Macrophages provide the first line of host defense with their capacity to react to an array of cytokines and bacterial components requiring tight regulation of protein expression and secretion to invoke a properly tuned innate immune response. To capture the dynamics of this system, we introduce a novel method combining pulsed stable isotope labeling with amino acids in cell culture (SILAC) with pulse labeling using the methionine analog azidohomoalanine that allows the enrichment of newly synthesized proteins via click-chemistry followed by their identification and quantification by mass spectrometry. We show that this permits the analysis of proteome changes on a rapid time scale, as evidenced by the detection of 4852 newly synthesized proteins after only a 20-min SILAC pulse. We have applied this methodology to study proteome response during macrophage activation in a time-course manner. We have combined this with full proteome, transcriptome, and secretome analyses, producing an integrative analysis of the first 3 h of lipopolysaccharide-induced macrophage activation. We observed the rapid induction of multiple processes well known to TLR4 signaling, as well as anti-inflammatory proteins and proteins not previously associated with immune response. By correlating transcriptional, translational, and secretory events, we derived novel mechanistic principles of processes specifically induced by lipopolysaccharides, including ectodomain shedding and proteolytic processing of transmembrane and extracellular proteins and protein secretion independent of transcription. In conclusion, we demonstrate that the combination of pulsed azidohomoalanine and pulsed SILAC permits the detailed characterization of proteomic events on a rapid time scale. We anticipate that this approach will be very useful in probing the immediate effects of cellular stimuli and will provide mechanistic insight into cellular perturbation in multiple biological systems. The data have been deposited in ProteomeXchange with the identifier PXD000600. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.030916, 792–810, 2014.

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Macrophages contribute to the establishment of innate immune responses toward invading pathogens by means of the phagocytosis of pathogens, recognition of pathogen-associated molecular patterns, secretion of proteins activating other immune cells, and presentation of antigens to lymphocytes. Depending on the nature of the stimulating agent (e.g. pathogen-associated molecular patterns or cytokines) they develop into classically (M1) or alternatively (M2) activated macrophages that are typically characterized by the secretion of pro- and anti-inflammatory cytokines, respectively. Yet this distinction most likely is an oversimplification, as macrophages in vivo dynamically respond to their environment by secreting a spectrum of M1 and M2 cytokines (1). Stimulation of macrophages with pro-inflammatory agents (e.g. lipopolysaccharides (LPS)) activates Toll-like receptor 4 (TLR4), inducing downstream signaling cascades that converge on transcription factors such as NF-κB, which in turn activate the genes encoding the proteins that are responsible for the immune response. Many of these factors are secreted (e.g. the cytokines Tnf and Il1) to propagate the inflammatory response in an autocrine or paracrine fashion, thereby attracting and activating other immune cells (2). The inflammatory response is counteracted in various ways such as action on NF-κB itself (3) or through the destabilization of transcripts of NF-κB target genes (4, 5). It is crucial that the immune response be carefully balanced to eradicate a bacterial infection without overactivation leading to severe syndromes such as septic shock, the major cause for mortality upon bacterial infection (6, 7). Therefore, accurate regulation of the timing, magnitude, and duration of pro- and anti-inflammatory genes and proteins is a fundamental property of properly functioning macrophages.

Macrophage activation with LPS has been investigated in several large-scale transcriptomic and qualitative or quantitative proteomic studies exploring different aspects of TLR4 signaling (8–18). For instance, Ramsey et al. combined mRNA expression analysis using microarrays with motif scanning of transcription factor binding sites, inferring a network of asso-
cations between transcription factor genes and clusters of co-expressed target genes (8). Similarly, transcriptome analyses revealed crosstalk between pathways downstream of TLR4 (9) and among the three transcription factors NF-κB, C/EBPΔε, and ATF3 that discriminates between transient and persistent TLR4-induced signals (10).

To investigate post-transcriptional events in macrophage activation, Bhatt et al. performed RNA sequencing of fractionated transcripts in a time-course study of lipid A–stimulated macrophages, elucidating a high-resolution map of coding and noncoding transcripts at three different cellular locations (chromatin, nucleus, and cytoplasm) (12). Although these studies provided detailed insights into the regulation of mRNA processing, abundance, and localization, regulatory processes at the level of translation were not investigated.

A proteomic analysis of macrophage activation by iTRAQ labeling revealed the differential expression of 36 proteins (11). In the most comprehensive proteome-wide quantitative study using SILAC and subcellular fractionation, Du et al. revealed signaling and regulatory networks that systematically operate in the early response to LPS (19). Weintz et al. and Sharma et al. performed quantitative phosphoproteomic analyses upon LPS treatment, identifying a highly dynamic phosphorylation pattern in established signaling routes and beyond that could be linked to kinases operating in canonical TLR pathways, but also in the cell cycle (ATM/ATR kinases) and cell growth (mTOR) (13, 14). The combination of phosphoproteomics and transcriptomic data at 15 min and 4 h after stimulation revealed novel signaling modules as well as the cytokeratin as key targets in LPS-regulated phosphorylation (13). Protein relocalization after LPS treatment was demonstrated by the rapid modulation of the microtubule cytoskeleton concomitant with an increase in secretory and migratory activity (15), and by the selective recruitment and activation of the proteasome to macrophage rafts (16).

Although several of the aforementioned studies used macrophage stimulation as a model system to investigate basic principles of gene expression and post-transcriptional or protein localization regulation, a full view of translational regulation achieved via the integration of mRNA expression and protein synthesis during LPS stimulation is still lacking. Furthermore, our view on the full repertoire of secreted proteins is incomplete despite the central role of secreted proteins in the immune response.

Although proteomic approaches have been widely used in the comparison of protein levels in samples obtained under static conditions, they do not capture the dynamics of protein expression determined by synthesis and degradation. Therefore, quantitative labeling protocols based on pulsed stable isotope labeling have been introduced to examine protein synthesis and turnover (20–24). Unfortunately, these approaches are hampered by their low temporal resolution, which is limited by the considerable level (5% to 10%) of stable isotope label that needs to be incorporated before protein synthesis can be determined reliably (25). In such cases, affinity purification of newly synthesized proteins has been recently facilitated by pulse labeling with noncanonical amino acids. Specifically, the replacement of methionine by its azide-bearing analog azidohomoalanine (AHA) allows the enrichment of newly synthesized proteins via click-chemistry using alkyne-functionalized beads (26, 27). The combination of this enrichment approach with stable isotope labeling methods allows the quantitative analysis of proteome dynamics, providing insight into the regulation of protein synthesis (28). Recently, we have demonstrated the utility of combining pulsed AHA and pulsed SILAC labeling for the detection and quantification of newly synthesized proteins in the secretomes of a range of cell types, including macrophages (29).

In the current study, we extended the use of this approach to probe proteome changes that are induced on a rapid time scale upon cellular stimulation. In a time course experiment, over the first 3 h of LPS-induced macrophage activation we determined expression profiles of transcripts, proteins, newly synthesized proteins, and secreted proteins.

