Modulation of the Phagosome Proteome by Interferon-γ

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Macrophages are immune cells that function in the clearance of infectious particles. This process involves the engulfment of microbes into phagosomes where these particles are lysed and degraded. In the current study, we used a large scale quantitative proteomics approach to analyze the changes in protein abundance induced on phagosomes by interferon-γ (IFN-γ), an inflammatory cytokine that activates macrophages. Our analysis identified 167 IFN-γ-modulated proteins on phagosomes of which more than 90% were up-regulated. The list of phagosomal proteins regulated by IFN-γ includes proteins expected to alter phagosome maturation, enhance microbe degradation, trigger the macrophage immune response, and promote antigen loading on major histocompatibility complex (MHC) class I molecules. A dynamic analysis of IFN-γ-sensitive proteins by Western blot indicated that newly formed phagosomes display a delayed proteolytic activity coupled to an increased recruitment of the MHC class I peptide-loading complex. These phagosomal conditions may favor antigen presentation by MHC class I molecules on IFN-γ-activated macrophages. Molecular & Cellular Proteomics 7:697–715, 2008.

The first line of defense against microbial infection involves the direct removal of pathogens by a variety of phagocytic cells including macrophages, polymorphonuclear neutrophils, and dendritic cells. These cells have evolved unique functions enabling them to engulf microorganisms in a specialized organelle, the phagosome, where they are killed and degraded (1). The innate ability of phagosomes to perform this task relies on the coordinated assembly of a variety of molecular machines through a complex process of organelle maturation (2). For instance, newly formed phagosomes acquire the vacuolar ATPase (V-ATPase)† complex, a proton pump that acidifies the phagosome lumen (3). This acidification process activates several lysosomal hydrolases delivered to phagosomes through fusion events with endosomes and lysosomes. Furthermore peptides derived from the degradation of microorganisms are loaded on both MHC class I and class II molecules for their presentation at the cell surface, a process that triggers an efficient adaptive immune response (4). Phagosomes are therefore pivotal platforms in linking both the innate and adaptive immune responses.

IFN-γ is a crucial factor in the clearance of infection as impaired production of IFN-γ or defects in the IFN-γ signaling pathway result in increased susceptibility to various bacterial (5) and viral infections (6). During the innate inflammatory response, IFN-γ is produced mainly by natural killer cells and subsets of T lymphocytes, including natural killer T cells and CD8+ T cells (7). Production of IFN-γ by these cells is stimulated by interleukin-12, a cytokine secreted by macrophages and dendritic cells in response to microbial stimulation of Toll-like receptors (TLRs) (8). On binding to its receptor, IFN-γ alters the expression of hundreds of genes in activated macrophages (5, 9, 10) by triggering complex signaling cascades, notably the JAK-STAT (Janus kinase-signal transducers and activators of transcription) signal transduction pathway (7). The ensuing IFN-γ-induced protein expression program enhances the microbicidal capacity of macrophages, which also respond to IFN-γ by increasing antigen presentation and by secreting inflammatory cytokines that contribute to the recruitment of immune cells to the site of infection (7). As the microbicidal capacity of macrophages involves many phagosome-associated functions, significant modifications in the protein content of phagosomes are thus expected to occur in response to IFN-γ. Several studies have examined the IFN-γ-induced expression program both at the transcriptional and the translational levels (5, 10, 11); however, the IFN-γ-induced protein expression profile has never been assessed on isolated phagosomes. In the present study, we took advantage of a unique proteomics platform developed to perform large scale comparative analyses and characterize the changes occurring to phagosomes in IFN-γ-treated cells. The results

†The abbreviations used are: V-ATPase, vacuolar ATPase; ER, endoplasmic reticulum; IFN-γ, interferon-γ; MHC, major histocompatibility complex; MDS, multidimensional scaling; TLR, Toll-like receptor; mAb, monoclonal antibody; pAb, polyclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 2-D, two-dimensional; IGTP, interferon-γ-induced GTPase; GBP, guanylate-binding protein; LRP, low density lipoprotein receptor-related protein; VAMP, vesicle-associated membrane protein.
obtained reveal a complex series of modifications in a wide range of phagosomal molecular machines coordinated to enhance both innate and adaptive immunity.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The rat anti-Lamp1 luminal ID4B mAb was from the Developmental Studies Hybridoma Bank. The rabbit anti-V-ATPase A1 subunit pAb was raised against peptide MFDSKLPKRIREDKEC. The rabbit anti-Rab7 pAb was a gift from Dr. Stéphane Méresse. The rabbit anti-calnexin pAb was a gift from Dr. John Bergeron. The rabbit anti-Erp57 pAb and the chicken anti-calreticulin pAb were from Abcam. The mouse anti-GRP78 and anti-EEA1 mAbs were from BD Biosciences. The mouse anti-transferrin receptor mAb was from Chemicon International. The rabbit anti-gp91phox pAb was from Upstate. The rabbit anti-Lmp2 (20 S proteasome subunit β1) pAb was from Affiniti.

**Cell Culture and Phagosome Formation**—RAW 264.7 murine macrophages were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 10 mM Heps, pH 7.3, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO₂. For cytotoxic-treated cells, 100 units/ml IFN-γ (PBL Biomedical Laboratories) was added in the medium 24 h prior to the isolation of phagosomes. Phagosomes were formed by the internalization of 0.8-μm blue-dyed latex beads (Estapor® Microspheres) (12). For 2-D gel or quantitative proteomics analyses, cells were allowed to internalize beads for 1 h, washed with ice-cold PBS, and incubated in new medium for 1 h. For the dynamic analysis of phagosome proteins, internalization and chase periods were as indicated in Fig. 5. Phagosomes were isolated on a sucrose gradient as described previously (13). Phagosome samples prepared using this method have been shown to be devoid of major subcellular contaminants (14).

**SDS-PAGE and Western Blotting**—Purified phagosomes or total cell lysates were resuspended in Laemmli lysis buffer. The amount of proteins and latex beads in the samples were quantified, respectively, by EZQ™ Protein Quantification kit (Molecular Probes) and spectrophotometry. Proteins were separated by SDS-PAGE prior to Western blotting, which was revealed using ECL reagent. The bands obtained from Western blotting were quantified using ImageQuant 5.2 software (GE Healthcare).

**Large Scale Quantitative Proteomics Analysis (CellCarta®)**—Phagosome samples (six control and four IFN-γ-treated samples) were resuspended in 50 mM ammonium carbonate, pH 8.1, containing 1 M urea and digested first with Lys-C (Wako Chemicals, Richmond, VA) for 4 h at 37 °C with subsequent reduction of disulfide bonds using 25 mM tris(2-carboxyethyl)phosphine at 37 °C for 1 h. The extracts were subsequently diluted to 0.1 M urea and 50 mM ammonium bicarbonate and then digested with Promega trypsin (Fisher Scientific) overnight at 37 °C with moderate shaking. Lys-C and/or trypsin were added to a mass ratio of 1:50 trypsin to total protein extract. Samples were evaporated to dryness and reconstituted at a concentration of 0.5 μg/μl in 0.2% formic acid and 5% acetonitrile. Chromatographic separations were performed using a modular CapLC liquid chromatograph with a Waters C18 Symmetry precolumn and a home-made analytical column (10 cm × 150-μm inner diameter, Jupiter 5-μm C18, Phenomenex, Torrance, CA). Peptide elution was achieved using a linear gradient of 10–60% acetonitrile (0.2% formic acid) in 60 min at a flow rate of 600 nl/min. The strong cation exchange columns were connected directly to the switching valve and were on line with the C18 precolumn during sample loading and toggled off line during reversed-phase peptide separation on the analytical column. Peptides were sequentially eluted from each strong cation exchange column onto a separate C18 precolumn at a flow rate of 20 μl/min for 3 min using 20-μl salt plugs of 0, 55, 65, 80, 100, 150, 400, and 1 M ammonium acetate, pH 3.5. Simultaneous peptide elution from both C18 precolumns and analytical columns was achieved using a linear gradient of 10–60% acetonitrile (0.2% formic acid) in 60 min at a flow rate of 600 nl/min.

Isotopic peak detection was automatically applied to every LC-MS and LC-MS/MS analysis and represented as isotope maps. Peak lists were generated with MassLynx version 4.0 using default parameters. The isotope maps were converted into peptide maps by Savitzky-Golay smoothing in both the mz and retention time dimensions followed by peak fitting to a four-dimensional (m/z, charge, retention time, and intensity) peptide isotope map. The identification of peptide features is based on two-dimensional correlation of time and isotopic peaks for peaks above a user-defined intensity threshold, typically set to 25 counts, to minimize the detection of MS background noise and interfering peaks. Co-eluting ions were matched to a model based on the average distribution of peptide isotopic ions similar to that described by Senko et al. (15) and provided a false positive detection rate lower than 25% at this level. Peptides ions showing up or down abundance changes were manually validated to ensure data consistency. The peptide maps were normalized for retention time using a dynamic and nonlinear correction algorithm applied across all comparable LC-MS and LC-MS/MS injections of the study. This software tool allows tracking between two or more LC-MS or LC-MS/MS injections independent of the LC column, mass spectrometer, or time of the analysis and is able to reduce the retention time variability to less than 7 s. Following normalization, peptides were matched across all samples in the study and clustered according to fraction, mass, retention time, and charge using standard hierarchical clustering techniques adapted to the proteomics context. A representative median mass and median retention time were calculated to represent the peptide clusters. Peptide clustering of the same peptide observed in different samples across the study enabled the detection of peptides that were modulated by IFN-γ. IFN-γ-modulated peptides were selected on the basis of their statistical significance (p ≤ 0.05 using a paired t test) and reproducibility of the control to IFN-γ-treated peptide intensity ratio (at least 1.6-fold in four of six samples). The false discovery rate for peptides selected with these parameters was determined by performing 1000 permutations of the expression data and hence estimated below 5%, a commonly accepted threshold. Multidimensional scaling (MDS) was used to visualize differences and detect bias using in-house software tools developed with Matlab (Mathworks). Peptides demonstrating a statistically significant change in intensity were targeted for sequencing by LC-MS/MS. For this purpose, the target peptides were compiled into an inclusion list containing retention time, charge state, and m/z for each target peptide.

Database searching of LC-MS/MS spectra for peptide identification was accomplished using Mascot version 1.8.01 (Matrix Science) and the non-redundant National Center for Biotechnology Information (nrNCBI) rodent protein database (April 1, 2004). A total of 288,692 protein entries were searched in that database. Mascot parameters specified trypsin proteolysis with one allowed missed cleavage and with variable modification of methionine (oxidation), asparagine/glu-tamine (deamidation), and serine/threonine/tyrosine (phosphorylation). Mass tolerances were 0.25 Da for both precursor and fragment ions. The identified proteins were clustered by sequence homology using BlastClust at 95% homology over 50% of the sequence length. Homology clustering groups proteins that are likely redundant but are not differentiated by the identified peptides. Proteins identified by a single peptide with Mascot scores lower than 25 were eliminated. The MS/MS spectra for single peptide-based protein identifications are available upon request.

