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REVIEW

Application of proteomics technology for analyzing the interactions between host cells and intracellular infectious agents

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Host–pathogen interactions involve protein expression changes within both the host and the pathogen. An understanding of the nature of these interactions provides insight into metabolic processes and critical regulatory events of the host cell as well as into the mechanisms of pathogenesis by infectious microorganisms. Pathogen exposure induces changes in host proteins at many functional levels including cell signaling pathways, protein degradation, cytokines and growth factor production, phagocytosis, apoptosis, and cytoskeletal rearrangement. Since proteins are responsible for the cell biological functions, pathogens have evolved to manipulate the host cell proteome to achieve optimal replication. Intracellular pathogens can also change their proteome to adapt to the host cell and escape from immune surveillance, or can incorporate cellular proteins to invade other cells. Given that the interactions of intracellular infectious agents with host cells are mainly at the protein level, proteomics is the most suitable tool for investigating these interactions. Proteomics is the systematic analysis of proteins, particularly their interactions, modifications, localization and functions, that permits the study of the association between pathogens with their host cells as well as complex interactions such as the host–vector–pathogen interplay. A review on the most relevant proteomic applications used in the study of host–pathogen interactions is presented.

Keywords:
Biomolecular interaction analysis / Host–pathogen interaction / Interaction profiling / Protein differential expression / Proteomics methods

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Abbreviations: CVB, coxsackievirus B; **HCMV,** human cytomegalovirus; **HCV,** hepatitis C virus; **HLA,** human leukocyte antigen; **ICAM,** intercellular adhesion molecule; **IFN,** interferon; **LeTx,** lethal toxin; **MAPK,** mitogen-activated protein kinase; **MHC,** major histocompatibility complex; **MudPIT,** multidimensional protein identification technology; **NF-κB,** nuclear factor κB; **NK,** natural killer; **RNP,** ribonucleoprotein; **SARS,** severe acute respiratory syndrome; **STM**, *Salmonella enterica* serovar *Typhimurium*; **TAP,** tandem affinity purification; **TCR**, T cell receptors; **TNFα,** tumor necrosis factor alpha

1 Host–pathogen interactions

In every intracellular parasite infection in complex organisms there are two levels of interaction between the pathogen and the host: a microcosm where the interaction between parasite and target cell is produced, and a macrocosm where the interaction of microbe populations and the immune system takes place. In the first scenario, the pathogen must adapt at multiple levels to infect a target cell: entry *via* specific receptors or endocytic mechanisms, generation of copies of its own genome, expression of parasite proteins, and protein assembly to produce an infectious progeny. In the second scenario, the pathogen must escape from the immune response, both the natural immunity mechanisms—interferon (IFN), phagocytosis, natural killer (NK)

cells—and the adapted immune response restricted by the human leukocyte antigen (HLA) system, thereby including antigen processing and presentation, antibody response, and helper and cytotoxic cellular response mechanisms mediated by CD_4 and CD_8 lymphocyte populations, respectively. For an infection to be productive, the pathogen should have certain characteristics in order to overcome all these barriers, from adaptation to receptors and biochemical cellular machinery, to evasion from mechanisms of intra- and extracellular immune response. Furthermore, in many cases intracellular parasites modify the cell environment at different levels to allow or enhance their own replication in the infected cell.

1.1 General characteristics of infectious microorganisms: Pathogenicity and virulence

Virulence and pathogenicity are closely related terms. Pathogeny includes the mechanisms by which a microorganism produces disease in the host, whereas virulence is the capacity to produce a more severe disease when compared to similar pathogens [1]. It is well known that infection by different variants of the same microorganism produces more or less aggressive forms of the disease depending on a series of features known as virulence factors. Increased virulence is related to the gaining of new proteins by a particular microbe or the modification of their characteristics leading to increased toxicity in the host. The study of these factors means establishing a genotype–phenotype correlation in the invading microorganism and has great importance for understanding cell interaction and adaptation as well as cell destruction mechanisms and escape from the immune response. Furthermore, the definition of virulence factors is also indispensable in understanding the physiopathology of infectious diseases and why microorganisms of generally low pathogenicity, such as *Meningococcus*, *Pneumococcus*, herpes, or influenza viruses, may generate catastrophic epidemic onset or severe illness in certain circumstances.

1.2 Interactions at the cellular level

Intracellular parasites, especially viruses, have extraordinarily compact genomes which do not encode all necessary functions for a complete infectious life cycle. For this reason, parasite adaptation to the host cell requires the use of numerous cellular factors so as to generate an infectious progeny [2, 3]. Apart from recruiting specific factors for the different phases of the infectious cycle, intracellular parasites must control the biochemical machinery of the cell to replicate their genome and generate their own proteins. This control of the cellular machinery takes places at different levels [4]. For example, different families of viruses such as *Rhabdovirus, Poxvirus, Paramixovirus,* and *Reovirus* inhibit gene transcription through interference with RNA polymerases or transcription factors. On the contrary, regulatory proteins of lymphotropic viruses such as Tax from human T-cell leukemia virus type 1 (HTLV-1), EBNA1 from Epstein-Barr, and Tat from human immunodeficiency virus type 1 (HIV-1) act through transcription of cellular genes. Many other viruses inhibit host protein synthesis while viral protein production is maintained [5]. These shut-off mechanisms include inactivation of translation factors, degradation of host cell mRNAs, or production of factors that specifically inhibit host protein synthesis or induce translation of viral mRNAs. All these mechanisms of interference consequently lead to the detriment of metabolic routes necessary for cell life. Consequently, the infection alters basic cell functions such as cell structure and viability, which leads in most productive infections to cell death *via* apoptosis [6]. Moreover, intracellular parasites can turn the cell machinery to their own advantage through modification of the cell proteome. The final goal of this process is to allow replication of the parasite despite the strong toxicity. As an example, HIV-1 infection induces the cellular transcription factor nuclear factor κB (NF-κB), which is not only an essential regulatory element for the virus, but also protects cells from apoptosis, thereby leading to both HIV-1 replication and prolonged cell survival [6, 7] (Fig. 1).

1.3 Immune response and immune escape mechanisms

There are three different levels of immune response against parasites: innate intracellular immunity, natural immunity, and specific HLA-mediated immune response. The innate intracellular immunity compiles warning mechanisms against microbial infections that include non-specific systems, such as endocytosis and parasite degradation in lysosomes, and specific mechanisms such as RNA-dependent protein kinase (PKR) or restriction factors of retroviral infections (*e.g.*, TRIM5α, APOBEC3G) [8]. However, many microorganisms have generated strategies to escape from non-specific mechanisms: *e.g.*, both *Mycobacterium tuberculosis* and *Mycobacterium leprae* are resistant to acidic pH in lysosomes; and *Francisella tularensis* can translocate from the phagosome and replicate in the cytosol. Moreover, pathogens can also escape from specific immune mechanisms. For example, the IFN-induced double-stranded PKR is a mammalian serine-threonine kinase that phosphorylates the eIF2alpha translation initiation factor in response to stress signals, mainly as a result of viral infections. eIF2alpha phosphorylation results in arrest of translation of both cellular and viral mRNAs, which is an efficient way to inhibit virus replication. As a consequence, several viruses have evolved to express PKR inhibitors, such as the vaccinia E3L and K3L proteins [9]. Other cellular factors such as TRIM5α or APOBEC3G restrict replication of retrovirus through interference in the uncoating process or inhibition of viral infectivity [10, 11].

Natural immunity represents a powerful mechanism of antimicrobial defense that pre-exists or is rapidly induced in the absence of previous contact with the infectious agent. Soluble elements such as complement, IFN, or inflammatory mediators (cytokines and chemokines) together with cell