Importantly, integration of these different datasets allowed us to gain insight into patterns and mechanisms of protein translation and export, and we observed negative feedback loops, increased protein degradation and turnover, and proteolytic ectodomain shedding of transmembrane proteins as integral aspects of macrophage activation.

**EXPERIMENTAL PROCEDURES**

Cell Culture—RAW 264.7 macrophages were grown at 37 °C in 5% CO₂ in DMEM (Invitrogen) with 10% FBS (Invitrogen), 100 mg/l Primocin (InvivoGen, San Diego, CA), and 4 mM/l L-glutamine (Invitrogen) added until ~70% confluence (~1 × 10⁶ cells for pSILAC experiments and ~0.5 × 10⁶ cells for full SILAC experiments). Cell viability was determined with trypan blue staining (Invitrogen 15250–061) in independent biological triplicates.

Pulse-labeling with AHA and SILAC—For complete SILAC labeling, RAW 264.7 cells were grown in SILAC medium (DMEM non-GMP formulation without methionine, arginine, and lysine; Invitrogen) supplemented with 100 mg/l Primocin, 4 mM/l L-glutamine, 10% dialyzed FBS (Invitrogen), 30 μg/ml methionine, and stable-isotope-labeled amino acids (84 μg/ml [¹³C₆]L-arginine and 146 μg/ml [¹³C₆,¹⁵N₄]L-lysine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) or the natural versions of these amino acids).

For the enrichment experiments, cells were depleted of methionine, lysine, and arginine by 30 min of incubation in depletion medium (DMEM non-GMP formulation without methionine, arginine, and lysine; Invitrogen) with 10% dialyzed FBS (Invitrogen), 4 mM/l L-glutamine, and 100 mg/l Primocin before incubation in the same medium supplemented with 0.1 mM L-AHA (AnaSpec, Inc., Fremont, CA). In the case of pSILAC, either 84 μg/ml [¹³C₆,¹⁵N₄]L-arginine and 146 μg/ml [¹³C₆,¹⁵N₄]L-lysine or 84 μg/ml [¹³C₆]L-arginine and 146 μg/ml [⁴,⁴,⁵,⁵-D₄]L-lysine (Cambridge Isotope Laboratories, Inc.) was added. RAW 264.7 cells were stimulated with 100 ng/ml LPS (Escherichia coli O111:B4; Sigma) for the indicated times. All assays were performed as independent biological duplicates with reversed SILAC labels. Collected media was centrifuged (5 min at 1000 × g), Complete EDTA-free protease inhibitor (Roche) was added, and samples were frozen at ~80 °C.
Cells were washed three times with warm PBS, detached using a cell scraper, and centrifuged (5 min at 1000 × g). After removal of the PBS, the cell pellets were frozen at −80 °C or directly subjected to cell lysis.

**Enrichment of Newly Synthesized Proteins and On-bead Digestion—** Newly synthesized proteins from concentrated media (Amicon Ultra® Centrifugal Filters, 3-kDa cutoff, Millipore, Billerica, MA) (250 μl) or cell lysates were enriched using the Click-iT® Protein Enrichment Kit (Invitrogen C10416), applying the vendor’s protocol with slight modifications. The enrichment from conditioned media was performed as described previously (29). Cells were lysed in urea buffer for 15 min and then sonicated (three times for 10 s each time with 1 min of cooling in between), agitated for 5 min, and centrifuged and then subjected to the catalytic reaction using 200 μl of agarose resin slurry. After the resin was washed with 900 μl water, 1 ml SDS buffer (supplied with the kit) and 10 μl 1 M DTT were added and agitated at 70 °C for 15 min. The supernatant was aspirated, 3.7 mg iodoacetamide in SDS washing buffer was added, and samples were incubated for 30 min in the dark.

The resin was transferred to a spin column (supplied with the kit) and washed with 20 ml of SDS buffer, 20 ml of 8 M urea/100 mM Tris/HCl pH 8, 20 ml of 20% acetonitrile. After the resin had been dissolved in digestion buffer (100 mM Tris/HCl pH 8, 2 mM CaCl₂, 10% acetonitrile), 0.5 μg of trypsin was added and samples were incubated overnight at 37 °C. The peptide solution was collected and the resin was washed with 500 μl of water. Both solutions were combined and acidified with 20 μl of 10% CF₃COOH.

**Sample Preparation for Mass Spectrometry—** Digestion of total cell lysates was performed according to a previously published protocol (30), with slight modifications. The concentration of DTT was adjusted to 0.1 M. After being heated to 95 °C for 5 min, the samples were cooled to room temperature and concentrated using Amicon Ultra® centrifugal filters (0.5 ml, 3-kDa cutoff, Millipore). 200 μl of 8 M urea in 0.1 M Tris/HCl pH 8.5 were added and concentrated. After alkylation using iodoacetamide (0.05 μg in 8 M urea/0.1 M Tris/HCl pH 8.5), proteins were digested overnight by 0.5 μg of endopeptidase Lys-C (Wako, Richmond, VA) in 40 μl of 8 M urea in 0.1 M Tris/HCl pH 8.0. Then trypsin (0.5 μg in 120 μl of 50 mM ammonium bicarbonate) was added and samples were incubated at room temperature for 4 h.

The acidified samples (10% CF₃COOH) and the samples prepared via on-bead digestion were desalted using Sep-Pak® cartridges (Vac 1 cc (50 mg) C₁₈, Waters, Milford, MA) as described elsewhere (31). The desalted peptide samples were either directly analyzed via nano-LC-MS/MS or fractionated using isoelectric focusing (cell lysates and conditioned media) or strong anion exchange chromatography (conditioned media). For isoelectric focusing, an Agilent 3100 OFFGEL Fractionator was used in combination with Immobiline TM DryStrips (pH 3–10 NL, 13 cm, GE Healthcare). Focusing was performed at a constant current of 50 mA with a maximum voltage of 4000 V. After reaching 20 kVh, the samples were collected, acidified with CF₃COOH, and desalted using StageTips (32). For media samples, 12 fractions were combined into 6 fractions. The peptide samples were dried and dissolved in 4% acetonitrile, 0.1% formic acid. Alternatively, strong anion exchange was used for the fractionation of the samples into six fractions, essentially as described elsewhere (33).