The minimum threshold for MS/MS has been identified previously through manual validations of several hundred MS/MS peptide spec-
tra and determination of the likelihood of proper MS/MS search engine peptide assignment correlated with search engine-reported peptide score values. The protein assignment of the IFN-γ-modulated peptides was verified by BLAST (Basic Local Alignment Search Tool) using UniProt. The peptide list was annotated to include UniProtKB accession numbers and remarks on protein function.

The relative protein abundance values were calculated using an experimentally determined differential intensity to differential abundance conversion formula ($dA = 2dI – 1$). The CellCarta platform measures peak height rather than peak volume. Peak volume is proportional to peptide and protein abundance. Through controlled spiking experiments we have established that the relationship between differential peak height ($dI$) and differential peak volume ($dA$) is $dA = 2dI – 1$. Peak height was used for the raw measurements, because it is less affected by co-eluting peptides and therefore a more accurate estimate. For example, for a peptide with a differential peak intensity of 3 between two samples, the estimated differential peak volume is 5. This is then the estimate of differential protein abundance between the samples.

High Resolution 2-D Gel Electrophoresis—Isolated phagosomal proteins were separated by 2-D gel electrophoresis and identified as described previously (16, 17). Briefly isolated phagosomes were re-suspended in lysis/rehydration buffer (8 M urea, 2 % thiourea, 4 % (w/v) CHAPS, 40 mM dithioerythritol, 20 mM Tris, 2 % IPG buffer, and bromphenol blue) and vortexed for 1 h. Sample loading in the first dimension was performed overnight by in-gel reswelling of linear immobilized 3–10 pH gradient 18-cm strips (Amersham Biosciences). Following the first dimension isoelectric focusing separation, the strips were equilibrated in a 13 mM DTT solution for 10 min and in a 2.5 % iodoacetamide solution for 5 min. The second dimension was performed using standard SDS-PAGE. The resulting gels were silver-stained, and the protein patterns were analyzed using the ImageMaster software (Amersham Biosciences). Densitometry measurements were obtained from silver-stained gels, to determine relative abundance changes in protein extracts. Protein spots were excised, destained, and trypsin-digested with the resulting tryptic peptides extracted with 0.2 % urea in 50 % aqueous acetonitrile. Each digested spot was analyzed by nano-LC-MS/MS using a Waters CapLC coupled to a Q-TOF Ultima (Waters).

RESULTS AND DISCUSSION

Large Scale Quantitative Proteomics Analysis—To characterize the phagosomal proteins that are modulated by IFN-γ, we used a quantitative proteomics platform (referred to as CellCarta) based on LC-MS intensity measurements of peptides matched across all samples according to $m/z$, charge, and retention time (18). CellCarta demonstrates high specificity, given its ability to easily differentiate between artificially introduced, low concentration, spiked proteins and the background protein content of a complex plasma sample; the platform is also highly sensitive as it can detect small differences (2–4-fold) in spiked protein concentrations in the plasma sample (supplemental Fig. 1). Large scale analysis of the proteome of phagosomes from control or IFN-γ-treated macrophages using the platform identified 8179 peptide precursors found in at least four of six control and IFN-γ-treated phagosome samples. Of these, 1253 peptide precursors (15% of all peptides) demonstrated statistically significant differences in their peptide intensities and were thus considered as modulated by IFN-γ.

MDS plots allow the observation of higher level structure within complex proteomics data sets by simplifying the order within those data sets and representing trends and relationships between variables (samples) in dimensionless space. The differences in peptide content between the control and IFN-γ-treated samples were reduced to one data point per sample based on the LC-MS intensity data for all peptides in the study. As shown in Fig. 1A, the separation between control and IFN-γ-treated samples is clearly evident. This suggests that the peptides detected, and the intensities measured for them, are distinct and unique to a high degree between the two groups of samples. When only the 1253 IFN-γ-modulated peptides between the two study groups were plotted (Fig. 1A, right panel) there is even greater evidence of group separation, underlining the unique and distinct nature of the IFN-γ-modulated peptides. Hence global proteomics analysis using CellCarta clearly distinguishes between control and IFN-γ-treated samples.

Further high level representation of the proteomics data using a heat map format for the intensity profile of each peptide allows illustration of similarities and differences between the control and IFN-γ-treated groups (Fig. 1B). The extremes of the plot highlight the peptides displaying the greatest difference in intensity between the control and IFN-γ-treated samples with the most up-regulated and down-regulated peptides found, respectively, on the left and right extremes. Fig. 1B clearly indicates that a majority of the IFN-γ-modulated peptides were up-regulated by IFN-γ. Fig. 1C illustrates the distribution of the values for the -fold intensity difference of the IFN-γ-modulated peptides as a function of their statistical significance. Of the 1253 peptides found to be modulated by IFN-γ in this study, 82% were up-regulated after IFN-γ treatment, whereas only 18% were down-regulated (Fig. 1C). A majority of the up-regulated peptides were increased by 4-fold or less with a $p$ value between 0.005 and 0.05, whereas most of the down-regulated peptides were decreased by 2.5-fold or less with a $p$ value between 0.01 and 0.05. Our study thus indicates that macrophage activation by IFN-γ takes place mostly through the up-regulation of proteins. However, the significant number of down-regulated peptides indicates a complex pattern of protein modulation by IFN-γ on the phagosome.

The sequences of the 1253 IFN-γ-modulated peptides were submitted to MS/MS, and the sequenced peptides were assigned to the proteins listed in Tables I and II. This process reduced the total number of IFN-γ-modulated peptides to 298 peptides due to the elimination of peptides with low Mascot scores and to redundant assignments with variable modifications such as deamidation and oxidation. These IFN-γ-modulated peptides could be attributed to 147 proteins, 135 (92%) that were up-regulated and 12 (8%) that were down-regulated by IFN-γ (Tables I and II). 63 proteins had more than one peptide identified, and of these, 10 proteins (16%) had peptides with -fold intensity values that were up-regulated along
**Interferon-γ-modulated Phagosome Proteome**

![Diagram of high level representation of the quantitative proteomics data from control and IFN-γ-treated phagosomes.](image)

**Fig. 1.** **High level representation of the quantitative proteomics data from control and IFN-γ-treated phagosomes.** A. MDS three-dimensional plot of intensity measurements from 8179 peptide precursors found in at least four of six control (−) or IFN-γ-treated (+) samples (left panel) or from the subset of 1253 peptide precursors identified as modulated by IFN-γ (right panel). The three dimensions (MDS1, MDS2, and MDS3) plotted represent the three most significant data trends within the study. B, heat map representation of LC-MS intensity measurements across the six control and six IFN-γ-treated study samples (y axis) from the subset of 1253 peptide precursors (x axis) identified as modulated by IFN-γ (bottom panel). Increasing LC-MS detection intensity is represented in a gradual color scale from lowest intensity (red) to medium intensity (yellow) to highest intensity (green). C, volcano plot of the log2 of the mean -fold intensity difference (x axis) between control and IFN-γ-treated sample pairs as a function of the −log of the p value (y axis) from a paired t test for the 1253 peptide precursors identified as modulated by IFN-γ (p ≤ 0.05). A majority (82%) of IFN-γ-modulated peptides were up-regulated by the IFN-γ treatment.

with a subset of down-regulated peptides. There are several reasons why peptides assigned to the same protein may differ in the direction of regulation including post-translational modifications, splice variants, or errors in peptide-to-protein assignment. The mean peptide -fold intensity value is shown for each protein in Table I, and the identified peptide sequences are shown in Table II. To illustrate the data obtained using CellCarta, two examples of up-regulated proteins in the IFN-γ-treated samples were chosen, GRP78/BiP and the V-ATPase subunit A1 isoform (Fig. 2). As shown in Fig. 2A, the seven peptides assigned to GRP78/BiP demonstrated a consistent pattern of modulation by IFN-γ. Similarly four up-regulated peptides were detected for the V-ATPase subunit A1 isoform A, and these showed a coherent pattern of up-regulation (Fig. 2C). Western blot analysis confirmed the up-regulation of both GRP78/BiP and V-ATPase subunit on phagosomes from IFN-γ-treated macrophages (Fig. 2, B and D).

**High Resolution 2-D Gel Electrophoresis**—To compare and further validate the IFN-γ-modulated proteins identified with CellCarta, we used high resolution 2-D gel electrophoresis. As shown in Fig. 3, phagosomes purified from control or IFN-γ-treated macrophages displayed complex protein patterns. Differential protein abundance measurements were obtained by comparing the peak volumes of aligned spots from 2-D gel analyses. A total of 395 spots were identified, and a subset of 121 spots showed at least a 2-fold change in protein abundance upon IFN-γ treatment. Of these, 47 spots corresponding to 40 different proteins were identified by MS/MS analysis (Table III). Notably half of the proteins identified by 2-D gel analysis had also been identified using the proteomics platform. The differences in the proteins identified by 2-D gel analysis and by quantitative proteomics analysis can likely be attributed to the distinct advantages and limitations of both proteomics approaches. For instance, the highly resolutive separation power of the 2-D gel approach highlighted an increase in discrete isoforms of cathepsin B on IFN-γ-treated phagosomes, most likely corresponding to differentially glycosylated forms of the enzyme (Fig. 3, inset). The 2-D gels, however, failed to identify membrane proteins, a known limitation of this approach. In contrast, more than 30% of the IFN-γ-modulated proteins identified using the platform were membrane proteins. The proportion of membrane proteins in phagosomes is expected to approximate 30%, indicating that the large scale approach was highly efficient in identifying membrane proteins. Together these results strengthen the validity of the IFN-γ-modulated proteins identified using CellCarta.

**IFN-γ-modulated Functions on the Phagosome**—The IFN-γ-modulated proteins identified in our proteomics analysis (Tables I, II, and III) were analyzed for their reported participation in functional biomodules as illustrated in Fig. 4. The protein networks that emerge from four of the IFN-γ-modulated proteins (LIMP2, VAMP8, NADPH oxidase gp91phox, and ERP57) were shared by other proteins identified in our proteomics analysis. This finding underscores the relevance of the proteins that we identified as regulated by IFN-γ and suggests that other members of these interactomes may be modulated by IFN-γ.
### Interferon-γ-modulated Phagosome Proteome

The number of different peptides identified, the mean peptide-fold intensity values (± mean deviation for proteins with multiple peptides) and the differential protein abundance values are indicated for each protein. Positive-fold intensity values indicate proteins that are up-regulated by IFN-γ (ratio of IFN-γ/control), and negative values indicate proteins that are down-regulated by IFN-γ (ratio of control/IFN-γ). The relative protein abundance values were calculated using an experimentally determined differential intensity to differential abundance conversion formula (ΔA = 2ΔI − 1). SNAP, N-ethylmaleimide-sensitive factor attachment protein; NEM, N-ethylmaleimide; LPS, lipopolysaccharide; ABC, ATP-binding cassette; TGN, trans-Golgi network.