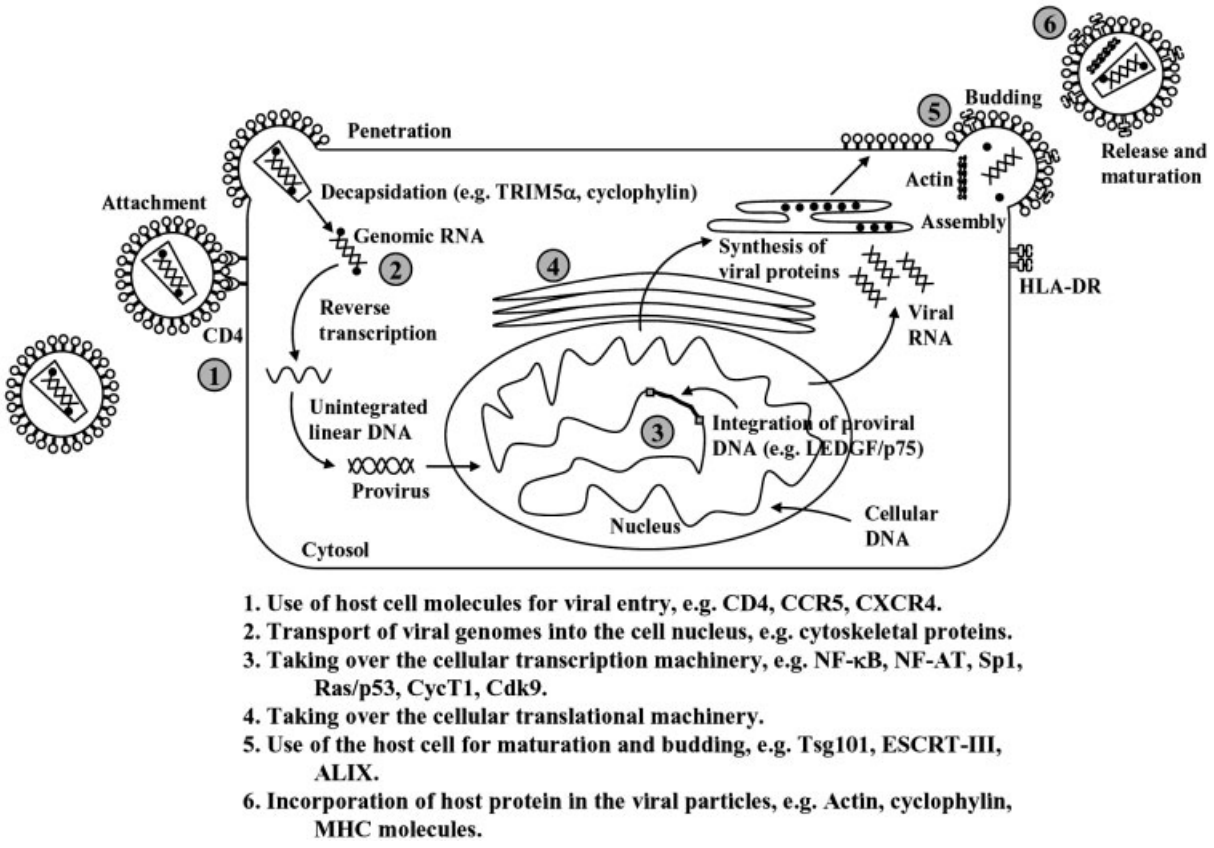

Figure 1. HIV-1 life cycle as an example of host–pathogen interaction. An intracellular pathogen is not only able to use the host proteins to replicate, but it can also incorporate cellular proteins in its structure to increase the spread or infectivity on adjacent cell hosts.

effectors, particularly macrophages and NK, can control or delay the replication and propagation of the pathogen. In the latter situation, natural immunity can provide enough time for the development of specific immune responses. However, parasites have also developed mechanisms to escape control of natural immunity. For example, large DNA viruses and parasites such as *Schistosoma mansoni* produce homologues of cytokines, chemokines, as well as their receptors, which are secreted to the medium and protect the infected cell from the action of inflammatory mediators [12]. Besides, human cytomegalovirus (HCMV) and HIV-1 escape from NK-mediated surveillance by down-regulating the expression of NKG2D ligands in infected cells [13].

Finally, major histocompatibility complex (MHC)-restricted immune response is highly specific and requires the following steps: (i) capture of microbes or their proteins by antigen-presenting cells; (ii) antigen processing and peptide presentation in class II MHC molecules; (iii) recognition by CD4 lymphocytes; and (iv) triggering of an effector response that will ultimately produce specific antibodies to neutralize soluble particles and cytotoxic lymphocytes to recognize and kill infected cells [14]. However, intracellular parasites, particularly viruses, have also developed multiple mechanisms to escape from this specific response [15]. MHC down-regulation is triggered by different viral families such as herpes, adenovirus, poxvirus, and retrovirus. Both epitope mutation and generation of a latent state in the infected cells are common mechanisms to escape from immune surveillance and make their eradication by the immune system extremely difficult [16].

All these changes generated in the pathogen as well as those provoked in the host cell can be studied by proteomic approaches in order to better characterize escape mutations of the infectious agent or modifications in the host immune machinery that lead to failure in antigen presentation or escape from humoral and cellular responses. Furthermore, by means of these studies, proteomic approaches can be applied for designing subunit or peptide-based vaccines [17].

1.4 Application of proteomics technology in the study of host cell and pathogen interaction

All mechanisms that are produced upon parasite–host interaction have in common the alteration of the normal cell proteome, both from a quantitative and a qualitative point of view: on the one hand, cellular biochemical machinery is

displaced to produce parasite proteins, therefore reducing synthesis of cell proteins; on the other hand, the generation of many PTMs in cell proteins render the cell more susceptible to parasite replication both at the viral protein transcriptional and translational level. Furthermore, new interactions between cell proteins and microbial proteins are produced and they direct different steps of the pathogen life cycle.

Proteomics is the large-scale, systematic study of proteins, particularly their interactions, modifications, localization, and functions. Usually, the tools of proteomics are used to analyze modifications induced during the course of cell activation, differentiation, or transformation. However, several shortcomings have been noted in the proteomic study of host–pathogen interactions. These restrictions are mainly due to technical challenges arising from the high complexity of these systems [18].

Nevertheless, infection by intracellular parasites is a privileged model for this type of study, as it permits one to have a precise experimental control (*i.e.*, a non-infected cell or low-virulence microbe variants) to compare protein profiles between parasite and host. In order to study this host–pathogen interaction by using proteomics, a global protein expression profile can be generated and compared using a 2-DE-based protein separation method and individual proteins can be identified when coupled to protein identification by MS. Besides, more specific interactions among host parasite proteins can be analyzed by immunoprecipitation techniques couple to MS.

Depending on the different levels of interaction between parasite and host, the use of proteomics is of particular use in the following aspects: (i) comparison of microbial proteomes from the same family but presenting a high or low virulence or with different phenotypic features; (ii) study of the modifications of cell proteins induced by viral infections, considering both productive infections and simple extracellular contact of the parasite with its receptors; the impact on the host proteome by selective expression of parasite proteins can be also analyzed to understand their function; (iii) analysis of PTMs caused by the infection, especially phosphorylation, which may result in the activation of transcription factors or induction of metabolic routes involved in apoptosis; (iv) study of interactions between parasite and host proteins at different levels: taking over the transcriptional and translational machinery, association with intracellular transport structures, generation of parasite protein production factories, or sequestering of cell factors involved in morphogenesis and cell exit processes; (v) both viruses and more complex parasites incorporate proteins from the infected cell once they emerge from it; proteomic analysis can define which proteins are incorporated into the parasite and their function in the life cycle; (vi) finally, by using proteomics, it might also be possible to study complex interactions such as those related to the host–vector–pathogen interplay, not only from the viewpoint of the vector, but also from that of the host and of the pathogens.

2 Proteomic approaches for the analysis of host–pathogen interactions

Proteomics has its roots in protein chemistry techniques used since the 1970s. By that time, the potential of 2-DE to separate hundreds of proteins had already been shown [19], but frustration also grew with the lack of tools to identify proteins of interest. It was not until the mid-1990s that MS became a mainstream technique for protein identification and characterization with the spread of soft ionization methods like ESI [20] and MALDI [21].

Despite technical challenges which pose some limitations in the application of proteomics technologies to the study of the host–pathogen interaction, these approaches are not only providing unparalleled information and understanding of the mechanism through which the host immune response is disarmed, but are also opening the way to biomarker discovery for early detection of pathogen exposure. In the following sections, a brief overview of proteomic technical aspects involved in the interaction between a host cell and an infectious agent is provided.

2.1 Sample preparation

Cellular proteins are commonly extracted from samples containing a wide variety of other biological molecules, namely, carbohydrates, lipids, nucleic acids, *etc*. Therefore, extraction protocols involve tissue and cell homogenization; protein solubilization with the use of such detergents as CHAPS, Tween, and SDS, and reducing agents such as DTT; protein denaturing with urea/thiourea to disrupt the bonds that are responsible for secondary and tertiary structure [22]; and addition of DNAse and RNAse enzymes to degrade co-extracted nucleic acids. A further complication specific to certain host–pathogen interaction studies is the need to include in the sample preparation protocols the necessary steps aimed at selectively releasing either host or pathogen proteins.

2.2 Protein separation

The incorporation of host-derived components in the microbial particles appears to be selective, since no cell molecule can be found embedded within the pathogen, but only a few of them are repeatedly found associated. Therefore, this is not a random process, because these proteins retain their functionality and could be related to a more effective spread and infection activity in the adjacent host cells.

To gain a comprehensive understanding of protein function and regulation in the host–pathogen interaction, proteins or peptides from a complex mixture must be separated, then identified and characterized. For this purpose, SDS-PAGE, 2-DE, or mono- or multi-dimensional LC are the techniques of choice for separating proteins or peptides. In the following sections, a variety of approaches aimed at the analytical separation of proteins in complex mixtures will be discussed (Fig. 2).