**LC-MS/MS—** Peptides were separated using a nanoAcquity UPLC system (Waters) fitted with a trapping (nanoAcuity Symmetry C₁₈, 5 μm, 180 μm × 20 mm) and an analytical column (nanoAcuity BEH C₁₈, 1.7 μm, 75 μm × 200 mm). The outlet of the analytical column was coupled directly to an LTQ Orbitrap Velos or Orbitrap Velos Pro (Thermo Fisher Scientific) using a Proxeon nanospray source (solvent A: 0.1% formic acid; solvent B: acetonitrile, 0.1% formic acid). Samples were loaded with a constant flow of solvent A at 15 μl/min onto the trapping column. Peptides were eluted via the analytical column at a constant flow of 0.3 μl/min. During the elution step, the percentage of solvent B increased in a linear fashion from 3% to 25% in 40 min, 110 min, or 210 min followed by an increase to 40% in 4 min, 10 min, or 5 min and an increase to 85% in 0.1 min, 1 min, or 1 min for the different gradient lengths. The peptides were introduced into the mass spectrometer via a Pico-Tip Emitter (360 μm outer diameter × 20 μm inner diameter, 10 μm tip, New Objective, Woburn, MA). Full-scan MS spectra with a mass range of 300–1700 m/z were acquired in profile mode with Fourier transform MS with a resolution of 30,000. The filling time was set at a maximum of 500 ms with a limitation of 10⁵ ions. The most intense ions (up to 15) from the full-scan MS were selected for fragmentation in the LTQ. A normalized collision energy of 40% was used, and the fragmentation was performed after the accumulation of 3 × 10⁵ ions or after a filling time of 100 ms for each precursor ion (whichever occurred first). MS/MS data were acquired in centroid mode. Only multiply charged (2+, 3+) precursor ions were selected for MS/MS. The dynamic exclusion list was restricted to 500 entries with a maximum retention period of 30 s and a relative mass window of 10 ppm. Lock mass correction using a background ion (m/z 445.12003) was applied.

**Sample Preparation for mRNA Expression Measurement—** Approximately 1 × 10⁶ RAW 264.7 cells were prepared for mRNA expression measurements and stimulated for 1, 2, and 3 h with 100 ng/ml LPS in DMEM (Invitrogen) with 10% FBS (Invitrogen), 100 mg/l Primocin (InvivoGen), and 4 μM L-glutamin (Invitrogen) (70% confluence) or left untreated. Each experiment was performed in triplicate. For RNA purification, the RNesy Plus Mini Kit (Qiagen, Hilden, Germany) was used according to the vendor’s protocol with cell lysis directly in the cell plates and homogenization using a QIAshredder (Qiagen).

**mRNA Expression Measurement—** Analysis of mRNA expression was performed using a GeneChip® Whole Transcript (WT) Sense Target Labeling Assay from Affimetrix, Santa Clara, CA. All sample preparation and measurement procedures were performed according to the vendor’s protocol. An Affimetrix exon expression chip (MoGene-1_0-st-v1) was used for hybridization and expression measurement.

**Dot Blot and Western blot—** For dot blot and Western blot analysis, the cells were suspended in 150 μl of 1% SDS in 50 mM Tris/HCl, pH 8.0, supplemented with Complete EDTA-free protease inhibitor (Roche) and lysed by sonication on ice (three times for 10 s each time). After centrifugation (13,000 × g for 5 min at 4 °C), protein in the supernatant was either directly labeled with biotin-alkyne or precipitated and stored at −20 °C. Labeling with alkyne-biotin was performed using the Click-iT™ Biotin Protein Analysis Detection Kit (Invitrogen C33372), applying the vendor’s protocol including protein precipitation.

For dot blots, the sample pellets were dissolved in 50 μl of 1% SDS in PBS. 200 μl of sample dilutions in PBS and a BSA dilution series were prepared for blotting on a nitrocellulose membrane. For SDS-PAGE prior to Western blotting, samples were mixed with 4× loading buffer (1% SDS, 40% glycerol, 20% β-mercaptoethanol, 150 mM Tris/HCl pH 6.8, 0.004% bromphenol blue) and heated for 5 min at 95 °C. Protein separation was performed using 12% SDS-PAGE gels with 4% stacking gel at a constant voltage of 140 V.

For Western blots, samples were incubated with anti-biotin antibody (ab53494, Abcam, Cambridge, UK) 1:10,000 and the secondary antibody ECL rabbit IgG HRP-linked whole Ab (from donkey) (NA934VS, GE Healthcare) 1:30,000. Proteins were detected using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore).

**Electron Microscopy—** Cells were fixed, dehydrated, and embedded in resin using a Ted Pella Biowave microwave. Briefly, cells were fixed in 2.5% glutaraldehyde for 30 min at 37 °C by adding equal amounts of a 5% glutaraldehyde solution in 50 mM cacodylate buffer with 50 mM KCl, 2.6 mM MgCl₂, 2.6 mM CaCl₂, and 2% sucrose to the
images were acquired at 3400x/H11003 surface of the block). Starting at a random position on the grid, image spacing of 10 μm was used. The thin sections were rotary shadowed with platinum/carbon and imaged using a Zeiss 902 cold stagescope (FEI, Eindhoven, The Netherlands) operated at 120 kV and equipped with a Cool CCD camera (Soft Imaging System, Muenster, Germany).

For the quantification of endosomal/lysosomal features, a sampling hierarchy was designed to ensure unbiased collections of images. Briefly, five and six independent culture dishes were prepared for control and LPS-treated cells. From each dish, two resin blocks were produced, from which one section was taken (2 to 3 μm below the surface of the block). Starting at a random position on the grid, images were acquired at 3400x magnification following a systematic and uniform scheme (according to Luccozq and Hacker (34)), with an image spacing of 10 μm. Frames containing no cell or frames occupied more than 80% by the nucleus were omitted in the counts. For each condition (control and LPS-stimulated macrophages), 100 images were considered.

Using a stereology approach and the point-counting method (34), the fractional volume of each endosomal/lysosomal compartment in the cytoplasm was estimated. The results are expressed as the average fractional volume for each compartment in the cytoplasm and for each condition.

Data Analysis—The mass spectrometric raw data were processed using MaxQuant (version 1.2.2.5) (35), and MS/MS spectra were searched using the Andromeda search engine (36) against mouse proteins in the UniProt database (53,623 entries, downloaded June 21, 2011) (37), to which 265 frequently observed contaminants and truncated versions of the proteins containing non-tryptic peptides were added. Cysteine carbamidomethylation was used as a fixed modification, and methionine oxidation, protein N-terminal acetylation, and replacement of methionine by AHA (in the case of AHA treatment experiments) were used as variable modifications. The minimal peptide length was set to six amino acids. The initial maximal allowed mass tolerance was 20 ppm for peptide masses, followed by 6 ppm in the main search and 0.5 Da for fragment ion masses. False discovery rates for peptide and protein identification were set to 1%. At least one unique peptide was required for protein identification. The protein identification was reported as an indistinguishable "protein group" if no unique peptide sequence matching a single database entry was identified.

For protein quantification, a minimum of two ratio counts was set and the "requantify" and "match between runs" functions were enabled. For pSILAC samples, a protein group was kept for further analysis if the number of identified peptide species carrying a median-heavy or heavy label divided by the total number of peptide species detected in the complete experimental setup was more than 0.2. For all other samples, proteins assigned to contaminants or reverse sequences were removed.

Average protein ratios were reported if they were quantified in two replicates each based on at least two ratio counts. Data are available via ProteomeXchange (38) with the identifier PXD000600.