#### TABLE I

| Protein name                  | UniProtKB accession no. | Remarks                                                                 | Peptide no. | Peptide -fold intensity | Relative protein abundance |
|-------------------------------|-------------------------|------------------------------------------------------------------------|-------------|-------------------------|----------------------------|
| α2-Macroglobulin              | Q6GQT1                  | Plasma proteinase inhibitor. Binds to LRP.                             | 1           | 3.4                     | 5.8                        |
| Acid ceramidase               | Q78P93                  | Lysosomal.                                                             | 3           | 3.5 ± 1.3               | 6.0                        |
| Acid sphingomyelinase-like phosphodiesterase | P58242 | Lysosomal. Belongs to the acid sphingomyelinase family.               | 1           | 1.7                     | 2.4                        |
| Arf6                          | P62331                  | ADP-ribosylation factor 6. Regulates endosomal membrane traffic.      | 1           | 1.7                     | 3.6                        |
| Arf10b                        | Q8VEH3                  | ADP-ribosylation factor-like 10B. Regulates lysosome motility.         | 5           | 2.3 ± 0.6               | 3.6                        |
| Aminopeptidase N              | P97449                  | Also known as CD13. Plasma membrane-bound metalloproteinase. Internalized into phagosomes. | 2           | 5.1 ± 1.2               | 9.2                        |
| Annexin A1                    | P10107                  | Ca²⁺-regulated membrane-binding protein.                              | 1           | 2.9                     | 4.8                        |
| Annexin A2                    | P07356                  | Ca²⁺-regulated membrane-binding protein.                              | 4           | 4.2 ± 1.2               | 7.4                        |
| Annexin A4                    | P97429                  | Ca²⁺-regulated phospholipid-binding protein.                          | 3           | 4.7 ± 0.9               | 8.4                        |
| Annexin A5                    | P48036                  | Ca²⁺-regulated phospholipid-binding protein.                          | 6           | 2.8 ± 1.0               | 4.6                        |
| Apolipoprotein D              | P51910                  | Plasma glycoprotein.                                                  | 1           | 5.1                     | 9.2                        |
| β₂-Microglobulin              | P01887                  | Light chain of MHC class I molecules. Functions in antigen presentation. | 1           | 4.8                     | 8.6                        |
| BasiGin                       | P18572                  | Membrane glycoprotein also known as CD147.                            | 1           | 2.5                     | 4.0                        |
| Calnexin                      | P35564                  | ER chaperone. Mediates the assembly of MHC class I molecules.         | 2           | 3.4 ± 1.2               | 5.8                        |
| Calreticulin                  | P14211                  | ER chaperone. Mediates the assembly of MHC class I molecules.         | 1           | 4.5                     | 8.0                        |
| Cathepsin A                   | P16675                  | Also known as Lysosomal protective protein and carboxypeptidase C.     | 2           | 2.6 ± 2.5               | 4.2                        |
| Cathepsin B                   | P10605                  | Lysosomal cysteine protease.                                          | 2           | 3.8 ± 1.6               | 6.6                        |
| Cathepsin C                   | P97821                  | Also known as dipeptidyl-peptidase I.                                | 1           | 1.8                     | 2.6                        |
| Cathepsin Z                   | Q9WWU7                  | Lysosomal cysteine protease.                                          | 1           | 3.2                     | 5.4                        |
| C-C chemokine receptor type 7 | P47774                  | Promotes cell migration.                                              | 1           | 3.8                     | 6.6                        |
| CD36                          | Q08857                  | Scavenger receptor. Functions as a phagocytic receptor for bacteria.  | 3           | 9.7 ± 10                | 18.4                       |
| CD45                          | P06800                  | May down-regulate interferon receptor activation.                     | 1           | 2.1                     | 3.2                        |
| CD98 heavy chain              | P10852                  | Also known as 4F2 cell surface heavy chain.                           | 1           | 3.9                     | 6.8                        |
| Coflin                        | P18760                  | Cytoplasmic. Regulates actin filament dynamics.                       | 1           | 2.0                     | 3.0                        |
| Cyclooxygenase-2              | Q5769                   | Functions as the rate-limiting enzyme in the synthesis of prostaglandins. | 5           | 10.2 ± 9.5              | 19.4                       |
| Cyclophilin C-associated protein | O35649               | Binds to Galectin-3. Down-regulates proinflammatory responses.       | 2           | 1.8 ± 1.4               | 2.6                        |
| Dendritic cell-associated transmembrane protein | Q99P91 | Type I transmembrane protein.                                        | 1           | 2.4                     | 3.8                        |
| Elongation factor 1-α         | P62631                  | Cytoplasmic. Functions in translation.                                | 1           | 1.9                     | 2.8                        |
| ERP57                         | P27773                  | ER chaperone. Functions in the assembly of MHC class I molecules.     | 7           | 3.5 ± 1.9               | 6.0                        |
| Ezrin/Moesin/Radixin protein  | P26041                  | Identified peptide shared by ERM family of proteins.                  | 1           | 2.0                     | 3.0                        |
| Ferritin heavy chain          | P09528                  | Functions as an intracellular iron storage protein.                   | 1           | −4.4                    | −7.8                       |
| Ferritin light chain          | P29391                  |                                                                           | 1           | −3.7                    | −6.4                       |
| Flotillin-1                   | O08917                  | Lipid raft-associated membrane protein.                               | 4           | 2.7 ± 0.2               | 4.4                        |
| Flotillin-2                   | Q60634                  |                                                                           | 3           | 4.8 ± 0.7               | 8.6                        |
| G protein G, α subunit        | Q9DC51                  | GTPase subunit of heterotrimeric G proteins.                          | 2           | 2.7 ± 0.3               | 4.4                        |
| G protein β1 subunit          | P62874                  | Subunit of heterotrimeric G proteins.                                 | 2           | 3.9 ± 0.7               | 6.8                        |
| G protein β2 subunit          | P62880                  | Subunit of heterotrimeric G proteins.                                 | 2           | 5.1 ± 0.5               | 9.2                        |
### TABLE I—continued

