproducibility based on repeated measurements. Biological validation carries with it more challenges. The exact definition of biological validation and what it constitutes remains a gray area. One approach is to use multiple sources of biological material such as primary cells from a number of donors. This approach, as mentioned previously, is difficult due to donor-to-donor variability. Another approach is to use orthogonal validation using a different method of detecting and/or confirming differences provided by high-throughput proteomic profiling. Many researchers have attempted to perform such validation.[55] Bioinformatic tools undoubtedly aid in avoiding false positive profiling results, but they do not solve problems with validation. In short, validation remains a very broad and unaddressed issue as profiling methods rapidly expand, and adequate discussion is beyond the scope of this review. In the following section, we discuss the proposition of "canonical proteins" - those that may be indicative of the ex vivo course of HIV-1 infection in hMDM. In our studies, we focus on biological validation using an orthogonal approach, which may be the best at reflecting the in vivo situation. All-in-all, we recommend that the method of validation, whether it be an already published method or a novel approach, be carefully considered for adequate interpretation of data.

9. Proteomic Approach to Study HIV-1 Latency

Latency of HIV-1 infection is a major obstacle in the search for a cure. Recently, along with the effective control of HIV-1 infection with cART, there has been a shift in focus to characterizing latently infected cells as an avenue to a cure. A "shock and kill" strategy aimed at reactivating latently infected cells to kill them in the following step did not yield the expected results. This pointed several researchers to use proteomics to find new targets. Many studies use cell lines, such as ACH2 or parental cells, which are not preferred models as they lack a direct connection to the in vivo situation. Nevertheless, such studies increase our general knowledge of HIV-1 pathobiology.[56] The lack of a widely accepted in vitro and/or ex vivo experimental model still presents a challenge.

The bi-specific T-cell engagers developed by Amgen as the BiTE Immuno-oncology platform was used by Brozy et al. to create two N-terminal domains of human CD4 fused to the proprietary human anti-human CD3ϵ scFv[57] and tested in CHO gp120 HIV-transfected cells. This is a novel approach to exploit the gp120 protein in prospective therapies, and it showed a promising outcome as proof-of-principle for eradication of HIV infection.

In summary, proteomic investigation of latently infected cells has yet to provide conclusive results about how homogeneous the population of latently cells is and how best to target them for HIV eradication therapy. One review that nicely summarizes what is known about latently infected cells has been published by Kok et al.[58]

10. Striving for Canonical Proteins: Normalization of Data

Due to the demand for analysis of high-throughput data, the field of bioinformatics has made unprecedented progress in developing tools, refining, and connecting databases, and creating repositories. While not everything has gone as smoothly as we would like, more and more such experiments are performed and more data with fewer false positives are generated. Surprisingly, the strongest links in systems biology experiments are in analytics. For example, the development of instrumentation for mass spectrometry and corresponding tools for database searches has improved over the last two decades to the extent that researchers can analyze samples with high precision and accuracy with quite predictable sources of systematic errors infused into each study. Biological interpretation, which is on the shoulders of individual investigators, remains at the center of many, if not all, scientific debates.

One aspect of experimental design that leads to discrepancy between studies is the use of cell lines versus primary cells. Cell lines are commonly used instead of primary cells to study biological systems for a number of reasons: 1) they are readily available and can be expanded to an unlimited supply, making them convenient and cost effective;[59] 2) use of cell lines also eliminates ethical concerns associated with using animal or human tissue; and 3) cell lines provide a pure population of cells, which is valuable for sample consistency and reproducibility of results.[60] However, the biggest disadvantage of cell lines is that they may not truly represent primary cells and the in vivo situation. The lack of tissue architecture and heterogeneous cell population eliminates cell-cell interaction, secretion, and other functions based on tissue context, causing cell line phenotypes to shift further from the primary cell phenotype. One study compared *M. tuberculosis* infection in primary macrophages and cell lines, and its results supported the idea that important differences exist between primary macrophages and cell lines.[61] Primary macrophages and J774 cells respond differently to infection with *M. tuberculosis*.[61] Another study also showed that THP-1 cells, the cell line most commonly used to study macrophage biology and primary monocyte–macrophages, have significant differences.[62] These differences should be considered when choosing a macrophage model to study host-pathogen interactions, and we feel that overall primary macrophages most closely represent the in vivo situation.