To align transmembrane domains, the first majority protein group I.D. having a transmembrane domain assigned in UniProt was selected as the identifier. Of all transmembrane-containing proteins, those annotated (Gene Ontology term) as residing in the plasma membrane, as well as the ones with no annotation, were manually selected. Furthermore, only proteins that were identified with at least one MS/MS spectrum were kept. "More I.D.s with LPS" or "more I.D.s untreated" were defined by MS/MS spectral counting. The log2 ratio of spectral numbers between LPS-treated and untreated samples was required to be greater than 0.75 or less than −0.75 and a minimal difference in the numbers of spectra of three was required in order for one of these terms to be assigned; otherwise the peptide was assigned to the "no difference" category. In the cleavage evidence, "potential cleavage" refers to proteins detected as targets of enzymes in large-scale enzyme interaction studies.

Evidence for protein processing was obtained from a two-step database search to identify and validate nontryptic peptides. First, the secretome MGF files prepared using MaxQuant (version 1.3.0.5) were searched in Mascot (version 2.2.0.7) against the UniProt mouse database, allowing one missed cleavage, using trypsin as the enzyme, setting a peptide tolerance of 5 ppm and an MS/MS tolerance of 0.5, and specifying cystein carbamidomethylation and methionine oxidation as fixed and variable modifications, respectively. The resultant list of proteins was loaded into Scaffold (version 3.6.3) and then exported as a subdatabase using peptide and protein probability scores of 95% and 90%, respectively. This subdatabase was searched by submitting all unmatched spectra from the first search, now omitting an enzyme specification. Nontryptic peptides were validated by means of repeated submission of the complete MGF files to a Mascot search using trypsin as the enzyme in the original full mouse database supplemented with truncated versions of the proteins containing non-tryptic peptides. A peptide was reported only if it was detected as an N- or C-terminal peptide of the truncated protein with a minimum mascot score of 20.

Statistical Analysis—Statistical analysis of mass spectrometric data was performed using the Limma package in R/Bioconductor (39). For a linear model had been fitted to the data, an empirical Bayes moderated t test was used and p values were adjusted for multiple testing with Benjamini and Hochberg’s method. If not stated otherwise, proteins with an adjusted p value of less than 0.01 and exhibiting a minimum fold change of 2 were considered to be differentially synthesized or secreted. Prior to statistical tests, values with high standard deviations were removed if the standard deviation of biological replicates was more than 1.2 (or 1.8 for pSILAC without AHA) or if the standard deviation was greater than the absolute log2 fold change for fold changes greater than 0.6 (or 1.2 for pSILAC without AHA). These filtering conditions were not applied to conditioned media samples. Correlations between replicates were calculated in R using Pearson correlation.

mRNA expression values were analyzed using Gene Spring GX Software (Agilent, Santa Clara, CA). An unpaired t test with asymptotic p value calculation followed by Benjamini–Hochberg p value correction for multiple testing was used to define statistically significant changes in mRNA expression between LPS-treated samples and untreated samples. After background determination, all RNAs with expression values of less than 50 were excluded from the analysis. Only RNAs with an adjusted p value greater than 0.01 and a minimum fold change of 2 were considered as significantly changing.

Bioinformatical Analysis—Functional annotation enrichment was performed using DAVID (40). MetaCore (GeneGo Inc., New York, NY) (41) was used for pathway map enrichment, transcription factor target gene enrichment, and network analysis. Functional classification was performed using MetaCore. Default parameters were used in all analyses. A connection to immune response was assigned if the protein was downstream of LPS, NF-κB, or AP1 based on the curated GeneGo database or Gene Ontology annotations contained any term with "immune," "inflammatory response," or "defense response."
RESULTS

In this study we aimed to investigate the molecular events underlying LPS-induced macrophage activation by combining transcriptome and proteome profiling with the analysis of protein synthesis and secretion. Whereas for the first two we used established techniques (microarray and SILAC-based proteomics, respectively), we employed a more novel approach for the detection and quantification of newly synthesized and secretory proteins. Specifically, we combined metabolic pulse-labeling of proteins with SILAC amino acids and L-AHA, an azide-bearing analog of methionine. AHA allows the selective and covalent capture of newly synthesized proteins to an alkyne-activated resin via click-chemistry (Fig. 1A), and the SILAC label allows quantification of protein levels via mass spectrometry, at the same time providing a mark to distinguish new from preexisting proteins. RAW 264.7 cells were treated with lipopolysaccharides (LPS) for the indicated times or left untreated. Protein synthesis and secretion were determined via parallel pulse labeling with AHA and stable-isotope-labeled amino acids, followed by newly synthesized protein enrichment and quantification using mass spectrometry after on-bead digestion and peptide fractionation. For the analysis of changes in overall protein abundance, completely SILAC-labeled cells were treated equally, omitting the enrichment step. RNA abundance differences were measured using Affimertix gene expression arrays. C, incorporation speed of AHA into newly synthesized proteins. Dot blot (using anti-biotin antibody) after treatment of RAW 264.7 cells with AHA or methionine (Met). Each sample was blotted two times in two dilutions each (as indicated next to the figure). D, selective incorporation of AHA into newly synthesized proteins. Western blot (using anti-biotin antibody) of SDS-PAGE-separated proteins extracted from RAW 264.7 cells that were grown for 2 h in the presence of AHA, AHA + cycloheximide, or methionine.

Fig. 1. Experimental design. A, principle of the enrichment of newly synthesized proteins. Azidohomoalanine (AHA) is incorporated into newly synthesized proteins, replacing methionine. Newly synthesized proteins are coupled to alkyne-functionalized agarose resin by 1,3-cycloaddition (click-reaction). Alternatively, labeling with a biotin-containing alkyne was used to prepare the samples for dot blot and Western blot. B, strategy for the global analysis of molecular events induced by lipopolysaccharide stimulation of mouse macrophages. RAW 264.7 cells were treated with lipopolysaccharides (LPS) for the indicated times or left untreated. Protein synthesis and secretion were determined via parallel pulse labeling with AHA and stable-isotope-labeled amino acids, followed by newly synthesized protein enrichment and quantification using mass spectrometry after on-bead digestion and peptide fractionation. For the analysis of changes in overall protein abundance, completely SILAC-labeled cells were treated equally, omitting the enrichment step. RNA abundance differences were measured using Affimertix gene expression arrays. C, incorporation speed of AHA into newly synthesized proteins. Dot blot (using anti-biotin antibody) after treatment of RAW 264.7 cells with AHA or methionine (Met). Each sample was blotted two times in two dilutions each (as indicated next to the figure). D, selective incorporation of AHA into newly synthesized proteins. Western blot (using anti-biotin antibody) of SDS-PAGE-separated proteins extracted from RAW 264.7 cells that were grown for 2 h in the presence of AHA, AHA + cycloheximide, or methionine.
pSILAC and pAHA Labeling Allows the Detection of Proteome Changes on a Rapid Time Scale—A prerequisite for the exact measurement of differences in protein synthesis during cellular signaling events is the rapid incorporation of AHA and SILAC amino acids. We detected newly synthesized proteins after only 5 min of AHA treatment (Fig. 1C), and across a wide range of molecular weights (Fig. 1D), indicating fast and proteome-wide incorporation of AHA only into newly synthesized proteins, thus demonstrating the applicability of the approach for this model system. Rapid SILAC labeling was demonstrated by the quantification of 4852 intracellular and 32 extracellular proteins after only 20 min of AHA and pSILAC incorporation (Table I, Fig. 2A). All of these proteins were identified carrying a SILAC label, proving that they were newly synthesized in this short time frame (20 min) (details below).