| Protein name                                          | UniProtKB accession no. | Remarks                                                                      | Peptide no. | Peptide fold-intensity | Relative protein abundance |
|-------------------------------------------------------|-------------------------|------------------------------------------------------------------------------|-------------|------------------------|---------------------------|
| Galectin-3                                            | P16110                  | Also known as Mac-2.                                                          | 2           | 2.2 ± 0.1              | 3.4                       |
| GAPDH                                                 | P16858                  | Cytoplasmic.                                                                 | 1           | 3.6                    | 6.2                       |
| GBP-5                                                 | Q8C8F8                  | Also known as interferon-induced guanylate-binding protein 5.                 | 4           | 9.1 ± 3.1              | 17.2                      |
| Glucosylceramidase                                    | P17439                  | Lysosomal.                                                                   | 6           | 3.1 ± 1.3              | 5.2                       |
| GRP78/Bip                                             | P20029                  | Glucose-regulated protein 78. Also known as Bip.                             | 7           | 4.0 ± 2.8              | 7.0                       |
| GRP94                                                 | P08113                  | Also known as gp96. ER chaperone. Involved in antigen cross-presentation.    | 2           | 1.9 ± 0.1              | 2.8                       |
| GTP-binding protein 1                                 | Q01514                  | Also known as interferon-induced guanylate-binding protein 1.                | 1           | 3.4                    | 5.8                       |
| Hexosaminidase B                                      | P20060                  | Lysosomal.                                                                   | 3           | 2.5 ± 1.2              | 4.0                       |
| HSC-71                                                | P63017                  | Heat shock cognate protein 71.                                              | 2           | 1.8 ± 1.2              | 2.6                       |
| HSP-90β                                               | P11499                  | Also known as HSP-84.                                                        | 2           | 2.5 ± 0.1              | 4.0                       |
| ICAM-1                                                | P13597                  | Cell surface membrane protein internalized during phagocytosis.              | 1           | 5.9                    | 11                        |
| IGTP                                                  | Q811M6                  | Interferon-γ-induced GTPase. Belongs to the p47 GTPase family.              | 1           | 4.5                    | 8.0                       |
| IIGP-1                                                | Q9QZ85                  | Interferon-γ-induced GTPase. Belongs to the p47 GTPase family.              | 2           | 16.8 ± 6.8             | 33                        |
| IIGP-2                                                | Q99K68                  | Interferon-γ-induced GTPase. Belongs to the p47 GTPase family.              | 2           | 6.6 ± 1.1              | 12                        |
| Interferon-induced transmembrane protein chain        | Q99J93                  | Belongs to the IFN-induced transmembrane protein family.                    | 2           | 2.8 ± 0.1              | 4.6                       |
| Lactate dehydrogenase A chain                         | P06151                  | Cytoplasmic.                                                                 | 1           | 2.0                    | 3.0                       |
| LAMP-1                                                | P11438                  | Lysosomal-associated membrane glycoprotein 1.                                | 2           | 2.2 ± 0.2              | 3.4                       |
| LAMP-2                                                | P17046                  | Lysosomal-associated membrane glycoprotein 2.                                | 1           | 1.8                    | 2.6                       |
| Leucyl-cystinyl aminopeptidase                         | Q8C129                  | Also known as placental leucine aminopeptidase (P-LAP).                     | 6           | 3.1 ± 1.3              | 5.2                       |
| LIMP-2                                                | Q35114                  | Lysosomal membrane protein 2.                                               | 4           | 3.2 ± 1.5              | 5.4                       |
| Lipoprotein lipase                                    | P11152                  | Functions in lipid uptake and metabolism.                                   | 3           | 5.3 ± 1.8              | 9.6                       |
| LRG-47                                                | Q60766                  | Interferon-γ-induced GTPase. Belongs to the induced p47 GTPase family.       | 9           | 6.3 ± 2.4              | 11.6                      |
| LRP                                                   | Q920Y4                  | Low density lipoprotein receptor-related protein. Also known as CD91.        | 2           | −2.9 ± 0.1             | −4.8                      |
| Lysosomal acid lipase                                  | Q9Z0M5                  | Functions in the hydrolysis of lipids. MD-1/Rp105 complex functions in LPS-mediated responses. | 2           | 1.8 ± 0.3              | 2.6                       |
| MD-1                                                  | O88188                  | Functions in antigen presentation.                                           | 1           | 3.0                    | 5.0                       |
| MHC class I heavy chain                               | Q61643                  | Functions in antigen presentation.                                           | 2           | 5.9 ± 3.0              | 11                        |
| MLN64 N-terminal domain homolog                       | Q9DC3                   | Also known as MENTHO. Localizes to late endosomes.                           | 1           | 4.1                    | 7.2                       |
| MP1                                                   | Q88653                  | Functions as a scaffold protein that binds MEK1 and facilitates ERK1 activation. | 1           | 2.0                    | 3.0                       |
| MP1-interacting protein                               | Q9JHS3                  | Also known as p14. Functions as a late endosomal adaptor protein for MP1.    | 1           | 4.0                    | 7.0                       |
| N-Acylethanolamine-hydrolyzing acid amidase            | Q9D7V9                  | Lysosomal. Belongs to the acid ceramidase family.                            | 4           | 11.7 ± 4.1             | 22                        |
| NADPH oxidase p22phox                                   | Q61462                  | NADPH oxidase generates superoxide, a bactericidal reactive oxygen species.   | 1           | 3.4                    | 5.8                       |
| NADPH oxidase gp91phox                                 | Q61093                  |                                                                              | 1           | 1.8                    | 2.6                       |
| Na+/K+-ATPase α 3 subunit                              | Q66C6                   | Sodium pump, catalytic subunit.                                              | 2           | 1.9 ± 0.1              | 2.8                       |
| Napsin A                                              | O09043                  | Aspartic protease.                                                           | 1           | 5.1                    | 9.2                       |
| NEM-sensitive fusion protein                          | P46460                  | Vesicle-fusing ATPase                                                       | 2           | 1.2 ± 0.7              | 1.4                       |
| Protein name                                      | UniProtKB accession no. | Remarks                                                                                           | Peptide no. | Peptide -fold intensity | Relative protein abundance |
|--------------------------------------------------|--------------------------|---------------------------------------------------------------------------------------------------|-------------|-------------------------|---------------------------|
| Neutral amino acid transporter ASCT2             | Q9ESU7                   | Transports neutral amino acids across cell membrane.                                               | 1           | 2.0                     | 3.0                       |
| Nicastrin                                        | P57716                   | Functions as a substrate-binding component in the γ-secretase complex.                            | 2           | 2.7 ± 0.9               | 4.4                       |
| Niemann-Pick C1 protein                          | O35604                   | Functions in intracellular sterol trafficking and regulation of cholesterol homeostasis.         | 1           | 4.5                     | 8.0                       |
| Palmitoyl-protein thioesterase                   | O88531                   | Removes fatty acyl groups during lysosomal degradation.                                            | 6           | 4.9 ± 1.3               | 8.8                       |
| Peroxiredoxin 1                                  | P35700                   | Cytoplasmic. Detoxifies reactive oxygen and nitrogen species.                                     | 7           | 2.9 ± 0.6               | 4.8                       |
| Peroxiredoxin 4                                  | O08807                   | Cytoplasmic. Detoxifies reactive oxygen and nitrogen species.                                     | 1           | 2.3                     | 3.6                       |
| Peroxosomal membrane protein 69                  | O89016                   | Belongs to the ABC transporter family.                                                             | 1           | 3.4                     | 5.8                       |
| Protein-disulfide isomerase A6                   | P09103                   | ER chaperone. Functions in the assembly of MHC class I molecules.                                | 2           | 4.7 ± 0.4               | 8.4                       |
| Pyruvate kinase, isozyme M2                      | P52480                   | Cytoplasmic.                                                                                        | 4           | 2.4 ± 1.0               | 3.8                       |
| Rab1                                             | Q5SW88                   | Localizes to the ER and Golgi.                                                                    | 2           | 1.4 ± 0.8               | 1.8                       |
| Rab2                                             | Q6PDZ3                   | Localizes in an ER/Golgi intermediate compartment.                                                | 1           | 2.1                     | 3.2                       |
| Rab5c                                            | P35278                   | Localizes to early endosomes.                                                                     | 1           | 1.7                     | 2.4                       |
| Rab7                                             | P51150                   | Regulates late endosomal and phagosomal traffic.                                                   | 7           | 2.8 ± 0.8               | 4.6                       |
| Rab9a                                            | Q9ROM6                   | Localizes to late endosomes and lysosomes.                                                        | 1           | 3.7                     | 6.4                       |
| Rab10                                            | P61027                   | Localizes to the trans-Golgi and TGN.                                                             | 1           | 2.4                     | 3.8                       |
| Rab14                                            | Q91V41                   | May function in membrane trafficking between the Golgi apparatus and endosomes.                  | 1           | 2.6                     | 4.2                       |
| Rab32                                            | Q9CZE3                   | Localizes to mitochondria.                                                                       | 1           | 3.1                     | 5.2                       |
| Rac1                                             | P63001                   | Associates with the plasma membrane.                                                              | 1           | −2.3                    | −3.6                      |
| Rac2                                             | Q05144                   | Associates with the plasma membrane.                                                              | 1           | 4.1                     | 7.2                       |
| Ral                                              | Q9JIW9                   | Belongs to the Ras-related small GTPase superfamily.                                              | 1           | 4.5                     | 8.0                       |
| Receptor-interacting protein Ser/Thr kinase 3    | Q9QZL0                   | Also known as RIP3. Functions as a proapoptotic protein.                                          | 1           | −2.0                    | −3.0                      |
| Rho-associated protein kinase 2                  | P70336                   | Also known as Rock-2. Functions in actin dynamics.                                                | 1           | 2.2                     | 3.4                       |
| Rp105                                            | Q62192                   | Rp105/MDF-1 complex functions in LPS-mediated responses.                                           | 1           | 4.1                     | 7.2                       |
| Sacsin                                           | Q9JLC8                   | May function in chaperone-mediated protein folding.                                               | 1           | 1.8                     | 2.6                       |
| Septin-7                                         | O55131                   | Distributed along stress fibers.                                                                 | 1           | 6.0                     | 11                        |
| Signal peptide peptidase-like protein α-SNAP     | Q9DB05                   | Functions in membrane fusion events.                                                              | 1           | 2.2                     | 3.4                       |
| α-SNAP                                           | Q9DB05                   | Functions in membrane fusion events.                                                              | 1           | 2.2                     | 3.4                       |
| SNAP-29                                          | Q9CWZ7                   | Functions in membrane fusion events.                                                              | 1           | −2.7                    | −4.4                      |
| SNAP-29                                          | Q9CWZ7                   | Functions in membrane fusion events.                                                              | 1           | 2.6                     | 4.2                       |
| Solute carrier family 2                          | Q5SXE0                   | Facilitated glucose transporter.                                                                 | 4           | 2.6 ± 1.6               | 4.2                       |
| Solute carrier family 6                          | Q5SXE0                   | Facilitated glucose transporter.                                                                 | 4           | 2.6 ± 1.6               | 4.2                       |
| Stomatin                                         | O88988                   | Lipid raft-associated membrane protein. Localizes to late endosomes and lysosomes.                | 2           | 14 ± 10                 | 27                        |
| Stomatins                                         | Q9MR3                    | Potassium/chloride transporter.                                                                   | 2           | 2.8 ± 0.1               | 4.6                       |
| Solute carrier family 12                         | Q9MR3                    | Potassium/chloride transporter.                                                                   | 2           | 2.8 ± 0.1               | 4.6                       |
| Solute carrier family 37                         | Q6P4P0                   | Glycerol 3-phosphate transporter.                                                                  | 2           | 2.1 ± 0.2               | 3.2                       |
| Solute carrier family 38                         | Q6P4P0                   | Glycerol 3-phosphate transporter.                                                                  | 2           | 2.1 ± 0.2               | 3.2                       |
| Solute carrier family 38                         | Q8CFE6                   | Amino acid transporter.                                                                           | 1           | −3.3                    | −5.6                      |
| Syntaxin                                         | O88988                   | Lipid raft-associated membrane protein. Localizes to late endosomes and lysosomes.                | 2           | 14 ± 10                 | 27                        |
| Strontal cell-derived factor receptor 2          | Q8K385                   |                                                                                                  | 1           | −2.8                    | −4.6                      |
| Cu,Zn-superoxide dismutase                       | P08228                   | Detoxifies radicals within cells.                                                                 | 1           | 3.0                     | 5.0                       |
| Syntaxin                                         | P70452                   | Functions in membrane fusion events. Involved in vesicle exocytosis.                             | 1           | 2.8                     | 4.6                       |

**TABLE I—continued**

Interferon-γ-modulated Phagosome Proteome

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| Protein name | UniProtKB accession no. | Remarks                                                                 | Peptide no. | Peptide -fold intensity | Relative protein abundance |
|--------------|-------------------------|------------------------------------------------------------------------|-------------|-------------------------|----------------------------|
| Syntaxin 7   | O70439                  | Functions in membrane fusion events in the endosomal pathway. Associates with maturing phagosomes. | 1           | 1.7                     | 2.4                        |
| Syntaxin 8   | O88983                  | Functions in membrane fusion events in the endosomal pathway.          | 1           | 2.5                     | 4.0                        |
| Syntaxin 12/13 | Q9ER00                | Functions in membrane fusion events in the endosomal pathway.          | 3           | 2.7 ± 0.4               | 4.4                        |
| Syntenin 1   | O08992                  | Localizes to endocytic compartments. Binds to syndecans, several receptors, and Rab5. | 2           | 2.4 ± 0.8               | 3.8                        |
| Thioredoxin  | P10639                  | Participates in various redox reactions and catalyzes dithiol-disulfide exchange reactions. | 1           | 1.8                     | 2.6                        |
| Toll-like receptor 3 | Q99MB1          | Functions in innate immunity. Binds to viral doubled-stranded RNA.     | 2           | 4.6 ± 0.6               | 8.2                        |
| Toll-like receptor 7 | P58681          | Functions in innate immunity. Binds to viral single-stranded RNA.     | 1           | 17                      | 33                         |
| Toll-like receptor 9 | Q9EQU3          | Functions in innate immunity. Binds to bacterial unmethylated CpG-containing DNA. | 1           | 7.9                     | 15                         |
| Transferrin receptor protein 1 | Q62351       | Functions in the cellular uptake of iron. Major constituent of microtubules. | 5           | −1.5 ± 1.0              | −2.0                       |
| Tubulin β-chain | P99024            | Functions in the cellular uptake of iron. Major constituent of microtubules. | 1           | 2.7                     | 4.4                        |
| Tweety homolog 3 | Q6P5F7           | Major constituent of microtubules.                                    | 1           | 2.9                     | 4.8                        |
| Uncharacterized protein C2orf18 | Q8VE96    | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 4.5                     | 8.0                        |
| Uncharacterized protein C12orf23 | Q9DAM7    | Functions in membrane fusion events involving late endosomes.           | 1           | 5.8                     | 11                         |
| VAMP7        | Q7Z409                  | Also known tetanus-insensitive VAMP. Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.9                     | 4.8                        |
| VAMP8        | O70404                  | Also known as Endobrevin. Functions in membrane fusion events involving early and late endosomes. | 2           | 3.0 ± 0.1               | 5.0                        |
| V-ATPase A subunit A (catalytic) | P50516     | Proton pump. Functions in ATP-dependent phagosome acidification.        | 4           | 4.4 ± 2.3               | 7.8                        |
| V-ATPase B subunit isoform 2 | P62814     | Functions in membrane fusion events involving late endosomes and lysosomes. | 5           | 1.4 ± 1.4               | 1.8                        |
| V-ATPase E subunit | P50518   | Functions in membrane fusion events involving late endosomes and lysosomes. | 4           | 3.5 ± 2.1               | 6.0                        |
| V-ATPase V₅ subunit a₃ isofrom | Q9JL12   | Functions in membrane fusion events involving late endosomes and lysosomes. | 5           | 2.5 ± 0.6               | 4.0                        |
| V-ATPase V₅ subunit d₁ isofrom | P51863   | Functions in membrane fusion events involving late endosomes and lysosomes. | 2           | 2.4 ± 0.1               | 3.8                        |
| V-ATPase V₅ subunit d₂ isofrom | Q80SY3   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | −1.7                    | −2.4                       |
| Voltage-dependent anion channel 1 | Q60932   | Functions in membrane fusion events involving late endosomes and lysosomes. | 2           | 2.5 ± 0.3               | 4.0                        |
| Voltage-dependent anion channel 2 | Q60930   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.4                     | 3.8                        |
| Voltage-dependent anion channel 3 | Q60931   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.2                     | 3.4                        |
| Voltage-dependent P/Q-type calcium channel, α₁A subunit | O00555  | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.4                     | 3.8                        |
| 40 S ribosomal protein S25 | P62852   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | −2.1                    | −3.2                       |
| 60 S acidic ribosomal protein P0 | P14869   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.1                     | 3.2                        |
| 60 S ribosomal protein L3 | P27659   | Functions in membrane fusion events involving late endosomes and lysosomes. | 2           | 2.4 ± 0.2               | 3.8                        |
| 60 S ribosomal protein L5 | P47962   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 6.9                     | 12.8                       |
| 60 S ribosomal protein L12 | P35979   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 1.6                     | 2.2                        |
| 60 S ribosomal protein L14 | Q9CR57   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.1                     | 3.2                        |
| 60 S ribosomal protein L31 | P62900   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 1.7                     | 2.4                        |
| A630077B13Rik protein | Q8C9E8   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 5.6                     | 10                         |
| 3110005G23Rik protein | Q8V8U8   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 3                       | 5.0                        |
| 4930506M07Rik protein | Q8K2Q9   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 1.9                     | 2.8                        |
| 9030624J02Rik protein | Q80XN3   | Functions in membrane fusion events involving late endosomes and lysosomes. | 1           | 2.4                     | 3.8                        |
### TABLE II