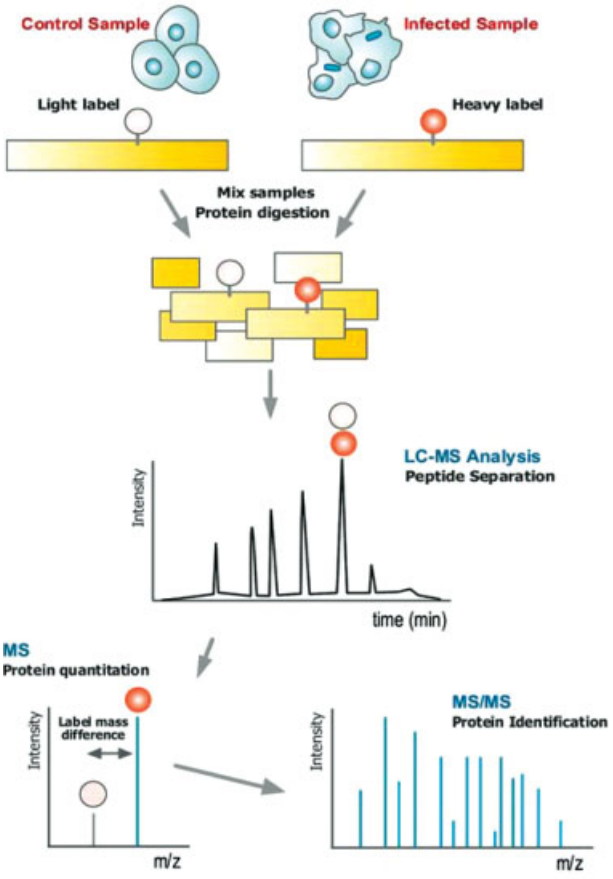

Figure 2. Proteomic analysis strategies. Proteins are extracted from biological samples, fractionated if needed, and separated using different techniques. In the gel-based methods (left) proteins are separated in 2-D gels based on their p*I* and size, and usually identified with MALDI-MS analysis. For relative quantitative comparison between two cell populations, samples are labeled with fluorescent tags, separated in 2-D gels and image analyzed (2-D DIGE technology) to detect differentially expressed proteins, which are identified by MALDI-MS. In the gel-free based methods (right) proteins are previously digested, usually with trypsin, and the peptide mixture is fractionated by LC. Usually, the peptides are identified *on-line* by ESI-IT MS. For relative quantitation analyses, proteins or peptides are previously tagged with stable isotopes.

2.2.1 2-DE

By using 2-DE, the components of protein extracts are resolved according to both their p*I* and their molecular weight [19]. Despite the 2-DE technique having some limitations mainly related to the solubilization of integral membrane proteins and the segregation of extreme p*I* and size proteins, this approach is currently the only technique that can routinely separate up to thousands of proteins in complex protein mixtures [23]. A number of works have supported the usefulness of the traditional 2-DE approach in understanding host–pathogen interactions [24].

2.2.2 HPLC

LC allows protein and peptide separation according to their affinity for a stationary phase when a mobile phase is forced through a fine capillary. Protein and/or peptide separation by HPLC is applied using a wide variety of stationary phases (*e.g.*, reversed, ion-exchange and affinity-based). The coupling of different protein and/or peptide separation devices such as multidimensional chromatographic approaches is a most powerful strategy. In particular, the ion-exchange/RP 2-D approach has revealed unprecedented capabilities for resolving complex peptide mixtures in a fully automated fashion with high sensitivity [25].

2.2.3 Subcellular fractionation

Regardless of the separation method employed, it is well known that proteins identified from highly complex samples (*e.g.*, whole cell lysates) are often high-abundant species, such as structural or house keeping proteins. The detection of less abundant proteins such as transcription factors is hampered by their low cellular concentrations and either the dominant signals from more abundant proteins (when using electrophoretic methods) or the much higher number of peptides thereof (if chromatography is preferred). In addition, hydrophobic proteins including membrane proteins are notably difficult to characterize by 2-DE mainly due to the low solubility in buffers commonly used for electrophoresis. Therefore, subcellular fractionation becomes necessary to reduce the complexity inherent to whole-cell lysates and for enriching subclasses of proteins (*e.g.*, membrane or nuclear proteins) [26].

No general strategies exist for enrichment in low-abundant proteins. Specific protocols must be based on the physicochemical properties of the analytes and their cellular location. Differential centrifugation and density gradient centrifugation are well-established procedures for subcellular fractionation compatible with protein solubilization and separation [26]. The membrane subfraction from either the pathogen or the host is a particularly relevant subproteome, since the interaction between surface proteins plays a pivotal role in the virulence and tropism of the pathogen. The membrane subproteomes of several organisms have been attained by using a combination of detergents and differential centrifugation procedures [27].

2.3 Protein identification

Proteins separated electrophoretically or protein mixtures are digested with a protease, typically trypsin, and the proteolytic peptides are subjected to MS analysis followed by database searching or *de novo* sequencing for protein identification. The advent of soft ionization techniques such as ESI [20] and MALDI [21] was a milestone in protein chemistry that revolutionized biological sciences. These MS techniques enable unambiguous and detailed characterization of pro-

teins resolved by the above-described separation methods with good throughput and high sensitivity at the femtomole level. If a top–down approach is followed, intact proteins are mass analyzed, normally after the induction of sequence-specific fragmentation in the mass spectrometer, while bottom–up proteomics focuses on peptides derived from proteolytically cleaved proteins. In the latter approach, the MS data are then analyzed using dedicated software algorithms that identify the proteins from which the mass-measured peptides were derived by searching protein databases [28]. This procedure can rely on intact peptide masses (PMF) or sequence-specific fragments thereof (MS/MS). Nevertheless, the under-representation of numerous pathogens in genome sequencing projects restricts the efficacy of database searching in host–pathogen interaction studies, often calling for the use of the *de novo* peptide sequencing approach [29].

2.3.1 MS/MS

MS/MS involves mass selection, fragmentation, and mass analysis and it combines at least two mass spectrometers in time and space with the objective of obtaining further information about the primary sequence of the analyzed peptides. This methodology was first used in the late 1960s [30], and the applications of the technique continue to grow. Most MS/MS configurations used are based on MALDI-TOF-TOF MS and ESI coupled to linear- or quadrupolar-IT-MS [31]. Given the high specificity of sequence-dependent fragmentation, MS/MS data enable more accurate database searching, *de novo* sequencing capabilities, and the characterization of PTMs.

2.3.2 Gel-based proteomics

In the gel-based approach, protein spots revealed by 2-DE or DIGE are cut out from the gel, proteolytically digested, and the resulting peptides are commonly analyzed by MALDI-MS followed by database searching or *de novo* sequencing. This approach allows the identification of differentially expressed proteins with good throughput and sensitivity at the femtomole level.

2.3.3 Gel-free proteomics: Multidimensional protein identification technology

Gel-free proteomics relies on the use of coupled chromatographic separation techniques for peptide separation after proteolytic protein cleavage in solution. Since ESI is ideally suited for on-line coupling to HPLC, this is the MS technique of choice in this approach, commonly referred to as multidimensional protein identification technology (MudPIT) [32]. MudPIT enables rapid identification of proteins that interact with tagged bait while bypassing some of the problems associated with analysis of polypeptides excised from SDS-polyacrylamide gels. It is based on the coupling of multidimensional chromatography and MS/MS. Using this methodology is possible to identify complex protein mixtures generated from biofluids, tissues, cells, organelles, or protein complexes. MudPIT has been employed for the identification of some interesting proteomes or subproteomes from pathogenic microorganisms [32–35]. In contrast to gel-based methods, MudPIT is not limited by the starting protein amount or the detection range, but by the complexity of the peptide mixture, which in turn leads to a decreased probability of identifying peptide from the less abundant proteins. The large number of data generated by these proteomics approaches has been combined with known biological networks to construct computer-aided models of the cellular processes of normal and diseased cell functions (network proteomics). This is an emerging area of the large-scale approaches (genomics, proteomics, metabolomics) to make sense of the analysis of differential expression experiments, by interconnecting expression-responsive genes, such as a catalogue of identified proteins through proteomic techniques, with protein interaction databases [36].

These tools could also be applied for the study of infectious diseases and host–pathogen interactions, for example, as has been described by Mawuenyega *et al.* [37]. A combination of this strategy with the subcellular protein profiling of *M. tuberculosis* has permitted the identification of proteins whose involvement in the reconstructed response networks for fatty acid degradation and lipid biosynthesis pathways were not previously suspected, thereby providing interesting insights into the compartmentalization of these pathways as well as its role.

2.3.4 Co-immunoprecipitation assays coupled to MS

Some standard methods for protein partner discovery are based on co-immunoprecipitation techniques. This approach represents a great advantage for identification of protein interactors that may reveal new functions or represent regulators of transcriptional factors. It implies the selection of specific antibodies against a target protein for the first immunoprecipitation. The result of this step is the semi-purification of the target protein and its bound partners. Subsequent steps include protein separation by SDS-PAGE and the transfer to an NC membrane. Finally, the membrane is revealed by immunoblotting using a limited set of selected antibodies against the putative interacting proteins. This limited method can now be combined with MS/MS to improve the identification results. This modification is based on the possibility of analyzing the immunoprecipitated pool of proteins by LC-MS. Either the eluted proteins or the solid immunoprecipitation support to which the retained proteins are attached must be previously digested in solution with trypsin. An interesting variation is the association of the tandem affinity purification (TAP) method and MudPIT. TAP allows rapid purification under native conditions of complexes by incubating the protein extract of interest with the target protein fused to the TAP tag, either N- or C-terminally. These combined approaches do not require prior knowledge

of the complex composition or function, and permit proteomic characterization of a pathway, organelle, or process to be made [38].