The incorporation of AHA into tRNA is slower than the introduction of methionine (42); however, cell viability was not affected after up to 4 h of AHA treatment (supplemental Fig. S1A). Most important, we did not detect any difference in proteome response in cells in the presence of AHA or methionine after LPS stimulation (supplemental Fig. S1B). Finally, the replacement of methionine by AHA and subsequent enrichment did not result in any bias in protein properties (e.g. methionine content, hydrophobicity, molecular weight) (supplemental Fig. S1C).

Next, we assessed how our approach, combining pSILAC with AHA labeling and protein enrichment, compared with pSILAC alone in its ability to detect differences in levels of newly synthesized proteins. Among ~5000 proteins quantified in LPS-activated macrophages that were pulse-labeled with SILAC for 2 to 3 h, none met our criteria of both a 1% false discovery rate and a fold change of >2 that would indicate differential synthesis (Figs. 2F and 2G). In contrast, when we included protein enrichment via AHA, we detected 189 and 246 differentially synthesized proteins after 2 and 3 h of LPS treatment, respectively (Figs. 2C and 2D). This was achieved by improved signal-to-noise ratios due to enrichment resulting in an increase of the correlation between replicates from 0.05 and −0.33 (without enrichment; Figs. 2F and 2G) to 0.84 and 0.86 (with enrichment; Figs. 2C and 2D). In addition, 35 proteins reached significance after only 1 h of LPS stimulation (Fig. 2B). This demonstrates that the selective enrichment of AHA-containing proteins greatly improves the sensitivity for the detection of differences in protein synthesis, enabling the detection of proteome responses on a rapid time scale.

### Table I

| time   | protein synthesis | secretome | protein abundance | RNA abundance |
|--------|-------------------|-----------|-------------------|---------------|
| 20 min | 4852 (8)          | 32 (0)    | 4042 (0)          | 7972 (79)     |
| 60 min | 4914 (35)         | 65 (24)   | 3508 (2)          | 7972 (79)     |
| 120 min| 5380 (189)        | 325 (41)  | 3355 (17)         | 7972 (380)    |
| 180 min| 5268 (246)        | 182 (95)  | 3357 (7)          | 7972 (424)    |

**Time Course Analysis of Macrophage Activation by Combining Transcriptome, Proteome, Protein Synthesis, and Secretome Profiles—**To obtain a detailed molecular understanding of the macrophage response during the first 3 h of activation by LPS, we complemented the protein synthesis data with a transcriptome analysis, a proteome analysis using full SILAC (to assess changes in overall protein levels), and a secretome analysis of conditioned media. The latter was performed utilizing pAHA and pSILAC labeling. Each of these analyses was performed in biological duplicate across the three time points (1, 2, and 3 h) (Fig. 1). In the transcriptome analysis, out of 11,660 detected transcripts, only those 7972 RNA expression values that mapped to our total list of 9131 identified proteins were used (supplemental Table S1). Combining all datasets, among the 9131 identified proteins, 782 showed a significant difference in at least one of the performed experiments. In all datasets, an increasing number of changing proteins/transcripts was detected after prolonged LPS stimulation, reflecting expansive macrophage activation. In absolute numbers, approximately twice as many transcripts as proteins changed significantly (Table I). On a relative scale, the proportion of changing secreted proteins was much higher than for the other datasets (e.g. 50% at 3 h), pointing to a strong effect of LPS on the macrophage secretory response (Table I). Of note, the enrichment enabled the detection of secreted proteins in growth media containing 10% serum, without the need for serum starvation, a condition that reduced macrophage viability and strongly perturbed the LPS-induced secretion pattern (supplemental Fig. S2). Notably, only a very small number of proteins changed significantly in overall protein abundance (Table I). There are at least three factors that may explain these observations. First, the enrichment strategy specifically augments the signal of newly synthesized proteins because it depletes existing (unlabeled) protein, thereby improving the capability for quantifying changes in protein synthesis rather than total protein abundance. Thus, the changing part of the proteome is targeted while the background ofunchanging proteins is excluded, leading to increased sensitivity. Second, because fewer proteins were quantified in the full proteome analysis, low-abundance proteins probably remained undetected. Third, a parallel increase or decrease in protein synthesis and degradation could lead to constant total protein levels, even though the levels of newly synthesized proteins might vary.

**Functionality of Proteins with Shared Temporal Expression and Secretion Profiles—**To compare general trends in transcriptome, newly synthesized proteome, and secretome data, we computed Pearson correlations between these datasets at each of the three time points (supplemental Fig. S3). The overall correlation (0.4 to 0.5) was in agreement with previous studies integrating mRNA abundance with protein abundance.
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datasets, although a wide range of correlation values has been reported (0.3–0.7) (43–46).

To systematically investigate trends in expression regulation, we derived temporal profiles for gene expression, protein synthesis, and protein secretion for the transcripts/proteins that showed a significant change in at least one time point. Profiles were then grouped by similarity, first populating three large groups (Fig. 3), termed “early induced” (showing an increase, meaning at least a log2 fold change of 0.75 in protein synthesis or RNA abundance, already after 1 h of LPS stimulation), “late induced” (showing increased protein synthesis or RNA abundance after 2 or 3 h of LPS stimulation), and “reduced” (showing reduced protein synthesis or RNA abundance).

Gene Ontology analysis revealed that “early induced” and “late induced” proteins were enriched in the expected terms for inflammation and response to LPS stimulation, such as “inflammatory response,” “toll-like receptor signaling pathway,” “regulation of cytokine production,” “apoptosis,” and “inflammatory response” (supplemental Table S1). Interestingly, among the proteins that are known to propagate the LPS-induced inflammatory response (e.g., Cc32, Stap1, Swap70, Jarid2, Ikbke), several proteins were induced that counteract this process. These included direct inhibitors of NF-kB (Nfkbid and Nfkbiz) and proteins that negatively regulate transcription or transcript stability of either NF-kB or its targets, such as Nr4a1 (47), Socsc3 (48), Zfp36 (Tristetraprolin) (49), Ier3 (50), and Cttnbn1 (β-catenin) (51). Strikingly, the latter two, together with Tnf, showed increased protein synthesis after only 20 min (Fig. 2E). This indicates that pro- and anti-inflammatory proteins are induced simultaneously from the early onset of the LPS response, and that these rapidly induced proteins can be effectively captured via our approach.