**IFN-γ-modulated phagosome proteins identified by the large scale quantitative proteomics analysis**

The identified peptide sequences, m/z, charge, and maximal Mascot score are indicated for each protein. SNAP, N-ethylmaleimide-sensitive factor attachment protein; NEM, N-ethylmaleimide.

| Protein name                                      | UniProtKB accession no. | Peptides                                           | m/z     | Charge | Score |
|---------------------------------------------------|-------------------------|----------------------------------------------------|---------|--------|-------|
| α2-Macroglobulin                                  | Q6GQT1                  | ALLAYAFALAGNQER                                     | 804.4217| 2      | 53.83 |
| Acid ceramidase                                   | Q78P93                  | WYYVQTNYDR                                          | 672.3703| 2      | 34.08 |
| Acid sphingomyelinase-like phosphiesterase        | P58242                  | DLVTYFLNLR                                          | 627.3804| 2      | 50.16 |
| Arf6                                              | P62331                  | ILMLGLDAAGK                                         | 559.3684| 2      | 30.11 |
| Arf10b                                            | Q8VEH3                  | LWDIGGQPR                                           | 521.3006| 2      | 51.27 |
| Aminopeptidase N                                  | P97449                  | AVNQQTAVQPPATVR                                      | 790.5159| 2      | 43.37 |
| Annexin A1                                        | P10107                  | GLGTDEDLIELLTR                                       | 873.9611| 2      | 50.78 |
| Annexin A2                                        | P07356                  | TNQEOLEINR                                          | 622.8378| 2      | 61.88 |
| Annexin A4                                        | P97429                  | DIESGSGDLFR                                         | 613.3285| 2      | 69.5  |
| Annexin A5                                        | P48036                  | GLGTDEDSILNLTSR                                      | 852.5292| 2      | 98.12 |
| Apolipoprotein D                                  | P51910                  | DILTSNNIDIEK                                        | 687.9158| 2      | 67.5  |
| β2-Microglobulin                                  | P01887                  | TPQIQVYSR                                           | 546.3511| 2      | 67.01 |
| Basigin                                           | P18572                  | VLOEDTPLDHTK                                         | 503.6281| 3      | 32.34 |
| Calnexin                                          | P35564                  | GSLSGWILSK                                          | 524.3249| 2      | 14.9  |
| Calreticulin                                      | P14211                  | VHVINFYK                                            | 510.3049| 2      | 42.26 |
| Cathepsin A                                       | P16675                  | LYQSMNSOYIK                                         | 687.8408| 2      | 56.97 |
| Cathepsin B                                       | P10605                  | HFGYTSYSNNSVK                                        | 788.8767| 2      | 24.16 |
| Cathepsin C                                       | P97821                  | NVQGVNVYSPVR                                         | 666.4252| 2      | 62.89 |
| Cathepsin Z                                       | Q9WUU7                  | NVNGVNASYTR                                         | 647.8851| 2      | 45.95 |
| C-C chemokine receptor type 7                     | P47774                  | DLGCLSEQLQR                                         | 655.8563| 2      | 47.73 |
| CD45                                              | Q08857                  | SSMFQTR                                             | 428.7483| 2      | 26.61 |
| CD98 heavy chain                                  | P06800                  | DLVSMIQDLEK                                         | 581.3459| 2      | 31.33 |
| Collin                                            | P10852                  | LLLSTDSAR                                            | 488.3124| 2      | 46.84 |
| Cyclooxygenase-2                                  | Q05769                  | YALYDATYETK                                          | 669.3823| 2      | 51.78 |
| Cyclophilin C-associated protein                  | O35649                  | VEIFYR                                              | 413.7286| 2      | 33.49 |
| Dendritic cell-associated transmembrane protein   | Q99P91                  | AVDOWSTETISHEDIER                                    | 696.3339| 3      | 60.42 |
| Elongation factor 1-α                             | P62631                  | IGIGGITVPGVR                                         | 513.3278| 2      | 51.11 |

**Interferon-γ-modulated Phagosome Proteome**

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| Protein name                                      | UniProtKB accession no. | Peptides                           | m/z     | Charge | Score |
|--------------------------------------------------|-------------------------|-----------------------------------|---------|--------|-------|
| ERp57                                            | P27773                  | FISDKDASVVGFFR, LSKDPNVIAK, FVMQEEFSR, TADGVSHLK, YGVSGYPTLK, EATNPPIIEEKPQ, FAHTNIESLVK | 529.9733 | 3      | 16.3  |
| Ezrin/Moesin/Radixin protein                     | P26041                  | ALELEQER                          | 494.3083 | 2      | 35.97 |
| Ferritin heavy chain                             | P09528                  | MGAPAEAGAMEYLFDK                  | 815.424  | 3      | 46.72 |
| Ferritin light chain                             | P28391                  | TOAMEAALAMEK                      | 711.9133 | 2      | 46.72 |
| Flotillin-1                                       | O08917                  | AQQVEAOQEIEAR, MRGEAEAFAGAR, TEAIAHALETLEGHQR, VTVGLILSRSR | 734.4863 | 2      | 37.96 |
| Flotillin-2                                       | Q60634                  | VDEIVLSDNKS, RAFELOK, MALVLEALPOIAAK | 687.9185 | 3      | 45.91 |
| G protein Gα, α subunit                          | Q9DC51                  | ISQNYTPIPTQDVLR, LLLLAGEGSK, IYAMHWGTDSCR, IYAMHWGTDSCR | 529.2919 | 2      | 64.6  |
| G protein β 1 subunit                            | P62874                  | KACDATLSQTNNDIPVGR, IYAMHWGTDSCR | 696.4256 | 3      | 15.76 |
| G protein β 2 subunit                            | P62880                  | KACGDSLTQTAGLDVPVGR, IYAMHWGTDSCR | 668.8292 | 2      | 57.08 |
| Galectin-3                                       | P16110                  | VAVNDAHLLQYNHR, QSAFFESGKFPK                             | 550.6506 | 2      | 60.1  |
| GAPDH                                            | P16858                  | RVIISAPSDAPMFVMGHNHEK, AIGHYQLMSEK, GTGAEVLOETLYNAK, IKAQEAOQLR, ALQQGQHRMHR | 795.7856 | 3      | 35.59 |
| GBP-5                                            | Q8CFB4                  | AIGHYQLMSEK, GTGAEVLOETLYNAK, IKAQEAOQLR, ALQQGQHRMHR | 464.6093 | 3      | 36.51 |
| Glucosylceramidase                               | P17439                  | GFGHAMTDATALNILASPTQK, LKIPLHQLALK, SYFSTNGIEYVR, NFVDSPVIDPK, IPLLHQLALK | 759.1015 | 3      | 15.16 |
| GRP78/BIP                                        | P20029                  | ITPSYVAFTPEGER, TFAPEESIAMVLT, VEIIANDQGNR, DAGTIAGLNVMR, ELEEIQPIK | 783.937 | 3      | 95.12 |
| GRP94                                            | P08113                  | FAOAQEAVNR, ELNASADLKD, IHMPTELQLELLDLHR, LOPALWPFPR, MVLEJR | 541.3016 | 2      | 34.72 |
| GTP-binding protein 1                            | Q01514                  | IHPMTETLQLELLDLHR                  | 649.3769 | 3      | 95.12 |
| Hexosaminidase B                                 | P20060                  | LOPALWPFPR, MVLEJR, VPEFDTPGHTQSGWKG | 614.885 | 2      | 69.57 |
| HSC-71                                           | P63017                  | MVNHFIAEFK, FEELNALFR, VEKVTISNR | 627.3250 | 3      | 39.84 |
| HSP-90β                                          | P11499                  | HELINPDPHVETL, VKEKTISNR           | 595.0472 | 3      | 22.94 |
| ICAM-1                                           | P13597                  | KADGALLPGVWK                      | 640.8979 | 2      | 30.43 |
| IGTP                                             | Q811M6                  | DLEAAEVSSEDDTANL                  | 918.0155 | 2      | 52.02 |
| IIGP-1                                           | Q9QZ85                  | TGVVEAOQFPR, TVFGVDETSLQR, IAVTGDGNSGMSSFINALR | 560.8367 | 2      | 60.8  |
| IIGP-2                                           | Q99K68                  | DGNLTLSVGVIK, IAVTGDGNSGMSSFINALR | 552.3447 | 2      | 44.84 |
| Interferon-induced transmembrane protein          | Q99J93                  | KMVDGVTGAQAYASTAK                 | 566.6761 | 3      | 49.83 |