Despite the important contributions of studies using macrophage cell lines and during our quest to find canonical HIV-infection proteins through literature mining, we chose for the remainder of this review to focus on the study of HIV-1 infection using primary macrophages. In "primary macrophages", we include MDMs, which are most commonly used in experimental systems that study macrophage responses

to virus infections.[11,53,63] Using primary cells comes with another unique set of challenges. The biggest obstacle is the high donor-to-donor variability of responses from different donors. Genetic and/or environmental factors can have a big impact on infection kinetics/dynamics. There is no "magic number" of donors, since increasing the sample size is costly and may even increase variation, making it hard to identify small yet meaningful changes in proteins or other features. On the other hand, small sample sizes are statistically challenging to analyze.[14] Thus, finding highly specific biomarkers, perhaps synonymous with "canonical proteins", is challenging because different individuals display a broad range of responses to infection, and these differences carry through to primary cell use.

Recognizing the somewhat fragmented knowledge base created from proteomic studies of HIV-1-infected macrophages, we propose to find a list of "canonical" host proteins. These so-called canonical proteins would serve as markers of a sort, allowing different research groups to better compare data based on these proteins. They would allow comparison despite different experimental approaches and could potentially increase our understanding of the effects of such different approaches. Furthermore, canonical proteins present the opportunity to model infection in humans if we can definitively determine how these canonical proteins behave during infection. This quest for canonical proteins has proven to be difficult, given the obstacles of infection dynamics, different experimental designs, the use of various models (cells and animals), sample preparation protocols, instrumentation, and statistical and bioinformatic data analyses. All of these factors will influence the characteristics that designate a protein as "canonical". Despite the vast amount of knowledge created by the recent boom in high-throughput data production in HIV studies, the need for a coherent knowledge base and for a way to compare these studies is evident. By raising this issue and combing through literature concerning HIV and the macrophage, we have made a step in the right direction.

There are several published reviews that offer good information concerning what has been done with HIV-1-infected macrophages.[5,14,64] While these reviews highlighted the great strides that have been made in proteomics and HIV research, it is surprising that there are so few overlapping results. These studies focus primarily on differentially expressed proteins between uninfected control and HIV-infected conditions in the hopes of finding biomarkers. Sometimes, hundreds of proteins per study are found to be up or down regulated, creating an immense amount of data to examine and compare. Despite this, some proteins have proven to be key players in HIV-infected macrophages, revealing themselves as potentially canonical proteins. One protein family that has had a bit of spotlight on it since the advancement of proteomic technology is the MMPs. Another protein that has been identified across multiple studies is STAT1 (signal transducer and activator of transcription 1). We discuss what has been learned about these proteins in the following sections.

11. Matrix Metalloproteinase-9 (MMP-9)

The role of MMPs during HIV infection of MPs (macrophages, microglia etc.) has been reported in many studies; however, most of them were reductionistic studies and only one publication employed early proteomics. Our early reductionistic study showed reduced secretion of MMP-9 by HIV-1 infected macrophages.[53] Colon et. al published a similar observation that used MPs isolated from HIV-1 infected women.[16] This latter study was the only other study utilizing high-throughput methods to investigate the secretome HIV-1 infected macrophages. The general consensus from these studies is that MMP secretion is significantly lowered in HIV-1 infected macrophages. The underlying mechanism of this effect is not clear, mostly due to the lack of systematic follow-up studies. Other reports show that gp120 alone or in combination with methamphetamine (Meth) has no effect on the MMP-9 transcript[65] and that Tat protein induces expression of MMP-9. The latter study, however, was performed using U937 cells and not human primary MPs. Other studies have shown the effect of various stimuli such as drug treatment or lipopolysaccharide stimulation on MMP-9 production. Our recent omics RNAseq study revealed that the expression of MMP-9 is not affected by Meth during monocyte to MDM differentiation, which is in line with observations published by Reynolds et al.[65] and Ghorpade et al.[66] In summary, decreased MMP-9 production by HIV-1-infected MDM has been reported in several studies and has been correlated with some clinical analyses.[67] Therefore, we propose that extracellular MMP-9 could serve as one canonical protein in models of HIV-1 infection of macrophage.