2.4 Quantitative proteomics

The methodologies described in Section 2.3 can be adapted to obtain additional quantitative results. Quantitative proteomic studies are directed at measuring relative differential expression between different cellular states. To minimize run-to-run variability, multiplex analyses allow the measurement of several differentially tagged samples in a single experiment. Currently used gel-based quantitative studies rely on protein labeling with fluorescent dyes for later image comparison, while gel-free approaches make use of isotopic or isobaric labeling of proteins/peptides amenable to LC-MS analysis (Fig. 3).

2.4.1 Gel-based approaches (DIGE)

Traditional 2-DE analysis has been criticized due to gel-to-gel variations. This variability makes the image analysis process necessary to assess differential protein expression a laborious, time-consuming process. Through the use of fluorescent dyes to label protein samples prior to 2-DE, fluorescence 2-D DIGE enables the multiplex analysis of multiple protein samples within one gel [39]. In this approach, protein extracts (*e.g.*, infected and control) are labeled with different fluorescent dyes, then combined and separated by 2-DE, and the corresponding gel images are obtained using the appropriate excitation wavelengths. These images are then merged and processed by dedicated image analysis software to reveal differences between them corresponding to differential protein expression between protein samples. The fluorescent dyes employed show a 5-log linear response to protein abundance, are sensitive enough to reveal subnanogram protein amounts, and are fully compatible with subsequent MS analyses.

2.4.2 Gel-free approaches

To circumvent the drawbacks inherent to gel-based approaches (Section 2.2.1), in recent years a variety of methods based on multiplex protein or peptide tagging for later MS quantification have been described.

2.4.2.1 Isotope-coded affinity tags

The isotope-coded affinity tag (ICAT) approach is aimed at relative protein quantification based on stable isotope labeling of proteins [40]. The ICAT™ reagent consists of (i) a thiol-reactive group that reacts with cysteine residues; (ii) a linker incorporating stable ^{12}C and ^{13}C isotopes; and (iii) a biotin tag for affinity purification. In this system, one sample (*e.g.*, infected sample) is labeled with a tag containing a heavy isotope, while the other sample (*e.g.*, control sample) is labeled with the light version. Then, both samples are mixed, proteolytically digested, and eluted from an RP-HPLC column as pairs. Differential protein expression is assessed by calculating the relative ratio of the two isotopically labeled peptides revealed by MS analysis.

2.4.2.2 Isobaric tags for relative and absolute quantitation

Recently, an improved approach analogous to ICAT has been developed called isobaric tags for relative and absolute quantitation (iTRAQ) [41]. The technique is based on chemically tagging the N-terminus of peptides generated from protein digests that have been isolated from cells in different states.

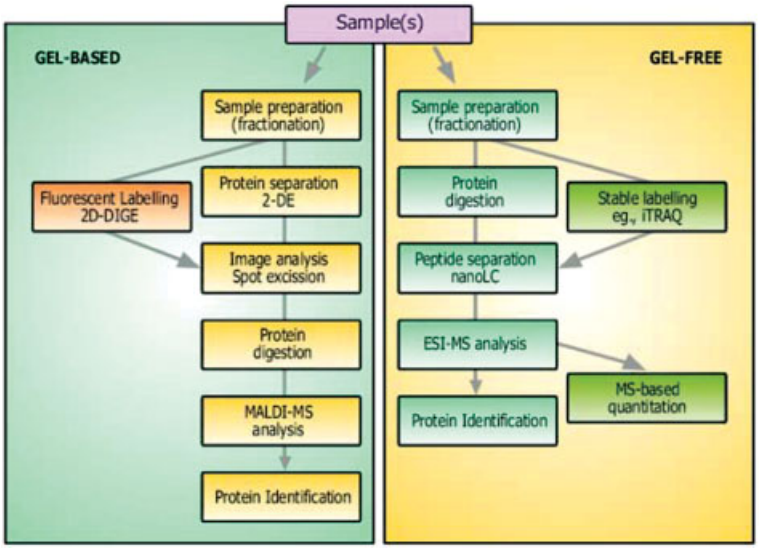

Figure 3. Isotopes in LC-based quantitative proteomics. Peptides containing "heavy" or "light" isotopes from two different protein conditions are separated by LC, co-eluting at the same retention time. During analysis (MS), the isotopic mass difference allows relative quantitation of the two different peptides, since the peak intensity is proportional to peptide abundance. Subsequently, either isotopic signal can be selected for fragmentation and sequence analysis and identification (MS/MS).

The labeled samples are then combined, fractionated by nano-LC, and analyzed by MS/MS. Fragmentation of the tag attached to the peptides generates a low-molecular-mass reporter ion, which is unique to the tag used to label each of the digests. Measurement of the intensity of these reporter ions enables relative quantification of the peptides in each digest and hence of the proteins from where they originate.

2.4.2.3 Stable isotope labeling with amino acids in cell culture

Another strategy for determining differential protein expression from two different cellular populations is the stable isotope labeling with amino acids in cell culture (SILAC) [42]. In this procedure, cells from two distinct biological conditions are cultured in parallel with culture media deficient in a natural amino acid, but supplemented with a different mono-isotopically labeled amino acid. After metabolic incorporation of light and heavy isotopes during cellular protein synthesis, proteins from each sample are isolated, mixed at a 1:1 ratio, proteolytically digested, and mass analyzed. Corresponding light and heavy peptides from each sample co-elute from the HPLC column, and the relative quantification of proteins is achieved by measuring the ratios of the mass peaks of the corresponding peptides by MS.

3 Application of proteomics technology for analyzing the host–pathogen interaction

The identification of pathogen proteins, particularly virulence factors and toxins, can be achieved by matching multiple virulent and non-virulent strains, mutants, knockouts, *etc.* Nevertheless, the pathogen proteome is often overwhelmed by the host proteome given the size differences regarding the proteomes, the host and the pathogen, and the pathogen/host ratio. To circumvent this inconvenience, host-free models have been considered for proteomic analysis of pathogens, normally by growing pathogens under physiologic *in vitro* conditions which mimic interaction with the host. Given the absolute dependence of viruses on target cells to replicate and the complexity of life cycle stages of fungi and parasites, to date proteomic reports in host-free models have been dominated by bacterial pathogen studies.

Presently, most proteomic studies are focused on describing the structural proteins from several microorganisms including viruses [43–46] and bacteria [47–50], while only a few reports are devoted to evaluating host–pathogen interaction by using proteomics. In the following sections, a brief overview of the application of proteomics in this field is provided, from the pathogen viewpoint as well as from the host viewpoint.

3.1 Modification of host cell by infection with intracellular pathogens

Intracellular pathogens can modify the host cell metabolic pathways for their own benefit. In fact, virulence often requires the usurpation of existing host cell signaling pathways or membrane traffic machinery. Therefore, pathogen exposure may elicit changes in host proteins that can be gathered in many categories, including cell signaling pathways, protein degradation, cytokine and growth factor production, phagocytosis, apoptosis, as well as cytoskeletal rearrangement. These effects may be exerted immediately after the binding of the pathogen to the receptor on the cell surface, either by a new protein encoded by modifying the activity of a cellular protein, or by a microbial protein mimicking a cellular protein. Some of the most important cellular pathways modified by intracellular pathogens are explained and are summarized in Fig. 4.

3.1.1 Modification of apoptotic pathways in the host cell

Most pathogens alter apoptotic pathways, either enhancing or inhibiting these processes. Apoptosis plays a critical role in inhibiting the proliferation of invasive organisms and viruses, thereby protecting uninfected cells and limiting damage to the host organism. Apoptosis is a complex mechanism that plays a critical role in the pathogenesis of several intracellular pathogens. Therefore, understanding the molecular basis of the host response to microbial infection, particularly anti-apoptotic responses, is essential for identifying targets to prevent disease and tissue damage resulting from the inflammatory response.

Chlamydia is a classical example of obligate intracellular bacteria. It causes eye infection, sexually transmitted diseases, and respiratory infections, depending on the species [51, 52]. During this cycle, *Chlamydia* alternates between an infectious extracellular pathogen and an intracellular dividing form surrounded by phagosome membrane termed inclusion [53]. Proteins secreted from the inclusion may interact with host cell proteins and modify the host cell response to infection. The identification of these proteins is difficult because the host cell cytoplasm of *Chlamydia*-infected cells cannot be purified. However, this problem has been solved by using comparative proteomics [53]. As a result, it was determined that *Chlamydia* either promotes or inhibits apoptosis of the infected host cell during intracellular development [54]. In fact, there are anti-apoptotic mechanisms that take place during the early stage of the development cycle as part of its intracellular survival strategy [55–57].