**Table II—continued**

Interferon-γ-modulated Phagosome Proteome
| Protein name                   | UniProtKB accession no. | Peptides                                                                 | m/z      | Charge | Score |
|-------------------------------|-------------------------|--------------------------------------------------------------------------|----------|--------|-------|
| Lactate dehydrogenase A chain | P06151                  | MVGDVTGAQAYASTAK                                                         | 785.4711 | 2      | 88.93 |
|                               | P11438                  | VTLTPEEEAR                                                               | 572.8466 | 2      | 59.25 |
| LAMP-1                        | P17046                  | YLDIFIAVK                                                               | 558.3606 | 2      | 37.76 |
| Leucyl-cystinyl aminopeptidase | Q8C129                  | ALQATVGNSYK                                                             | 576.3551 | 2      | 82.25 |
|                               |                         | YFAATQFEPLAAR                                                            | 742.8694 | 2      | 39.7  |
|                               |                         | IGVQVHALDITIK                                                            | 716.9418 | 2      | 52.4  |
|                               |                         | ILEALASSEVDHKE                                                          | 706.3557 | 2      | 34.46 |
|                               |                         | SYLSEDVFR                                                               | 558.3096 | 2      | 41.43 |
|                               |                         | YFETAVSRPGLGEPR                                                         | 550.3593 | 3      | 15.35 |
|                               |                         | AALSANVLTLJEKE                                                          | 700.4776 | 2      | 50.38 |
|                               |                         | IETAAAMDTGLR                                                            | 589.3498 | 2      | 48.3  |
| Lysosomal acid lipase         | Q9Z20M5                 | NGAPIIMSFPHFYQADEK                                                       | 689.3701 | 3      | 48.92 |
|                               |                         | QIOQSNLNR                                                               | 543.3179 | 2      | 42.06 |
|                               |                         | YFETAVSRPGLGEPR                                                         | 550.3593 | 2      | 13.53 |
|                               |                         | LLLVQDASER                                                              | 572.3749 | 2      | 71.59 |
| MHC class I heavy chain       | Q61643                  | KLTDVGFIK                                                               | 581.9071 | 2      | 32.67 |
|                               |                         | VESFLPQPTDEIR                                                           | 839.5182 | 2      | 34.92 |
|                               |                         | HYDPDFLR                                                                | 531.8079 | 2      | 55.3  |
|                               |                         | TAVAQVIGDR                                                              | 515.3365 | 2      | 51.79 |
| NADPH oxidase p22phox         | Q61462                  | ERPOVQGTIK                                                              | 542.8339 | 2      | 48.53 |
| NADPH oxidase gp91phox        | Q61093                  | GHHFIFNK                                                                | 481.2672 | 2      | 42.97 |
| Na"/K"-ATPase α 3 subunit     | Q6PCIC                  | NMPVQQALVIR                                                             | 634.894  | 2      | 48.17 |
| Napsin A                     | Q60943                  | FAIQYGTGR                                                               | 506.7857 | 2      | 32.53 |
| NEM-sensitive fusion protein  | P46460                  | AENSNLNIJK                                                              | 573.3654 | 2      | 52.59 |
| Neutral amino acid transporter | Q9ESU7                  | EIVLDSFLDTVRS                                                            | 653.4058 | 2      | 86.65 |
| Niaastrin                     | P57716                  | LENIDSFVELGOVALR                                                         | 902.0338 | 2      | 77.7  |
| Niemann-Pick C1 protein       | O35604                  | ALANAVTLVAR                                                             | 549.8902 | 2      | 54.14 |
| Palmitoyl-protein thioesterase | O88531                  | TSDADYTDAMK                                                             | 551.8035 | 2      | 30.57 |
| Peroxiredoxin 1               | P35700                  | ATAVMPDGQFK                                                             | 582.8516 | 2      | 62.77 |
|                              |                         | QITINDLPVGR                                                             | 613.4182 | 2      | 44.63 |
|                              |                         | TIAODYGVKL                                                             | 554.3593 | 2      | 21.0  |
### TABLE II—continued

| Protein name | UniProtKB accession no. | Peptides | $m/z$ | Charge | Score |
|--------------|-------------------------|----------|------|--------|-------|
| Peroxiredoxin 4 | O08807 | SVDEILR | 416.2751 | 2 | 21.7 |
| Peroxisomal membrane protein 69 | O89016 | DISLSEYK | 477.7984 | 2 | 18.7 |
| Protein-disulfide isomerase | P09103 | KQGLGPNIPLISDPK | 594.3902 | 3 | 18.3 |
| | | QGGLGPNIPLISDPK | 819.0374 | 2 | 41.92 |
| Protein-disulfide isomerase A6 | Q922R8 | TGEIAVIDAALSAR | 693.9184 | 2 | 28.25 |
| Pyruvate kinase, isozyme M2 | P52480 | P52480 | 613.4182 | 2 | 44.63 |
| Rab1 | Q5SW88 | EFADSGLPFELETSAK | 862.9417 | 2 | 46.65 |
| Rab2 | Q6PD23 | GAAGALLVYDTR | 660.3863 | 2 | 86.74 |
| Rab5c | P35278 | QASPNIIVALNGK | 698.4722 | 2 | 86.7 |
| Rab7 | P51150 | TSLMNQYVNNKK | 442.5623 | 3 | 32.47 |
| Rab9a | Q9R0M6 | DSTDNAAAFEAADVR | 740.3617 | 2 | 47.78 |
| Rab10 | P61027 | FHTTTSYRY | 644.8179 | 2 | 35.66 |
| Rab14 | Q19V41 | NLTNPNTVIILNGK | 812.5272 | 2 | 65.96 |
| Rab32 | Q9CZE3 | DNINIDEAAT | 580.8464 | 2 | 29.74 |
| Rac1 | P63001 | LTPITYQGGLAMAK | 752.4446 | 2 | 52.04 |
| Rac2 | Q05144 | KLAPITYQGGLAMAK | 528.7022 | 3 | 36.23 |
| RaI | Q6IJW9 | VIMVSGGGSVGGK | 502.2769 | 2 | 44.25 |
| Receptor-interacting protein Ser/Thr kinase 3 | Q9QZL0 | DSDKVDAVESV | 580.8036 | 2 | 42.16 |
| Rho-associated protein kinase 2 | P70336 | KDQAQPSFQNLHLLK | 580.0491 | 2 | 55.25 |
| Rp105 | Q62192 | KYKGGAPSFQNLHLLK | 488.2644 | 2 | 36.76 |
| Sacsin | Q9JLC8 | MVDVLLD | 481.2899 | 2 | 30.34 |
| Septin-7 | O55131 | LKDEESALQ | 594.8431 | 2 | 28.86 |
| Signal peptide peptidase-like protein | Q9JFJ9 | ETLGDSVT | 545.8401 | 2 | 36.35 |
| α-SNAP | Q9DB05 | NSQFSGLFGSGSA | 775.4192 | 2 | 38.73 |
| γ-SNAP | Q9CWZ7 | LIENVDPEK | 528.8044 | 2 | 33.38 |
| SNAP-29 | Q9EBR0 | DLPDPGDPADIDR | 640.8755 | 2 | 32.81 |
| Solute carrier family 2 | Q5SEX0 | VSWAAR | 409.7102 | 2 | 29.64 |
| | | ADSEHWEFEIQQR | 701.3907 | 3 | 31.36 |
| | | TEGLYDTPPEPVPATPG | 1047.599 | 2 | 34.62 |
| | | SLEIEAEFFHTT | 493.2777 | 3 | 35.32 |
| Solute carrier family 6 | Q35316 | EGATPFHSSR | 501.3051 | 2 | 45.69 |
| Solute carrier family 12 | Q99MR3 | AFVIDTLSPS | 652.9274 | 2 | 39.54 |
| Solute carrier family 37 | Q6PA04 | EGGSACISL | 763.4035 | 2 | 69.81 |
| Solute carrier family 38 | Q8CF6E | SHYADVDPQNSNLGESLQGK | 797.4274 | 3 | 59.25 |
| Solute carrier family 38 | O89988 | VIAAEGMNASR | 624.3629 | 2 | 54.6 |
| Stomatin | O89988 | VIAAEGMNASR | 624.3629 | 2 | 54.6 |
| Stromal cell-derived factor receptor 2 | Q8K385 | HSOQPLTVEK | 672.3431 | 2 | 37.75 |
| Cu,Zn-superoxide dismutase | P08228 | VISLGGHGGIR | 684.4114 | 2 | 45.28 |
| Syntaxin 4 | P70452 | HSEIQLQER | 570.2924 | 2 | 32.76 |
| Syntaxin 7 | O70439 | TNLQGTPQDSPAR | 835.0197 | 2 | 95.81 |
| Syntaxin 8 | O89983 | QNLLDDLVR | 593.8851 | 2 | 47.58 |
| Syntaxin 12/13 | Q9ER00 | ISQATAQIK | 480.323 | 2 | 41.68 |
| Interferon-γ-modulated Phagosome Proteome | 708 | Molecular & Cellular Proteomics 7.4 | | | |
| Protein name | UniProtKB accession no. | Peptides | m/z  | Charge | Score  |
|--------------|-------------------------|----------|------|--------|--------|
| Syntenin 1   | O08992                  | SLMDHTIPEV | 571.2788 | 2 | 22.29 |
|              |                         | SIDNGIFVLQVANSAPLSVGLR | 1200.221 | 2 | 99.34 |
| Thioredoxin  | P10639                  | EAFQEAALAAAGDK | 660.8969 | 2 | 64.21 |
| Toll-like receptor 3 | Q99MB1    | LFAOZNALQPLNHLTEK | 683.7533 | 3 | 39.6 |
|              |                         | SFYGLSNLR | 528.7764 | 2 | 44.32 |
| Toll-like receptor 7 | P58681    | LEVLPGLTNFIK | 681.4485 | 2 | 39.46 |
| Toll-like receptor 9 | Q8EQU3    | SAGALPYDAFVFDK | 836.0295 | 2 | 24.18 |
| Transferrin receptor protein 1 | Q62351 | VEYHFLSPYVSPR | 797.4265 | 2 | 47.3 |
|              |                         | SSYGTGLLLK | 487.8438 | 2 | 24.46 |
|              |                         | VPQLQGNOVR | 542.8553 | 2 | 30.22 |
|              |                         | LDLTEALGQ | 591.3636 | 2 | 42.99 |
|              |                         | QLSQNTYTPR | 604.2576 | 2 | 51.23 |
| Tubulin β-chain | P99024         | ISVYYNEATGGK | 651.3888 | 2 | 29.58 |
| Tweety homolog 3 | Q6P5F7    | ALVEMODWAEWL | 793.5172 | 2 | 75.49 |
| Uncharacterized protein C2orf18 | Q8VE96    | WRLPTQEOEG | 510.3114 | 3 | 34.99 |
| Uncharacterized protein C12orf23 | Q9DAM7   | VTGGIFSVTK | 504.8397 | 2 | 50.34 |
| VAMP7        | Q7Z409                  | FQOTTYSGR | 480.2396 | 2 | 29.02 |
| VAMP8        | O70404                  | NIMQTGNVER | 552.8273 | 2 | 62.03 |
| V-ATPase A subunit A (catalytic) | P50516   | ALDEYDKHTEFVPLR | 536.5445 | 4 | 27.09 |
|              |                         | FSMVQWVPVR | 624.8929 | 2 | 75.47 |
|              |                         | EHMGEILYK | 506.3077 | 2 | 30.01 |
| V-ATPase B subunit isoform 2 | P62814    | IPISFAAHLPHNEAAICR | 707.7505 | 3 | 66.99 |
|              |                         | TPVESDMLGR | 552.8332 | 2 | 52.94 |
|              |                         | RIPQSLSEFYP | 797.4686 | 2 | 74.55 |
| V-ATPase E subunit | P50518   | IMEYVYK | 468.2249 | 2 | 22.52 |
|              |                         | GALFGANANK | 559.8666 | 2 | 29.88 |
|              |                         | KQDFPLVK | 487.8902 | 2 | 29.47 |
| V-ATPase V$_0$ subunit a3 isoform | Q9JL12   | LFKEYK | 700.7505 | 3 | 66.99 |
|              |                         | GFLGANANK | 559.8666 | 2 | 29.88 |
| V-ATPase V$_0$ subunit d1 isoform | P51863   | LYPREGYK | 615.8457 | 2 | 41.14 |
|              |                         | SIAELVK | 428.7575 | 2 | 26.02 |
| V-ATPase V$_0$ subunit d2 isoform | Q80SY3    | TLEDVFYER | 586.3655 | 2 | 34.57 |
| Voltage-dependent anion channel 1 | Q60932   | LTLALSADLG | 515.8151 | 2 | 99.65 |
|              |                         | VNNSSLIGLGYTQLKPGIK | 701.7679 | 3 | 54.39 |
| Voltage-dependent anion channel 2 | Q60930   | YQLDPTASIK | 647.3296 | 2 | 54.68 |
| Voltage-dependent anion channel 3 | Q60931   | LTLTIFIPNTGK | 709.9737 | 2 | 48.99 |
| Voltage-dependent P/Q-type calcium channel, α-1A subunit | O00555 | DPCGSAGLDAR | 523.3228 | 1 | 28.72 |
| 40 S ribosomal protein S25 | P62358   | LTPAASVR | 542.8225 | 2 | 30.22 |
| 60 S acidic ribosomal protein P0 | P14869   | GHLHNPENAEL | 611.3461 | 2 | 35.12 |
| 60 S ribosomal protein L3 | P27659   | HQSLGFLPR | 492.3196 | 2 | 57.85 |
| 60 S ribosomal protein L5 | P47962   | RFPQVDSEK | 593.3472 | 2 | 26.85 |
| 60 S ribosomal protein L9 | P35979   | IGPLGSPK | 441.3189 | 2 | 32.13 |
| 60 S ribosomal protein L14 | Q8CR57   | LVAVVIDONR | 677.9423 | 2 | 70.76 |
| 60 S ribosomal protein L31 | P62900   | SAINEXIT | 494.8081 | 2 | 29.56 |
| A630077B13Rik protein | Q8C9E8   | DWQESIALYTFNPK | 856.4212 | 2 | 35.78 |
| 3110005G23Rik protein | Q8VDU8   | AKPLPVDLLEEK | 684.4012 | 2 | 37.45 |
| 4930506M07Rik protein | Q8K2Q9   | LENEALKH | 477.3475 | 2 | 32.23 |
| 9030624J02Rik protein | Q80XN3   | ISSMCVDSR | 523.3381 | 2 | 34.14 |
Our proteomics analysis identified many proteins associated with the microbicidal function of phagosomes, consistent with the previously described effects of IFN-γ on macrophages (5, 7). The IFN-γ-up-regulated proteins shown in Tables I, II, and III include more than 10 different lysosomal hydrolases; four subunits of the V-ATPase complex, the proton pump that acidifies the phagosome lumen; and two subunits of NADPH oxidase, the protein complex that generates reactive oxygen species within phagosomes. IFN-γ treatment validated IFN-γ-modulated proteins identified by the quantitative proteomics analysis. A, plot of the normalized mean MS intensity evaluated across the six control and six IFN-γ-treated samples for each of the seven peptides assigned to GRP78/Bip. The mean values were normalized to the lowest intensity measured for all peptides such that the base value becomes 1 with which all other peptide intensities are compared. B, Western blot validation of GRP78/Bip up-regulation by IFN-γ. The relative abundance of GRP78/Bip was compared in total cell lysates (TCL) or in phagosome preparations from control (−) or IFN-γ-treated (+) cells. Equal amounts of proteins from total cell lysates or phagosome preparations were loaded in each lane prior to Western blotting. The intensities of the bands obtained were quantified, and the values were normalized to the highest value. Histograms represent the mean of two independent preparations of total cell lysates and phagosomes. Representative blots, corresponding to identical expositions for total cell lysates and phagosomes, are shown. C, plot of the normalized mean MS intensity evaluated as in B for each of the four peptides assigned to the catalytic subunit A1 isomor of V-ATPase. D, Western blot validation of V-ATPase up-regulation by IFN-γ as in C. E, Western blot of GAPDH as in C. GAPDH was not significantly modulated by IFN-γ on phagosomes.