Of note, among the proteins exhibiting “reduced” kinetics, only a minor proportion have been associated with inflammatory response (green symbols in Fig. 3). Indeed, functional annotation for all proteins in this group resulted in very different enriched terms (supplemental Table S1), primarily associated with “chromosome organization” and “transcription regulation” (e.g., Taf1b, Elf2, Il16, E2f7, Lmo4, E2f8, Pax6,

Fig. 2. Quantification of differences in protein synthesis. Plots indicate log2 fold changes of two biological replicates comparing LPS-treated cells to untreated control cells. A–D, comparison of protein synthesis with and without LPS treatment for the indicated times using pSILAC in combination with AHA labeling and enrichment of newly synthesized proteins. E, as in A, highlighting the proteins with significant changes after 60 min of LPS treatment from B. F, comparison of protein synthesis with and without LPS treatment for the indicated times using pSILAC. Gray dots represent proteins quantified with high standard deviation and therefore excluded from significance tests.
Trib3, Elk3, Nr1d2, Hbp1, Bcl6, Rab9, Bhle41, Asf1b, Thap11, Txnip, Klf13, Cebpg, Rbl1, Atad2), as well as “cell cycle,” pointing to a repression of specific transcriptional programs and the reduction of proliferation, which has been reported before (52, 53). The reduction of several proteins involved in cell cycle progression (e.g. Cdk2, Cdc25a, Cdc25b, Orc5) in combination with the induction of some of its inhibitors (e.g. Mdm2, Myc, Ccnd1) is visualized in the KEGG pathway map (17 mapped genes, corrected p value: 3.1 × 10^{-10}) (supplemental Fig. S4). Therefore, the inhibition of cell cycle activity appears to be an integral and immediate effect of macrophage activation.

In addition, among both up-regulated proteins (e.g. Rgl1, Lamc1, Flnb, Cpeb4, Dusp16, Hmga2, Homer1, Vps37c, Sile4a7, Arl5b, Csnnp1, Eif2, Tgm2, Hexim1, Phlda1, and Ehd1) and down-regulated proteins (e.g. Lyt1; the Kelch-like proteins Kih16, -24, -25, and -30; and Zfp161, a transcriptional repressor of Myc) were many that have not been associated with immune response that might represent novel candidates with possible functions in macrophage activation (supplemental Table S1).

Mechanistic Insights Obtained from Temporal Patterns of Gene Expression, Protein Synthesis, and Secretion—Next, groups of profiles were further subdivided based on temporal differences in mRNA abundance and protein synthesis/secretion. This produced 14 categories (A to N), each containing proteins/transcripts with highly similar expression/secretion patterns (Fig. 3). Proteins within each category were then examined for shared functional properties and for regulatory mechanisms at the level of transcription, translation, or secretion. Category A consisted of 24 proteins that were rapidly induced in response to LPS stimulation on both the protein and the RNA level (supplemental Table S1), encompassing the major hallmark proteins of M1 macrophage activation.
(Jun, Fos, Tnf, Ccl2, and Cxcl10), as well as some of its inhibitors as mentioned above (Ier3, Zfp36, and Nfkbia). Both categories were also firmly represented in Category B (e.g. Nfkbia, Ikbke, Rel, Gadd45, Il1b indicating activation, and Socs3 indicating counteracting inflammation). The increase in protein synthesis of the 40 proteins in this category showed a delay relative to RNA abundance changes, suggesting suspended translational activity compared with category A. Category C contained proteins that did not show a change in abundance or synthesis, despite an increase in transcript levels. This may be explained by stalled translation, by translation that is counteracted by simultaneous protein degradation, or by a delay in protein synthesis beyond the 3-h window of this experiment. Interestingly, several proteins in this category, including Tgbf and Ldr, showed an increase in protein secretion, suggesting that they were externalized immediately upon synthesis. Members of categories D and E showed a peak in mRNA expression at 1 h that resulted in increased and sustained protein synthesis in category D but no observable effect at the protein level in category E. Seven of the 21 proteins in category D were transcription factors, including Btg2, Jun, Fos, and all early growth response proteins (Egr1, Egr2, and Egr3) detected in this experiment. An increase in protein synthesis followed by a reduction in transcription is indicative of regulation by negative feedback, which has been shown for various transcription factors (54), either in a direct autoregulatory fashion as for Fos (55) or indirectly by proteins destabilizing specific mRNAs. Such a function has been shown for Zfp36 (Tristetraprolin), which is strongly induced by LPS (category A in Fig. 3), targeting a wide range of proteins including serine/threonine-protein kinase PLK3 (Plk3) (56) and immediate early response gene 5 protein (Ier5) (57), both of which were members of category D. One may postulate a similar mode of negative feedback regulation for the other proteins in this category, many of which (13 out of 21) have been linked to inflammation before (supplemental Table S1). This would be in line with the recent finding that many proteins involved in immune response have unstable RNAs but stable proteins (46). Category F consisted of 70 proteins for which protein synthesis and mRNA levels largely coincided (with a delay relative to category A), suggesting that protein expression was controlled at the transcriptional level. Similarly, both transcripts and proteins in category I go down following comparable kinetics. Interestingly, overall transcript levels in categories G and J did not change, but protein synthesis was markedly increased or decreased, respectively, indicating that protein expression was regulated at the post-transcriptional level. Furthermore, these effects clearly exceeded the abundance change for the proteins that were detected in the full-SILAC experiment, indicating a minimal change in overall protein abundance (i.e. not measurable by SILAC) or, alternatively, a change in protein turnover. This was particularly evident for 12 proteins in category G and 3 in category J (supplemental Fig. S5). Interesting cases in category G are Ubc (poly-ubiquitin) and Rps27a, the precursor of both ubiquitin and 40S ribosomal protein S27a (58). Ubiquitylation plays a central role in the regulation of NF-κB signaling, not only by targeting proteins for proteasomal degradation, but also as a signaling tag (59). Other examples are β-catenin (mentioned before), Fosb, Jak1, Eph2a, Relb, Sin3b, Brd2 (category G), Bci6, E2hf, Il17ra, and Ifnar2 (category J), indicating that crucial signaling proteins and transcription factors are subject to positive and negative post-transcriptional control.

In contrast to categories G and J, transcripts in categories H and K showed a marked increase or decrease, respectively, but without a concomitant change in protein synthesis. The most likely explanation is a delay in protein translation with the main effect beyond the 3-h window of our experiment. This is supported by the trend in protein synthesis at the 3-h time point that is upward in category H (average log2 ratio 0.343, S.D. 0.383) and downward in category K (average log2 ratio −0.289, S.D. 0.318). This tendency was amplified at 8 h (average log2 ratio 0.704, S.D. 0.581 and average log2 ratio −0.622, S.D. 0.817 in categories H and K, respectively). The functionality of the 225 transcripts in category K is associated with chromatin (functional annotation clustering score: 4.2), including most of the detected histones (Prim1, Dsn1, H1f0, Chek1, Rfc4, Xpo1, Hist1h1a, Hist1h4a, Hist2h2a, Hist2h2aa1, Hist1h2b3, Ncapd3, Hist1h2bf). In addition, seven proteins exhibit “GTPase regulator activity” (score: 2.0) (e.g. Fdg4, Rasa3, Racgap1, Wdr67) and nine proteins are associated with the functional term “cell cycle” (score: 1.8) (e.g. Chek2, Dsn1, Rassf2, Anln, Chek1, Kif11). The 101 mRNAs in category H are enriched in “immune system development” (score: 2.96), “immune response” (score: 2.66), “cell death” (score: 2.05), and “response to organic substance” (score: 2.04). This category also includes IL6 and IL23a, which indeed are known to be secreted after immediate-response cytokines such as Tnf (60). Overall, the expression profiles in category H, as well as the functionalities of the proteins, seem to indicate that these proteins are not in the forefront of the LPS response and instead embody secondary effects to fine-tune or dampen this response.