On the other hand, *Neisseria gonorrhoeae*, the causative agent of gonorrhea, inhibits apoptosis in human monocytic cells by preventing tumor necrosis factor alpha (TNFα)-dependent processes [58]. TNFα plays a central role in initiating apoptosis and therefore in protecting against bacterial infections [59]. Beck and Meyer [58] proposed that *N. gonorrhoeae*

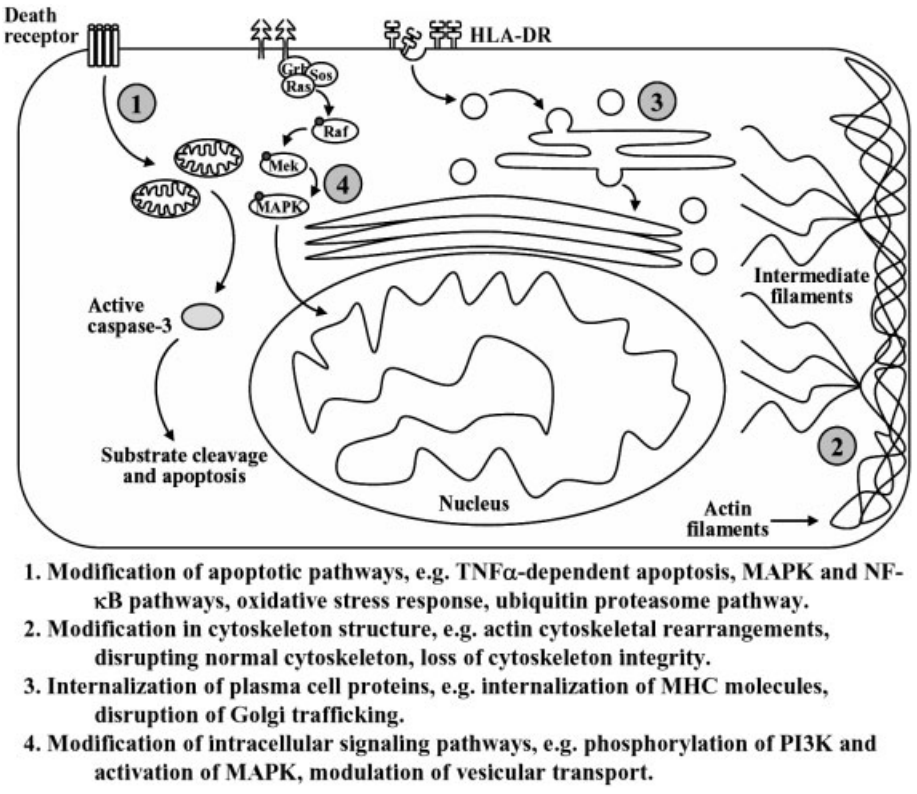

Figure 4. Modification of host cell by infection with intracellular pathogens. Intracellular pathogens can modify the host cell metabolic pathways for their own benefit, thereby modifying critical pathways such as apoptosis, phagocytosis, cytoskeletal rearrangement, cell signaling, protein degradation, *etc.* Some of the most important cellular pathways modified by intracellular pathogens are summarized.

IgA1 protease directly cleaves the extracellular ligand-binding domain from the TNF receptor type II (TNF-II), preventing its activation and subsequently the TNFα-dependent apoptosis. Besides, the inhibition of the host inflammatory response *via* both the mitogen-activated protein kinase (MAPK) and the NF-κB signaling pathways facilitates the cellular invasion [60]. Accordingly, recently studies have also identified two deubiquitinating proteases from *Yersinia* (YopJ and YopP), which are virulence factors directly involved in the block of inflammatory response by inhibiting these MAPK and NF-κB pathways. This mechanism might be important for evasion of the host immune response and may aid in establishing a systemic infection [61–63].

Another example of apoptotic induction is shown by *Bacillus anthracis*, a Gram-positive bacterium that produces the anthrax lethal toxin (LeTx) [64]. Inhalation of anthrax occurs when *B. anthracis* endospores enter the body through the respiratory tract. They are then phagocytosed by alveolar macrophages, which are the main target of LeTx [65]. Recent proteomics approaches such as DIGE followed by nano-LC-MS have identified downstream proteins that are targeted by LeTx [66–68]. Interestingly, most of the cell proteins modified after LeTx phagocytosis are related to events that ultimately lead the macrophage into apoptosis directly or through decreasing reactive oxygen species production [67, 68]. Failure in oxidative stress response leads to apoptosis, possibly through the action of caspases such us caspase-9, an initiator of an apoptotic pathway that occurs through release of cytochrome *c* from the mitochondria, which is up-regulated in macrophages after treatment with LeTx [68, 69].

Other pathogens are also able to alter the apoptotic pathway that occurs through release of cytochrome *c* from the mitochondria in response to oxidative stress within the cell [69]. In fact, reactive oxygen species generation and changes in the control of mitochondrial membrane potential have also been involved in severe acute respiratory syndrome (SARS) coronavirus (CoV) pathogenesis, as ATP synthase and cytochrome *c* oxidase were differentially up-regulated in cells that express the SARS CoV 3C-like protease (3CLpro) [70]. This protease plays an essential role in virus replication as it mediates the proteolytic processing of the viral replicase. In human promonocyte cells, 3CLpro has been demonstrated to induce apoptosis *via* caspase-3 and caspase-9 [71]. A comparative proteome analysis of human promonocyte cells expressing or not expressing 3CLpro, performed by 2-DE and nano-LC/ESI-quadrupole-TOF MS, showed that viral protease expression results in alteration of the ubiquitin proteasome pathway [70]. This pathway plays a central role in several cellular processes including antigen processing, apoptosis, cell cycle, inflammation, and response to stress [72] and its up-regulation might be involved in SARS pathogenesis. Consequently, both ubiquitin protein degradation and modification have also been shown to be essential for the host immune response to pathogens [60].

3.1.2 Modification in the cytoskeleton structure of the host cell

Targeting the cytoskeleton of the host cell is a common strategy among pathogens and reflects the alteration of many cellular activities [73]. In addition, the actin cytoskeleton system is crucial for active recruitment in cells such as lymphocytes and macrophages that rapidly have to move to the infection and inflammation sites [74]. For example, comparing protein profiles of gastric epithelial cells infected or not infected with *Helicobacter pylori* by using 2-DE and PMF reflects that infected cells up-regulated tropomodulin 3 (TMOD3) [75], a protein involved in negative regulation of pointed-end disassembly and turnover of free barbed ends of actin [76] as well as in focal adhesion complex signaling and remodeling [77]. Consequently, TMOD3 up-regulation may exert an influence on *H. pylori* inducing actin cytoskeletal rearrangements associated with cell elongation [78–80]. Moreover, *H. pylori* induces cell motility, which is a key element in tumor progression.

Another example is *Listeria monocytogenes*, a Gram-positive bacterium that causes the disease listeriosis. The primary site of infection is the intestinal epithelium where the bacteria invade non-phagocytic cells. After uptake, this bacterium is able to rupture endocytic vesicles in order to replicate within the cytosol of host cells [81]. Moreover, *Listeria* can move within eukaryotic cells by driving polymerization of actin filaments and, therefore, it can infect adjacent cells without exposing to the extracellular environment [82]. This ability is mainly due to the surface protein ActA, which can promote actin polymerization at the bacterial cell surface [83]. Therefore, this unique protein makes it possible that *Listeria* has the driving force required for intracellular movement.

With regard to causing pathogenesis by disrupting the normal cytoskeleton, enterovirus 71 infection (EV71) induces the up-regulation of CFL1, a protein that increases the rate of actin depolymerization and facilitates actin filament turnover [84, 85]. Furthermore, low levels of the protein stathmin 1 (STMN1), involved in assembly regulation of the microtubules filament system [86], have been associated with neuropathogenesis in both EV71 [85] and Alzheimer disease [87].

There is also evidence that infection produced by Coxsackievirus B3 (CVB3), a member of the family *Picornaviridae*, leads to a loss of cytoskeleton integrity [88] by affecting the regulation of γ-actin, lamin, and various subunits of tubulin [89]. Nevertheless, proteome profiling based on 2-DE and MALDI-MS cannot discriminate whether the degradation of lamin, the main component of the nuclear lamina, is a direct effect of the viral protease or a side effect due to viral-induced apoptosis. Lamin modifications could be the cellular basis for virus-mediated myocarditis, given that the essential nuclear architecture is altered [90].

The host cell proteome can even be altered by the intracellular expression of a single protein from a specific pathogen. For example, the regulator protein Tat from HIV-1 is a potent activator of viral gene expression and replication [91]. Recently, the effect of constitutive intracellular Tat expression in a lymphoid T cell has been analyzed by performing DIGE followed by MS analysis [92]. Tat-expressing cells showed down-regulation of proteins involved in cytoskeletal activities such as actin, β-tubulin, annexin, cofilin, and gelosin, responsible for causing profound cytoskeletal reorganization in the host cell. Interestingly, it has been reported that these cytoskeletal proteins are essential to activate mitochondrial-dependent apoptotic pathways [93], and therefore, its down-regulation may explain the resistance to apoptosis found in Tat-expressing cells [92]. Moreover, this could explain the survival of certain subsets of HIV-infected CD_4^+ T cells leading to long-term reservoirs and continuous producers of HIV-1 virions.