Fig. 2. Validation of IFN-γ-modulated proteins identified by the quantitative proteomics analysis. A, plot of the normalized mean MS intensity evaluated across the six control and six IFN-γ-treated samples for each of the seven peptides assigned to GRP78/Bip. The mean values were normalized to the lowest intensity measured for all peptides such that the base value becomes 1 with which all other peptide intensities are compared. B, Western blot validation of GRP78/Bip up-regulation by IFN-γ. The relative abundance of GRP78/Bip was compared in total cell lysates (TCL) or in phagosome preparations from control (−) or IFN-γ-treated (+) cells. Equal amounts of proteins from total cell lysates or phagosome preparations were loaded in each lane prior to Western blotting. The intensities of the bands obtained were quantified, and the values were normalized to the highest value. Histograms represent the mean of two independent preparations of total cell lysates and phagosomes. Representative blots, corresponding to identical expositions for total cell lysates and phagosomes, are shown. C, plot of the normalized mean MS intensity evaluated as in B for each of the four peptides assigned to the catalytic subunit A1 isomor of V-ATPase. D, Western blot validation of V-ATPase up-regulation by IFN-γ as in C. E, Western blot of GAPDH as in C. GAPDH was not significantly modulated by IFN-γ on phagosomes.

Our proteomics analysis identified many proteins associated with the microbicidal function of phagosomes, consistent with the previously described effects of IFN-γ on macrophages (5, 7). The IFN-γ-up-regulated proteins shown in Tables I, II, and III include more than 10 different lysosomal hydrolases; four subunits of the V-ATPase complex, the proton pump that acidifies the phagosome lumen; and two subunits of NADPH oxidase, the protein complex that generates reactive oxygen species within phagosomes. IFN-γ treatment
### TABLE III

**IFN-γ-modulated phagosome proteins identified by high resolution 2-D gel electrophoresis**

The proteins identified and their relative abundance (based on 2-D gel densitometry measurements) are indicated for each spot. TNF, tumor necrosis factor; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.

| Spot no. | Protein name | UniProtKB accession no. | Remarks | Relative protein abundance |
|----------|--------------|-------------------------|---------|---------------------------|
| 1        | Calreticulin | P14211                  | ER chaperone. Mediates the assembly of MHC class I molecules. | 2 |
| 2, 3, 4, 5, 9 | Cyclophilin C-associated protein precursor | O35649 | Binds to Galectin-3. Down-regulates proinflammatory responses. | 2 |
| 6        | Reticulocalbin 2 precursor | O70341 | ER Ca\(^{2+}\)-binding protein. | 2 |
| 7, 30, 31, 33 | Cathepsin D | P18242 | Lysosomal aspartyl protease. | 2 |
| 8        | Hypothetical protein | Q9CQ22 | Unknown. | 3 |
| 9        | Cytochrome b\(_6\) | P68395 | ER hemoprotein. Functions as an electron carrier. | 2 |
| 10, 11, 12 | Cathepsin C | P97821 | Also known as dipeptidyl-peptidase I. Lysosomal cysteine protease. | 2 |
| 13       | Cathepsin S | O70370 | Lysosomal cysteine protease. Functions in the removal of the invariant chain from MHC class II molecules. | 2 |
| 14, 15   | Cathepsin B | P10605 | Lysosomal cysteine protease. | 4 |
| 16       | Cathepsin Z | Q9WU7 | Lysosomal cysteine protease. | 2 |
| 17, 18   | HSP-60 | P63038 | Mitochondrial. Heat shock protein 60 kDa. | 3 |
| 19       | Protein-disulfide isomerase A6 | Q922R8 | ER chaperone. | 2 |
| 20       | ATP synthase subunit β | P56480 | Mitochondrial. | 3 |
| 21       | Protein-disulfide isomerase | P09103 | ER chaperone. Functions in the assembly of MHC class I molecules. | 3 |
| 22       | GRP94 | P08113 | Also known an gp96. ER chaperone. Involved in antigen cross-presentation. | 2 |
| 23       | GRP78/BiP | P20029 | Glucose-regulated protein 78. Also known as BiP. ER chaperone. | 2 |
| 24       | Ferritin light chain | P29391 | Functions as an intracellular iron storage protein. | 3 |
| 25       | Ferritin heavy chain | P09528 | Cytoplasmic. Adenine phosphoribosyltransferase. | 2 |
| 26       | CREG1 | O75629 | Cellular repressor of E1A-stimulated genes 1. | 2 |
| 27       | Peroxiredoxin 4 | O08807 | Cytoplasmic. Detoxifies reactive oxygen and nitrogen species. | 3 |
| 28       | TRAIL | P60592 | TNF-related apoptosis-inducing ligand. | 2 |
| 29       | ERp29 | P57759 | ER chaperone. | 2 |
| 30       | Coronin-1A | P31146 | Cytoplasmic. Actin-binding protein. | 2 |
| 31       | GRB2 adaptor protein | P62994 | Links growth factor receptors to the Ras signaling pathway. | 2 |
| 32       | β-Glucuronidase | P12265 | Lysosomal hydrolase. | 2 |
| 33       | ERp57 | P27773 | ER chaperone. Functions in the assembly of MHC class I molecules. | 2 |
| 34       | T-complex protein 1 epsilon | P80316 | Cytoplasmic chaperone. | 2 |
| 35       | T-complex protein 1α | P80314 | Cytoplasmic chaperone. | 2 |
| 36       | T-complex protein 1α B | P11983 | Cytoplasmic. Adenine phosphoribosyltransferase. | 2 |
| 37       | Hexosaminidase B | P20060 | Lysosomal. | 3 |
| 38       | V-ATPase B subunit isoform 2 | P62814 | Proton pump. | 2 |
| 39       | MEK-binding partner 1 | O88653 | Enhances the efficiency of the MAP kinase cascade. | 2 |
| 40       | Cathepsin A | P16675 | Also known as lysosomal protective protein and carboxypeptidase C. | 2 |
| 41       | Phosphoglycerate mutase 1 | P25113 | Cytoplasmic chaperone. | 2 |
| 42       | Annexin A2 | P07356 | Ca\(^{2+}\)-regulated membrane-binding protein. | 3 |
| 43       | Palmitoyl-protein thioesterase | O88531 | Removes fatty acyl groups during lysosomal degradation. | 2 |
| 44       | Nucleoside-diphosphate kinase B | Q01768 | Cytoplasmic. Functions in the synthesis of nucleoside triphosphates. | 2 |
| 45       | V-ATPase E subunit | P50518 | Proton pump. | 4 |
| 46       | Proteasome subunit α type 6 | Q9QUM9 | Cytoplasmic. Functions in antigen processing for MHC class I molecules. | 2 |
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Fig. 4. IFN-\(\gamma\)-modulated functions on the phagosome. The IFN-\(\gamma\)-modulated proteins identified in the current study are in color. Four of the up-regulated proteins were used as input nodes to build interactomes using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database (proteins are identified by their gene name). Dots corresponding to proteins identified in this study are in green. PDI, protein-disulfide isomerase; \(\beta_2\)-microglobulin.

up-regulated several phagosomal proteins involved in fusion with endocytic compartments, including syntaxin 7, syntaxin 13, Rab7, LIMP2, VAMP8, annexin A2, and Arl10b (also known as Arl8a). Syntaxin 7, syntaxin 13, and Rab7 function in phagosome maturation (19, 20), whereas LIMP2 appears to be involved in the maintenance and biogenesis of lysosomes (21), and VAMP8 functions in the fusion between early and late endosomes (22). Actin polymerization has been shown to occur on isolated phagosomes and to facilitate fusion with late endosomes (23), a process that may necessitate annexin A2, a protein that probably organizes actin assembly sites on membranes (24). The up-regulation of Arl10b on phagosomes from IFN-\(\gamma\)-treated cells may also promote phagosome maturation as overexpression of Arl10b and Arl10c (also known as Arl8b) results in increased lysosome motility (25). Taken together, these findings suggest that IFN-\(\gamma\) alters the fusogenicity of phagosomes toward late endosomes and lysosomes and therefore regulates phagosome maturation.