Categories M and N are the only two in which changes in transcript and protein levels showed an anticorrelation. The fact that combined these categories contained only 17 proteins illustrates that this happens only in a minority of cases. Still, they included some important players in LPS response. For instance, NF-κB inhibitor α (Nfkbia) and Cdc25a showed increased transcription but a decrease in protein synthesis. Nfkbia traps NF-κB/REL dimers in unstimulated macrophages and is degraded in response to TLR signaling (61). The maintenance of high transcriptional activity might represent a mechanism to rapidly counteract NF-κB signaling to avoid overactivation that would be damaging for the host. Similarly, ubiquitin-mediated proteolysis of M-phase inducer phosphatase 1 (Cdc25a), resulting in delayed cell-cycle progression.
(62), accompanied by continued transcription may preserve the ability to quickly produce Cdc25a. A similar mechanism might apply to Zcchc2 and Irf2bpl in category N. Reduced RNA expression in combination with induced protein synthesis (category M) was observed for only 11 proteins with diverse functions ranging from apoptosis and stress response to transcriptional regulation (e.g., Atf4, Chac1, Gch1, Hpgds, Ndg1, Sesn2), suggesting enhanced protein synthesis. Finally, an interesting category was formed by proteins that were newly synthesized and secreted without an appreciable change in either transcription or intracellular protein synthesis (category L). The observed pattern suggests that translation was induced upon LPS stimulation and followed by the immediate secretion of newly synthesized proteins. Of the 67 proteins in this category, 24 carry a signal peptide or are annotated as secreted in UniProt, and 25 proteins are established factors in immune response (supplemental Table S1). Strikingly, the secreted proteins in this category are externalized by various routes. For instance, unconventional secretion has been established for four proteins in this group (Mif, Ybx1, Lgals1, and Lgals3) (63–65). Furthermore, functional annotation of proteins in category L assigned the highest enrichment score (7.54) to a cluster containing the terms “vesicle” and “pigment granule,” including App, Capg, clathrin, Coro1a, Gpnmb, Hsp90ab1, Hsp90b1, Prdx1, Hspd1, Hsp90aa1, P4hb, Pdia, Sdcbp, Ywhae, and Ywhaz. All of these are proteins known to be secreted via melanosomes. Strikingly, 18 out of the 782 proteins in our dataset are associated with the Gene Ontology term “melanosome,” 15 of which are in category L (supplemental Table S1). A third cluster within category L contained lysosomal proteins (Atp6ap1, Arsb, Ctsb, Cltc, Gusb, Ifi30, Napsa, Psap, Srgn, Ppia), similarly pointing to an LPS-dependent induction of vesicular protein release, but without the detection of the major lysosomal enzymes. These observations are in line with a recent study (66) in which an increase in lysosomal proteins appearing in the secretome of macrophages was observed without a concordant increase in mRNA expression. We used electron microscopy to perform a quantitative analysis comparing the fractional volumes of endosomes and lysosomes, but we failed to observe a difference between LPS-activated and control macrophages (supplemental Fig. S6). A fourth group in category L consisted of plasma membrane proteins (e.g., Ca12, Alcam (CD166), Eno1, H2-D1, H2-K1, Havcr2, Hyou1), most of which have been associated with macrophage activation before. Finally, a remaining set of proteins had main functions in the cytosol with no direct evidence of being secreted (i.e., lacking a signal peptide). However, a large proportion of these may be secreted via exosomes. For example, 49 proteins, representing 51% of the proteins with this profile, have been observed in exosomes based on the curated database ExoCarta (67), and 36 of these do not carry a signal peptide. This represents a clear enrichment of exosomal proteins in category L when comparing to the assignment of only 149 (34.7%) of all 429 proteins detected in our supernatant of mouse macrophages in this database (supplemental Table S2).

The Role of Transcription Factors in LPS Response—Transcriptional regulation of gene expression is one of the main mechanisms by which a cell responds to changing environmental conditions, putting transcription factors at center stage. Overall, we detected 424 transcription factors, 53 of which (14%) changed in abundance, representing one of the most prominently regulated classes of proteins (supplemental Fig. S7). We found 36 transcription factors that increased in abundance, 30 of which have an established function in inflammation, including 25 downstream of LPS activation. This includes several robustly induced members of the NF-κB and AP1 transcription factor complexes (Rel, Nfkb1, Nfkb2, Relb, Junb, Fosl1, Fosl2, Jun, Fos, Jund, Fosb) but also Atf3 (68), Egr1/2/3, Ets2, Cebpd (10), Irf7, and Irf4 (Fig. 4). Of the 17 proteins with reduced expression, only 5 are established inflammatory proteins (Fig. 4). Several of the induced transcription factors with unknown functions in immune response are involved in cellular stress response or apoptosis, both of which are well known to be activated by LPS (69) (e.g., Csmrp1, Csmrp2, Maff, Atf4, and Plag(2)). In contrast, many of the transcription factors with reduced expression or synthesis are transcriptional inhibitors (Tcfap4, E2f7, E2f8, Hbp1, and Bhlhe41), most likely indicating that active transcription of their target genes is required for the LPS response.

To further investigate the correlation between the expression of transcription factors and their target genes, we performed a transcription factor target enrichment applied to the mRNAs and proteins showing a significant change within our dataset. At the transcriptional level, strong enrichment of many NF-κB target genes was found after only 1 h of LPS stimulation (Fig. 5). Lower target gene enrichment was observed at the protein level, but this was enhanced from 2 h onward (Fig. 5), most likely reflecting the time needed for protein synthesis. The members of the AP1 transcription factor family exhibited moderate enrichment of target proteins at all time points.

For several transcription factors, a strong enrichment of target genes was observed on the RNA level at 1 h after stimulation, but the enrichment score at later time point decreased (e.g., Jun and three members of the ATF transcription factor family). This coincides with the observations reported by Bhatt et al., who scanned for transcription factor binding motifs in RNA expression data obtained from lipid A-stimulated macrophages (12). Similarly, the observed pattern for STAT family members, being enriched across all time points, matched their observations.

Strong enrichment of downstream targets at the protein level at the 1-h time point was found for only a few transcription factors, including Tcfap4, Myc, Srf, Elk3, and Elk4. Thus, these transcription factors may be involved in the regulation of early responses to LPS stimulation. ETS domain-containing protein Elk-3 (Elk3) and activator protein 4 (Tcfap4) protein synthesis was reduced after 3 h of LPS stimulation.
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**Fig. 4.** Regulated protein classes in LPS-activated macrophages. Significant proteins are sorted by protein function assigned in MetaCore (41) and by regulatory pattern. Proteins above the dotted lines could be assigned to "immune response" based on the following parameters: regulation downstream of LPS, AP1 or NF-κB described in the MetaCore database, or any Gene Ontology term containing "immune," "defense response," or "inflammatory response."