On the other hand, the obligate intracellular parasite *Toxoplasma gondii* of the phylum Apicomplexa has the capacity to infect almost any warm-blooded animal and is one of the most successful protozoan parasites [94]. The apical complex that defines this protozoan has been implicated in attachment, invasion, and growth of the parasite within the various cell types that are invaded [95, 96]. The complex consists of a microtubular spiral (conoid) and two sets of apical secretory organelles (micronemes and rhoptries) that release their contents upon contact with the host cell. They include several molecules that span the plasma membrane of the parasite and provide a connection between the host cell and the actin/myosin-based motor of the parasite [97]. Analyses performed by MS of rhoptry proteins resolved by SDS-PAGE have permitted the identification of some proteins which could play a key role in the ability of these parasites to invade and adapt the host cell for their own survival and growth [98].

3.1.3 Internalization of plasma host cell proteins

The MHC (or HLA) encodes proteins that are expressed on the surface of cells. These proteins have a vital role in the complex immunological interaction that occurs between T lymphocytes and other cells of the host organism. MHC molecules display short polypeptides to T cells, *via* the T cell receptors (TCRs). The polypeptides may have originated from a protein created by the organism itself, or they may have originated from bacteria, viruses, protozoans, *etc*. The MHC–TCR interaction allows that T cells ignore self peptides while reacting appropriately to the foreign peptides. Some intracellular pathogens are able to internalize these molecules, thereby blocking the host cell communication with other cells.

For example, CVB3 encodes two non-structural proteins named CVB3 2B and 2BC that are able to increase internalization of MHC class I complexes from the cell surface [99]. This may explain why infected cells remained invisible to CD_8^+ T cells. However, the dramatic trafficking inhibition imposed by CVB3 3A protein by disrupting the Golgi apparatus is incomplete, and there may exist a cycling of pre-

existing MHC class I molecules to and from the infected cell surface because the overall levels of cellular protein synthesis are not affected [100, 101]. An increased internalization of MHC class I molecules has been also observed in HIV-1-infected cells [102]. In particular, the regulatory protein Nef down-regulates the cell-surface expression of MHC class I proteins by inducing endocytosis re-localized to the *trans*-Golgi network [103], thereby promoting immune evasion. The mechanisms implicated in MHC endocytosis have not yet been completely elucidated, but are known to require the binding of Nef to phosphofurin acidic cluster sorting protein 1 (PACS-1) [102], which enables the formation of transport vesicles from membranes and controls the endosome-to-Golgi trafficking of specific proteins [104, 105].

3.1.4 Modification of cell host intracellular signaling pathways

Many pathogens have evolved sophisticated mechanisms to imitate host proteins and to hijack the intracellular signal transduction systems for their own benefit [81]. In fact, the binding of pathogens to target cells is known to be mediated by several intracellular signaling events. For example, HIV-1 binding to a target cell induces changes such as phosphorylation of phosphoinositide 3-kinase (PI3K) and activation of MAPK [106, 107]. This process has a role in the regulation of HIV-1 transcription and virion production [108, 109]. Therefore, these virus-mediated signal transduction events might be partly due to the additional interactions between host-encoded glycoproteins on virions and their physiological counter-receptors on the target cell surface [110]. Besides, virion-associated host MHC class II glycoproteins could initiate a wide array of different cellular responses ranging from activation, anergy, and apoptosis through interaction with the TCR/CD3 complex located on the target cell [110].

Furthermore, the majority of the hepatitis C virus (HCV) proteins modified during replication are involved in vesicular transport, protein trafficking, and cell signaling, as was assessed by DIGE coupled to MS and by SILAC combined with SDS-PAGE separation and MS [111]. Differential protein expression in HCV-replicating cells was largely concordant with both proteomics approaches. Up-regulation of several Rab GTPases, proteins involved in vesicular transport by facilitating fusion between membrane compartments, was found [112]. This might reflect a complex modulation of vesicle formation and traffic driven by HCV. Finally, down-regulation of proteins belonging to the Rho family of small GTPases (*e.g.*, Cdc42) might be responsible for increasing HCV replication. In fact, it has been shown that Cdc42 stimulates the PI3K pathway as well as the molecular target of rapamycin (mTOR), a member of the phosphoinositide 3-kinase related kinase (PIKK) family and a central modulator of cell growth, which in turn constrains HCV replication [113, 114].

3.1.5 Modification of cell host metabolic pathways

Proteomic approaches permit the identification of proteins interacting with the host cell metabolism. The obligate intracellular pathogen *M. leprae* proteome has been subfractionated in its major subcellular compartments and analyzed by DIGE combined with ESI-MS, MALDI-MS and N-terminal sequencing [115]. Results indicated that most of the novel proteins found are involved in intermediary metabolism, such as enzymes required in fatty acid β-oxidation. This finding reinforces the idea that *Mycobacterium* receives most of its energy from the degradation of host-derived lipids [116]. Besides, it has been suggested that the HCV core protein plays a crucial role in liver pathogenesis by affecting lipid metabolism [117]. A comparative proteomic analysis of lipid droplet proteins in core-expressing and non-expressing hepatoma cell lines, based on SDS-PAGE and MALDI-MS or direct nano-LC-MS, has revealed that core proteins participate in lipid droplet biogenesis by reducing adipose differentiation-related protein through a highly increased tail-interacting protein of 47 kDa (TIP47) [118].

Proteomics also constitutes a powerful tool for the study of plant stress response. For example, the plant pathogenic fungus *Fusarium graminearum* causes one of the most destructive diseases of wheat (*Triticum aestivum*) and it results in serious grain yield and quality losses [119]. Mycotoxin contamination in harvested grain is also a major health concern for both humans and animals [119, 120]. A proteomic study on the interaction between *F. graminearum* and wheat by using nano-LC-MS reported a complex cellular network in the wheat cells in response to fungus infection [121]. The analysis showed an oxidative burst inside the tissues infected by this fungus, given that proteins with antioxidant function were up-regulated, as well as a direct interaction of *F. graminearum* with the glycolysis pathway, because the pathogen obtains carbon from wheat [121].

3.1.6 Identification of cellular interaction partners

Only a few proteomic studies on the interaction with cellular factors have been described, *e.g.*, the identification of cellular partners of the influenza virus ribonucleoprotein (RNP) complex and polymerase complex by proteomics approaches [122]. In fact, most of the previously described virus-interacting cellular factors were identified by either yeast two-hybrid screens with individual components of the viral RNP, including polymerase subunits or co-immunoprecipitation experiments using specific antibodies directed against a target cellular interacting partner [123–127]. In this work, for the identification of cellular factors associated to native viral complexes, the authors purified reconstituted viral RNPs by strep-tagged viral RNP (NP-Strep) and the viral polymerase by TAP-tagged polymerase subunits from human cells. Co-purified cellular factors were identified by LC coupled to ESI-IT MS, resulting in the identification of 41 cellular-interacting proteins, including five of the seven previously described

interaction partners. Taylor *et al.* [128] used immunoprecipitation and MS to identify over 50 cellular and viral proteins as partners of the herpes simplex virus 1 (HSV-1) ICP 8 ssDNA-binding protein. Some of the identified partners were hypothesized to be involved in HSV-1 replication.

3.2 Modification of pathogen proteome after infection

During the interaction of host cell and pathogen in the course of an infection, not only is the host cell proteome adapted to benefit the pathogen, but there are also pathogens that can evade host resistance mechanisms by altering their own proteome following cell infection. This is the case of *Salmonella enterica* serovar *Typhimurium* (STM), a facultative intracellular pathogen that infects macrophages to cause typhoid fever. LC-MS-based methods have been used to identify STM proteins that contribute to the replication of these bacteria inside macrophages. Shi *et al.* [129] suggested that proteins STM3117-3119, whose coordinated expression modulates the peptidoglycan layer, a major component of the STM cell wall that serves as the first line of defense against the antibacterial agents produced in host cells [130], function as critical components in mediating macrophage colonization and subsequent systematic infections.

Other pathogens can modify their own metabolism or proteome to adapt to the host cell, thereby increasing virulence or inducing a latent state within the infected cell to remain unnoticed for the immune system.