Phagosomes from IFN-\(\gamma\)-treated cells were found to have increased levels of several IFN-\(\gamma\)-induced GTP-binding proteins, notably four members of the p47 GTPase family (IGTP, IIGP-2, IIGP-1, and LRG-47). These GTPases play essential roles in innate immunity against bacterial and parasitic infections (26, 27). The underlying mechanisms for the protective effect of p47 GTPases against infections are unclear; however, p47 GTPases are suggested to function in controlling phagosome maturation (26, 28). The p47 GTPases studied so far are mostly membrane-bound and localize to endoplasmic reticulum (ER) or Golgi compartments in IFN-\(\gamma\)-treated cells; however, during phagocytosis, p47 GTPases relocalize rapidly to nascent phagosomes (29). GBP-5 is another IFN-\(\gamma\)-induced GTPase that we identified for the first time on phagosomes. GBPs belong to the dynamin family of large GTPases and display high GTPase activity. The function of GBPs is uncertain, although they demonstrate mild antiviral activity (30). Thus IFN-\(\gamma\) up-regulates several phagosomal proteins that are specifically involved in the control of infections.

The IFN-\(\gamma\)-modulated proteins on phagosomes include several receptors and signaling proteins, such as syntenin 1, a scaffolding protein suggested to couple receptors to the cytoskeleton (31). Two receptors, LRP/CD91 and the transferrin receptor, were found to be down-regulated by IFN-\(\gamma\) on phagosomes. The decrease in LRP/CD91, a receptor involved in the phagocytosis of apoptotic cells (32), may signify that activated macrophages reduce their uptake of apoptotic cells in inflammatory conditions. The down-regulation of the transferrin receptor is consistent with the role of IFN-\(\gamma\) in reducing the uptake of nutrients such as iron, an effect that limits the intraphagosomal growth of bacteria (33). The IFN-\(\gamma\)-modulated receptors on phagosomes also included three Toll-like receptors (TLR-3, TLR-7, and TLR-9), essential players in the immune response to microbes. These TLRs bind to bacterial or viral nucleic acids present in endocytic compartments in contrast to other TLRs such as TLR-4 that bind to microbial ligands at the cell surface. In line with the up-regulation of TLRs, GRP94/gp96, a protein recently shown to function as an essential chaperone for TLRs (34), was up-regulated by IFN-\(\gamma\) on phagosomes. Cyclooxygenase-2, a key enzyme in the production of proinflammatory mediators (35), was also up-regulated on phagosomes following IFN-\(\gamma\) treatment. These results indicate that phagosomal proteins play a role in several aspects of the macrophage immune response to IFN-\(\gamma\).

The up-regulation of antigen presentation is a well-described effect of IFN-\(\gamma\) on macrophages and phagosomes from IFN-\(\gamma\)-treated cells contained increased levels of several proteins involved in MHC class I antigen presentation (7). As illustrated in Fig. 4, phagosomes from IFN-\(\gamma\)-treated cells contained increased levels of a proteasome subunit (subunit \(\alpha\) type 6), MHC class I molecules (composed of an \(\alpha\)-heavy chain and of \(\beta_2\)-microglobulin), and components of the MHC class I peptide-loading complex (GRP78/BiP, ERP57, protein-disulfide isomerase, and calreticulin) (36, 37). Several studies have demonstrated that the contribution of these ER proteins to phagosomes defines a pathway that leads to the loading
and presentation of exogenous peptide antigens on MHC class I molecules, a process referred to as antigen cross-presentation (17, 38, 39). IFN-γ-activated macrophages are thus expected to demonstrate enhanced antigen cross-presentation.

**Dynamic Analysis of IFN-γ-regulated Proteins on the Phagosome**—The large scale proteomics analysis has allowed us to highlight important functions modulated on phagosomes by IFN-γ, such as microbe inactivation, protein degradation, and antigen presentation. To simplify the readout, our analysis focused on a single temporal window (phagosomes formed by a 1-h pulse period followed by a 1-h chase period) in the phagosome maturation process. Phagosomes, however, continuously exchange material with various intracellular compartments as they mature into phagolysosomes, a process that makes their proteome highly dynamic (2). To obtain a more comprehensive view of the changes occurring on IFN-γ-treated phagosomes, a selection of relevant markers on phagosomes from control or IFN-γ-treated macrophages was analyzed dynamically by Western blotting. As shown in Fig. 5, IFN-γ-regulated proteins were differently modulated during phagolysosome biosynthesis, and some proteins responded to IFN-γ only at specific stages during phagosome maturation. Hence EEA1 was up-regulated on early phagosomes from treated cells but returned to control levels at later stages in phagosome maturation (Fig. 5). The increase in EEA1, an early endosomal marker that functions in membrane fusion, indicates that IFN-γ may stimulate phagosome fusion with early endosomes.

*Resistance to infection is critically dependent on the function of the NADPH oxidase in phagocytic cells* (40). In macrophages, the NADPH oxidase (gp91phox subunit) was also up-regulated on early phagosomes by IFN-γ (Fig. 5). The NADPH oxidase complex produces reactive oxygen species that consume protons in the phagosome lumen, thereby restricting the acidification of phagosomes in various phagocytic cells (41, 42). The recruitment of this complex to early phagosomes may thus limit the acidification caused by the V-ATPase proton pump. In agreement with this suggestion, Russell and co-workers (43) have recently shown that IFN-γ treatment reduced the acidification rate of early phagosomes. The function of the NADPH oxidase in controlling phagosomal pH has also recently been shown to maintain phagosome conditions that limit antigen degradation and favor antigen cross-presentation (42). In relation with this finding, our proteomics analysis demonstrated the up-regulation of several components of MHC class I peptide-loading complex (Fig. 4). In the dynamic analysis, two of these proteins, calreticulin and ERp57, were increased by IFN-γ throughout the phagosome maturation process (Fig. 5). In contrast, calnexin, an ER protein not directly involved in antigen loading, displayed a very different dynamic profile with little variation in abundance between control and IFN-γ-treated phagosomes (Fig. 5). These results thus indicate that IFN-γ specifically up-regulates the components of the MHC class I peptide-loading complex on early phagosomes.

**MHC class I peptide antigens are generated by the proteolytic activity of proteasomes**. IFN-γ treatment is known to up-regulate immunoproteasomes, subsets of proteasomes that contain three substituted β subunits, including Lmp2 (44). As shown in Fig. 5, IFN-γ treatment increased the recruitment of the immunoproteasome subunit Lmp2 on early phagosomes as shown previously (17). IFN-γ macrophages demonstrated a delayed acquisition of cathepsin B and of the lysosomal marker Lamp1, consistent with the recent finding that IFN-γ down-regulates the degradative capacity of early phago-

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**Figure 5. Phagosome-associated proteins are dynamically modulated by IFN-γ**. The relative abundance of the indicated proteins was compared between phagosomes isolated from control (−) or IFN-γ-treated (+) macrophages following the indicated internalization/chase periods in minutes. Equal amounts of proteins from each sample were loaded prior to Western blotting for the indicated proteins. The intensities of the bands obtained were quantified, and the values were normalized over the highest value. Graphs represent the mean of three independent experiments, which were analyzed separately. Error bars indicate the mean deviations. Representative blots, corresponding to identical expositions for control or IFN-γ-treated samples, are shown. GAPDH was not significantly modulated by IFN-γ on phagosomes.

| Time (min) | Early Markers | ER Markers | Late Markers |
|-----------|---------------|------------|--------------|
| 150/300 | + IFN-γ | EEA1 | Calnexin | Rab7 |
| 60/240 | + IFN-γ | - | - | - |
| 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| gp91phox | + IFN-γ | Lamp1 | - | - |
| 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| Cathepsin B | + IFN-γ | Lmp-2 | - | - |
| 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| GAPDH | + IFN-γ | - | - | - |
| 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
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Fig. 6. Integrated model of the IFN-γ-modulated functions that favor antigen cross-presentation. The increased recruitment of the NADPH oxidase complex may limit the acidification of the phagosome lumen by the V-ATPase proton pump. Delayed acidification and phagolysosome fusion, together with the increased association of immunoproteasomes, may limit protein degradation by lysosomal proteases and favor protein processing by the immunoproteasomes. The resulting peptide antigens could then associate with the upregulated MHC class I peptide-loading complex (composed of transporter associated with antigen processing (TAP), tapasin, calreticulin, ERpS7, protein-disulfide isomerase (PDI), and the MHC class I heterodimer), leading to enhanced antigen cross-presentation by IFN-γ-activated macrophages. β2M, β2-microglobulin; CathB, cathepsin B.

The delay in phagosome maturation occurred despite the efficient recruitment of Rab7, a small GTPase that regulates phagolysosome fusion (43). The delay in phagosome maturation occurred despite the efficient recruitment of Rab7, a small GTPase that regulates phagolysosome fusion (Fig. 5), suggesting that other factors may regulate phagosome fusion events in IFN-γ-treated cells. Rab14, an upregulated protein in our study (Table I), is one possible factor as the recruitment of Rab14 has been suggested to block phagosome maturation (45). Taken together, the dynamic analysis of this selection of phagosomal proteins suggests that IFN-γ enhances the conditions that favor the production of peptide antigens and their loading on MHC class molecules as illustrated in Fig. 6. The current study demonstrates the efficiency of a quantitative large-scale proteomics approach to outline general features of the response to cytokines. In addition, our study has allowed us to point out specific functions that are targeted by IFN-γ on phagosomes and to identify susceptible players in these processes. IFN-γ indeed modulates proteins involved in phagosome maturation, microbe degradation, innate immune response, and antigen presentation. Using these findings, we could focus on the dynamic behavior of a selection of IFN-γ-modulated proteins during phagosome maturation to refine the model inferred from the proteomics data. Globally, this approach led us to propose a new working hypothesis whereby the production of antigen-loaded MHC class I molecules increases in phagosomes of IFN-γ-activated macrophages.

Acknowledgments—We thank Matthias Trost for helpful discussions and Guillaume Goyette for the Web link.

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