12. Signal Transducer and Activator of Transcription 1 (STAT1)

Recent investigations that shed light on the upregulation of STAT1 in HIV-1 infected macrophages designated this protein as a candidate for a canonical protein.[9] A higher level of STAT1 protein can be recognized as a hallmark of innate immune activation and active HIV-1 replication. The biological function of STAT-1 is mediating the transcription of interferon (IFN)-stimulated genes that drive the cell into an antiviral state. Genomic research on the non-human primate model of neuroAIDS (simian immunodeficiency virus-infected monkeys) and on post-mortem human neuroAIDS samples revealed a shared set of IFN-inducible/pro-inflammatory genes, including STAT1. STAT1 was found to be significantly elevated in all studies, regardless of species, microarray, and means of analysis.[68]

It is known that in vivo STAT1 occurs in two active forms: tyrosine-STAT1PY (associated with HIV-1 inhibitory activity) or serine-phosphorylated STAT1PS (postulated to induce blood-brain barrier damage). An increased expression of STAT1 was observed in one study on MDM and placental macrophages regarding the influence of cystatin B/STAT1PY interaction during HIV-1 infection.[9] Furthermore, there is evidence that the level of STAT1PY increases beginning 6 days after HIV-1 infection and up until 20 days post-infection.[69] Another study demonstrated that STAT1PY expression differs between placental macrophages, which are restrictive cells for HIV replication, and MDM,[70] in which STAT1PY was more abundant in comparison to MDM at 12 days after infection.[71]

13. Closing Remarks

As much as reductionistic studies have contributed new knowledge to the area of biological sciences, we realize now that

biological systems are composed of such complicated machinery that one intracellular process cannot be considered in isolation and rather must be placed in the context of other processes. All of them intertwine within a biological system to the extent that fragmented data from even a hundred studies can appear conflicting. High-throughput approaches, which started with full-unbiased gene sequencing followed by mRNA profiling in conjunction with protein profiling, have created an immense appetite for thousands of pieces of new information derived from a single systems biology study. Rather quickly, we have learned that there are many limitations and obstacles to be overcome before we start benefiting from such high-throughput experiments. Every study that considers "failed" is beneficial in that we realize the critical importance of experimental design, the connection between all elements of such studies, and maybe most importantly, the challenges in the analysis of high-throughput multidimensional data.

What have we learned from high-throughput studies? It is inevitable that the application of proteomics will expand as researchers ask more questions about the pathophysiology of HIV and other viral infections. These studies will likely be met with challenges elegantly discussed by Zhao et al. for adenoviruses[72] and Scaturro et al. for flaviviruses.[73] Thus, a broad range of literature emphasizes the importance of the rate of infection in studying the dynamics and effects of viral infection. Regardless of the experimental approach, data analyses will need to address the dynamic response of a biological system if meaningful findings, but not false positives, are to be obtained. One way is to find proteins proven to show stable responses in any given experimental model and infection, proteins we dub as "canonical". Two potentially canonical proteins, one intracellular (STAT1) and one extracellular (MMP-9), have been discussed. We strongly believe that more such "canonical proteins" will be identified and will aid data analyses in reducing the number of false positives. For example, Helikar and Rogers are trying to create a dynamic model of changes generated in cells due to infection, malignant transformation, and so on.[74] This publicly available tool can be found in https://cellcollective.org/#.

One avenue to finding more canonical proteins is the study of extracellular vesicles (EVs). EVs have become recognized as important pathological factors, and several reports have aimed to characterize the EVs found in the plasma of HIV-1 infected individuals. In some instances, studies have focused on EVs isolated from plasma but not on the proteomic profiles of plasma itself.[75] Expanding on these studies may strengthen the findings presented in this review.

The proteomics approach, which has been proven to be a powerful tool in biomarker discovery, also has limitations. As discussed above, not every platform is equally applicable to investigating non-dividing cells. In the near future, we expect that MRM will gain more use in the context of investigating viral infections by mass spectrometry as shown by Foster et al.[76]

Acknowledgements

The authors would like to thank Dr. Nicole Haverland for her help in preparing graphics for Figure 1, Mr. Douglas Megis for his help in preparing graphics for Figure 2, and Ms. Robin Taylor for editorial support. Financial support was provided by National Institutes of Health grants: R01 DA043258 and P30 MH062261.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

human immunodeficiency virus, innate immunity, macrophage, proteomics, systems biology, virus

Received: May 18, 2020
Revised: September 4, 2020
Published online: October 14, 2020

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