3.2.1 Modification of pathogen metabolism

Some other pathogens modify their metabolism to adapt to their specific host. For example, *Streptococcus pneumoniae* is a major pathogen causing very serious and often fatal diseases such as meningitis, sepsis, or pneumonia [131]. The wide variety of invasive diseases caused by this microorganism implies that it is able to adapt to the environmental conditions encountered at several sites of infection [132]. Analysis of the modification in the *S. pneumoniae* proteome caused by blood has been performed using a combination of highly sensitive 2-DE and MALDI-MS and/or LC-MS [133]. It was determined that *S. pneumoniae* altered its metabolism when exposed to blood as an adaptation to its host. Besides, analysis of the switch in protein expression by *S. pneumoniae* at the transition from log growth phase to stationary phase has revealed protein species involved in central intermediary metabolism, amino acid synthesis, nucleotide and fatty acid metabolism, cell wall synthesis, protein degradation, and stress responses, suggesting previously unrecognized relationships between proteins along the life cycle of this pathogen [134]. The heat shock response of *S. pneumoniae* at the protein level has been addressed by comparing protein expression patterns at 37°C and 42°C. This study revealed several factors amenable to play a role in the physiopathology of this pathogen [135].

3.2.2 PTMs in pathogen proteins

The interaction between host and pathogen not only entails alteration of protein expression levels, but also post-translational processing of the specific proteins involved. Thus, it is known that some proteins of intracellular pathogens need the interaction with other host-cell proteins to be modified. This is the case of PTMs in some proteins from bacteria and viruses, which are mediated by the cellular protein machinery. Such modifications are generally reversible and play key roles in protein functional regulation. Among many reported types of PTMs, phosphorylation in serine, threonine, and tyrosine residues has received significant attention, since this reversible modification is a key event in signaling processes [136]. Antibodies directed toward phosphorylated residues have been used to enrich protein extracts for phosphoproteins by immunoprecipitation [137]. IMAC allows enrichment of phosphopeptides in more large-scale studies [138]. Phosphorylation is also common in virus proteins implicated in the control of virus replication, transcription, and assembly. This is the case for Rubella virus capsid protein [139], influenza virus nucleoprotein [140], and Rabies virus nucleoprotein [141]. Therefore, great effort has been directed towards developing methods for detecting and characterizing these modifications. However, identification of phosphorylated residues was a difficult task, involving direct labeling of proteins with ^{32}P [142]. ESI is one of the softest ionization techniques for MS [20], which allows the detection and characterization of numerous PTMs, as direct sequence information is obtained. Highly complex protein mixtures can be identified directly, and the possible PTM characterized by MS [32]. For example, MudPIT coupled to MS has been used to study the nucleocapsid protein phosphorylation of transmissible gastroenteritis virus (TGEV) in infected cells [143], which led to the identification of at least four phosphorylations in the TGEV nucleocapsid protein. Interestingly, previous work had suggested that phosphorylation/dephosphorylation processes could play an important role in coronavirus assembly [144].

The phosphorylation state of the human respiratory syncytial virus (HRSV) phosphoprotein (P protein) has been also assessed by LC-MS [145]. Further experiments demonstrated that phosphorylation in a threonine residue on the HRSV P protein plays an important role in the interaction with another viral protein (M2-1), which is therefore implicated in viral transcription and replication.

3.2.3 Pathogenicity and virulence factors

As described in Section 1.1, virulence factors make different variants of the same microorganism cause a more severe disease, whereas pathogenicity is the ability of an organism to cause disease in another organism. Both can be influenced by the immunological state of the host organism. For example, *Candida albicans* is one of the most commonly encountered human pathogens, causing a wide variety of infections

ranging from minor mucosal infections in generally healthy persons to life-threatening systemic infections in individuals with impaired immunity (*e.g.*, oral and esophageal *Candida* infections are frequently seen in AIDS patients). Few classes of drugs are effective against these fungal infections, and all of them have limitations with regard to efficacy and side effects. Yeast and hyphal forms of *C. albicans* have been compared upon sequential solubilization of the cell wall [146]. This study revealed, among others, glycolytic and fermentation enzymes believed to be essential in hyphal formation as well previously unreported hyphal-specific proteins. Since virulence is believed to be related to *C. albicans* hyphal morphologic form, these proteins are potential targets for antifungal drugs.

Another example is *F. tularensis,* a facultative intracellular bacterium that is the causative agent of tularemia and rabbit fever. It spreads easily by aerosol routes and is highly virulent. Multiple strains of *F. tularensis* spp. were studied by 2-DE and MALDI-MS to determine subspecies tularensis-specific proteins, which represent putative virulence factors. This led to the identification of several tularensis-specific proteins, including PilP, a component necessary for the biogenesis of the type IV pilus, and virulence and adhesion factors for many human pathogens [147]. Confirmation of specific gene transcription in this highly virulent pathogen in response to iron restriction was recently made at the protein level based on metabolic labeling [148].

Pseudomonas aeruginosa is a clear example of an opportunistic pathogen that causes serious infections in burn victims, cystic fibrosis patients, and the immunocompromised. To this aim, it uses several virulence factors that modify the physiology of the host cell [81]. For example, the toxin exoenzyme S (ExoS) is injected directly into the cytosol where it modifies crucial components of the intracellular signaling machinery. As a consequence, this pathogen alters the actin cytoskeleton and the cell survival machinery, thereby resulting in cell death [149].

Proteomics technology has been also applied to analyze the virulence of the proteome of *Yersinia pestis,* an intracellular pathogen that is the causative agent of the systemic invasive infectious disease classically referred to as bubonic plague. The effect of temperature and calcium concentration on the proteome of *Y. pestis* was investigated in a DIGE-based study [150]. Proteomic analyses of *Y. Pestis* grown under temperature and calcium concentration conditions mimicking host bloodstream, and host intracellular environment permitted the identification of a variety of virulence-associated factors such as catalase-peroxidase, murin toxin, plasminogen activator, and F1 capsule antigen.

Besides, the comparative proteomic study of pathogenic and non-pathogenic strains facilitates elucidation of the biological mechanisms underlying their virulent phenotypes. This approach has been followed in the identification of the *Listeria* cell wall subproteome [151]. A comparative analysis of the cell wall subproteome between two different species of *Listeria,* pathogenic and non-pathogenic (*L. monocytogenes* and *L. innocua,* respectively), was described in order to discover the surface proteins involved in the adhesion to the host cell surface receptors. Although no protein interaction between pathogenic bacteria and the host cell was described, a subset of proteins strongly associated to the peptidoglycan was identified. This subgroup of surface proteins plays an important role in the adsorption of the pathogen to the host cell and therefore in the virulence of the bacteria.

Another example is the proteomic analysis of the outer spore surface or exosporium of *B. anthracis.* Concerns regarding these bacteria as a biological weapon focus on the spore because of its resistance to heat, radiation, *etc.*, and its ability to survive in the dormant state for a prolonged period [152]. Killing dispersed spores effectively has proven difficult and it would be beneficial, therefore, to be able to selectively inactivate *B. anthracis* spores. This could be achieved by finding components unique to these spores which are critical for resistance and targeting them. For this purpose, the exosporium of *B. anthracis* has been characterized by electrophoresis and peptide mapping [153]. Several spore coat proteins were identified that are targets for functional studies. Huang *et al.* [154] performed a comparative study of dormant and germinating *B. anthracis* spores that enabled identification of several spore proteins with germination-associated differential expression. These proteins are targets for the development of prophylactic and therapeutic agents against anthrax. The secretomes of virulent and avirulent strains have also been compared to conclude that CO_2-responsive chromosome- and plasmid-encoded regulatory factors modulate the secretion of potential novel virulence factors, most of which are associated with extracellular proteolytic processes [155].

3.2.4 Mechanisms of latency

Tuberculosis remains a leading cause of mortality worldwide due to the ability of *M. tuberculosis* to adapt to a wide range of conditions both inside and outside the human host. *M. tuberculosis* is a globally successful pathogen due to its ability to persist for long periods of time unrecognized by the human immune system. The panoply of genes that allows the organism to enter latency and then re-emerge later during endogenous re-infection are now being elucidated [156]. Proteomic studies aimed at deciphering the molecular basis of latency in tuberculosis started with the work of Starck *et al.* [157] on the cytosolic proteome of *M. tuberculosis* under aerobic and anaerobic conditions. Several proteins related to the latent phase were identified and it was estimated that this pathogen induces expression of *ca.* 1% of its genes in response to latency. A study on the challenging membrane proteome of *M. tuberculosis* has been carried out that revealed a number of proteins potentially associated with immunogenicity [158]. Proteins specific to the intraphagosomal phase of the pathogen have been determined by matching protein patterns of intraphagosomally grown *M. tuberculosis* against those of broth-cultured

mycobacteria, albeit the role of these proteins as factors enabling intracellular survival of the pathogen is under discussion [159].

3.3 Incorporation of host proteins by pathogens after infection

The incorporation of host-derived components into microbial particles is selective, and therefore only specific host proteins can be found in association with the pathogen. These associated proteins retain their functionality, depend on the producer cell system, and could be related to a more effective spread and infection activity in the adjacent host cells.

3.3.1 Host-derived proteins incorporated into pathogens

In the life cycle of enveloped viruses, assembly of new infectious virus particles involves interactions between viral and host cell proteins [110]. Although the majority of host cell-surface proteins are excluded when enveloped viruses are released by budding through cell membranes, the incorporation of host proteins into the nascent virion is a common phenomenon. This is because the host cellular machinery is actively participating during assembly of the newly formed viral entity. For example, the avian myeloblastosis virus acquires membrane ATPase [160]; H-2 antigens are incorporated by murine leukemia virus and Friend virus particles [161, 162]; HLA-A and -B antigens are embedded in the envelope of feline leukemia virus [163]; cellular histones, Thy-1, and receptors from human interleukin 2 (IL-2) are incorporated in simian virus 40 (SV-40), murine leukemia virus (MuLV), and HTLV-1, respectively [164–166]; complement control proteins such as decay-accelerating factor (CD55) and the membrane inhibitor of reactive lysis (CD59) are also associated with HTLV-1 and HCMV [167]; human CD4 is assembled into avian leucosis virus (AVL) particles [168]; MHC class II molecules are present on the surface of Epstein-Barr virus [169] and both class I and II proteins are associated with feline leukemia virus (FeLV) [163] and Friend virus [162]; host-encoded annexin II and protein phosphatases PP1 and PP2A are associated with HCMV [170, 171].

Acquisition of host cellular constituents is also a property of HIV-1. The proteome from this retrovirus has been exhaustively analyzed and most of the main proteins incorporated from the host cells are already known. HIV-1 can incorporate a vast array of host proteins while budding out of infected cells [110]. Furthermore, viral envelope protein was shown to represent less than 0.1% of the total protein, whereas the virion-bound MHC class II DR molecule (HLA-DR), which is incorporated into the envelope of HIV-1 as it buds from the host cell plasma membrane, accounted for 20% of the total protein [172, 173]. In addition, more than 20 different host-cell derived proteins have been identified in the HIV-1 envelope. On the other hand, cytoskeletal host proteins [174] and proteins with co-stimulatory effects [175–178] or enzymatic properties [179, 180] have been also found inside the virions. An example of the application of proteomics technology for such studies is the analysis of cyclophilin A associated with HIV-1 virions by using 2-DE and MALDI-MS [181].

3.3.2 Functional role of host-derived proteins incorporated into pathogens

The biological significance of the incorporation of host-derived molecules in viruses is important in determining their effects on the pathogen cell cycle. Moreover, pathogen-associated host cell proteins are most likely functional and could play a role in pathogenesis. In fact, a contribution of virally acquired host cell proteins in virus infectivity has been suggested [110]. For example, many acquired cellular proteins retain their biological function in HIV-1 particles: CD44 on the virion has been shown to bind hyaluronic acid [182]; CD55 or CD59 can provide resistance to complement-mediated lysis [183, 184]; CD86 has been related to activation of the HIV-1 promoter region in human T cells and engagement of the TCR [177]; and antibodies to lymphocyte function-associated antigen-1 (LFA-1), intercellular adhesion molecule 1 (ICAM-1), ICAM-2, ICAM-3, and MHC classes I and II have been shown to affect the infectivity of HIV-1 *in vitro*, suggesting that these cellular molecules are involved in virus–cell interactions [185]. ICAM-1 has also been reported to be involved in HIV-1 mediated syncytium formation [186] as well as in a faster and extensive depletion of $CD4^+$ T cells, considered as the major reservoir of HIV-1 [187]. ICAM-1 also renders HIV-1 particles less susceptible to antibody-mediated neutralization [188]. On the other hand, when HLA-DR is incorporated in HIV-1 it increases the process of virus attachment to target cells and enhances HIV-1 activity [189–191]. HLA-DR also renders HIV-1 more refractory to neutralization by mAbs directed against Env proteins [188, 192]. All these results suggest that virion-bound cellular constituents might accelerate the kinetics of virus entry, most likely by interacting with their corresponding counter-receptors displayed on the target cells, and thus act as additional ligands for virus attachment [110]. As a consequence, incorporation of some specific host molecules into nascent virions modifies the intrinsic properties of HIV-1 and it might play a role in the HIV-1-mediated immune dysfunctions [188, 190–192].

The functional ability of many host cell-acquired molecules has also been demonstrated in other viruses. For example, the phosphatases PP1 and PP2A on HCMV were recently shown to cause hypophosphorylation of the cellular proteins to which virus binds [171], and the IL-2 receptor on HTLV-1 was demonstrated to retain the ability to bind its ligands [166].

On the other hand, the incorporation of host-encoded proteins into viral particles is affected by the virus producer cell system. Therefore, the progeny viruses could be differ-

ent according to the cells where they are produced. Hence, the adhesion molecule profile of the envelope of HIV-1 primary isolates is dependent on the host cell in which the virus is grown [185]. For example, the donor source has a marked influence on the insertion of HLA-DR in clinical variants of HIV-1 produced in peripheral blood mononuclear cells and monocyte-derived macrophages [193]. Moreover, the incorporation of HLA-DR by HIV-1 *in vivo* may correlate with the state of immunological activation of the cell supporting viral replication [173]. Therefore, insertion of host-derived proteins within mature HIV-1 particles is a complex process that occurs under the control of several still-undefined factors.

4 Genomics *versus* proteomics

The term "proteome" stands for *prote*in complement of the geno*me* [194]. Proteomics can be defined as the study of gene expression at a functional level. Unlike the genome, the proteome is dynamic: it varies according to the cell type and the functional state of the cell. In addition, the proteome shows characteristic perturbations in response to disease and external stimuli. Nevertheless, proteomics is to a large extent dependent on genome sequencing projects, since these provide the necessary protein sequences annotated in protein databases [23]. These databases are necessary so that they may be matched against MS data for both protein identification and sequence alignment.

Both proteomics and genomics are useful technologies for studying differential gene expression in the context of host–pathogen interactions. However, since mRNA expression levels frequently fail to correlate with the corresponding protein expression levels, proteomic and genomic studies tend to produce differing results [195]. This is particularly significant in the host protein shut-off, *i.e.*, the reduced synthesis of host cell proteins as a consequence of poxvirus infection [196]. In contrast to transcriptome analyses, which allow the quantification of gene expression in a global manner, quantitative and qualitative differences of a given protein can be monitored in different situations using proteome techniques. Proteomics therefore further extends transcriptome studies, despite significant technical challenges arising from the complexity that results from often numerous splice variants of the same gene.

Only proteomic approaches permit the study of functional issues related to the host–pathogen interaction that will clarify the underlying mechanisms of infection diseases: (i) PTMs, which provide a widespread machinery for regulating a broad range of cellular processes and reflect fundamental aspects of bacterial pathogenesis and virulence, can be assessed mainly through MS-based methods after enriching for modified proteins; (ii) protein–protein interactions, a promising approach to search for alteration in the composition of protein complexes upon infection, can be studied by characterizing these complexes through affinity purification of tagged proteins [197]; (iii) the abundance of protein isoforms; (iv) protein location, whose correlation with protein interactions of pathogen proteins in hosts systems as a function of time can ultimately lead to a better knowledge of the infection process [198]; and (v) identification of host protein incorporated in the pathogen structures, which could play a role in pathogenesis. On the other hand, the pathogen proteome is often overwhelmed by the host proteome given the size differences regarding the proteomes, the size differences regarding the host and the pathogen, and the pathogen/host ratio, thus hampering the differentiated study of both proteomes separately. Genomic approaches are capable of circumventing this inconvenience by using specific gene arrays.

Furthermore, combination of genomics, transcriptional profiling, and proteomics can be applied for the development of vaccines against pathogenic agents [199]. For example, proteomic analyses of cell surface-associated proteins from *Staphylococcus aureus* [200], *Staphylococcus epidermidis* [201], *Streptococcus pyogenes* [199, 202], *Streptococcus agalactiae* [203], *Streptococcus pneumoniae* [204], and *Clostridium difficile* [205] have been performed by using MALDI-TOF MS or MS/MS. The identification of these surface-associated proteins has provided a valuable set of proteins that may be used in the development of effective vaccines to prevent infections in humans caused by these pathogens.

5 Conclusion

Infection results from close interaction between the pathogen and host, which involves cross-talk at the protein level. Pathogen exposure induces changes in host proteins that are responsible for modifications in cellular metabolic processes and critical regulatory events. In fact, pathogens can not only manipulate the host cell proteome to obtain an optimal replication, but can also modify their own proteome to get a better adaptation to the host cell. Earlier descriptive reports on the influence of infection on cells have given way to more refined proteome studies, and are shedding light on the role of host–pathogen interplay in pathogenesis.

As genome sequencing projects and the sensitivity and resolution of proteome analytical techniques continue to increase, proteomic research is foreseen to increase our knowledge of host–pathogen interactions. Proteomics technology is the most suitable tool for investigating this interplay as it allows the study of protein–protein interactions, protein isoforms and PTMs, as well as protein location. By using proteomics it is now possible to separate very complex protein mixtures with high resolution, extract the proteins of interest, study them with MS, and identify them with high reliability. Moreover, proteomic analysis of host–pathogen interactions may provide useful information for identification of virulence factors, analysis of antimicrobial substances, or targets for vaccine design.

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