(supplemental Table S1), possibly indicating a suppression of inflammatory responses at prolonged times after infection, known as endotoxic tolerance (70).

Target proteins downstream of transcription factor E2F1 (E2F1) were enriched across the complete 3-h time course. Notably, transcription factors E2F7 and E2F8 were reduced after 3 h of LPS stimulation. As both E2F7 and E2F8 inhibit E2F-dependent transcription in a synergistic fashion, in turn repressing E2F1-dependent apoptosis (71), their down-regulation may promote ongoing E2F1-dependent transcription. Together, the high percentage of regulated transcription factors and the tight temporal regulation of their target genes in our data demonstrate the rapid and fine-tuned response program that is induced after LPS treatment.

**Regulation of Kinase and Phosphatase Levels in Stimulated Macrophages**—The inflammatory response involves multiple signaling cascades, and indeed major changes in phosphorylation levels on many target proteins have been demonstrated in recent large-scale phosphoproteome analyses (13, 14). We identified 33 kinases and 10 phosphatases with a significant change in expression or synthesis, only 11 of which are known to be involved in immune response, including 8 that are induced in response to LPS (Fig. 4).

Rapid induction upon LPS stimulation was observed for Polo-like kinase 2 (Plk2) and 3 (Plk3) and choline kinase α (Chka), three proteins classically known to be involved in cell cycle regulation, as well as inhibitor of nuclear factor κ-B kinase subunit ε (Ikbe). Although a rapid increase in RNA abundance was observed for dual-specificity mitogen-activated protein kinase kinase 3 (Map2k3), no significant change in protein synthesis was detected.

Slower induction kinetics were observed for Stk40, a possible negative regulator of NF-κB (72); Gk, the key enzyme in the regulation of glycerol uptake and metabolism; Brd2; Mknk2; and Jak1 and -2, which both support signal transduction of cytokine receptors lacking intrinsic kinase activity (73).

Among the kinases with an established role in immune response, reduced RNA expression or protein synthesis was detected only for Stk31, a component of the signal transduction cascade involved in NF-κB activation, and for Tk1. Reduced expression was observed for several cell cycle kinases (Cdk2, Masti, Chek1, Chek2, Clk1, and Nek7) and phosphatases (Cdc25a, Cdc25b), as well as for Stk38, an inhibitor of Map3k1 (74). Six of the seven induced phosphatases are dual-specificity phosphatases (Dusp1, Dusp2, Dusp4, Dusp5, Dusp14, Dusp16), but several additional dual-specificity phosphatases showed no regulation (Dusp3, Dusp6, Dusp7, Dusp11, Dusp12, Dusp19, Dusp22). All regulated dual-specificity phosphatases are mitogen-activated protein kinase phosphatases, which can dephosphorylate MAPKs and therefore act as antagonists of MAPK signaling cascades (75).
In conclusion, LPS activation of mouse macrophages results in the regulation of multiple kinases and phosphatases. Apart from their well-described functions downstream of TLR4, the detection of several proteins involved in cell cycle control suggests regulation of proliferation in response to macrophage activation.

Signaling and Signal Recognition Are Tightly Orchestrated in Activated Macrophages—Receptors and receptor ligands represent key functionalities in macrophage activation, and both classes were prominently regulated in our data. The interplay of receptors and their ligands is highlighted by the enrichment of the pathway “cytokine–cytokine receptor interaction” (Benjamini corrected p value: 1 × 10^{-11}) encompassing 27 significant proteins in our dataset (supplemental Fig. S8). Interestingly, induced expression of ligand/receptor pairs was observed only in TNF signaling (e.g. Tnf/Tnfrsf1b), and the remaining receptors showed reduced profiles (e.g. Il6/Il6ra and Tgifb1/Tgfb1) illustrating the pro-inflammatory conditions during the early period of LPS signaling.

In total, 44 receptors and 31 receptor ligands, including 20 cytokines and 3 growth factors, were regulated in response to LPS stimulation of macrophages. Most of them have established functions in immune response (e.g. Cd14, Sdc4, Ccr1, Icam1, Gpr84, Tnf, Cxcl10, Ccl4). In addition, several of the receptors are involved in cell adhesion (e.g. Ptporj, Itgav, Itgax, Emr1, Pvr, Icam1, Neo1, Plknb2), low-density lipoprotein binding (Msr1, Ldr, Orf1), and antigen processing and presentation (e.g. H2-L, H2-K1, H2-D1, Clec4d, Lirrb4), representing known processes highly regulated in inflammation. Among the receptors with a poorly defined role in LPS signaling are the down-regulated Toll-like receptors Tlr7 and Tlr13. Transcription of Tlr13 is inhibited by NF-κB (76), fitting with the strong induction of this protein, but Tlr7 has not been associated with LPS stimulation before.
Although most of the receptors are induced in protein synthesis only after prolonged LPS stimulation, multiple ligands exhibited very strong and fast intra- and extracellular induction (Cxc10, Tnf, Serpine1, Il1m) (Fig. 4). A delayed response to LPS stimulation was observed for Ccl4, Tnfsf9, Jag1, Ltb, Lyz2, and Csf3. Finally, Spn1, Ccl9, Ccl5, Il6, Il23a, and Ccl7 responded very slowly (8 to 19 h; information derived from our previously published dataset (29)). Interestingly, mRNA abundance in all of these cases was already regulated within 3 h of LPS stimulation, showing that translation is adapted to cellular needs, and inflammatory cytokines are synthesized more rapidly than factors with a modulating functionality.

Notably, only two signaling proteins were reduced after LPS stimulation (Il16 and Tifa) (Fig. 4). Reduction of Il16, a ligand of CD4 (77), in response to LPS stimulation could prevent cell cycle arrest, known to be induced after translocation of its N-terminal part to the nucleus (78). Alternatively, the previously described desensitization of chemokine receptors in target cells (79) like Ccr5 could be omitted. Ccr5 binds the cytokines Ccl4, Ccl5, and Ccl7 that were strongly induced in the presented dataset.

In summary, the high number of differentially expressed, synthesized, or secreted receptors and receptor ligands highlights their importance in the regulation of inflammation. The time-resolved synthesis and secretion profiles indicate individualized rather than bulk secretion of these factors, suggesting a sophisticated interplay of protein synthesis and secretion.

LPS-induced Protein Shedding from the Plasma Membrane—Because we observed many membrane proteins in the secreted fraction, we next looked for evidence in our data indicative of shedding of transmembrane proteins via proteolytic cleavage or alternative mechanisms. To systematically investigate this, we considered all 134 plasma membrane proteins detected in the secretome data and grouped them in four classes based on the positions of detected peptides in the intracellular or extracellular region (Fig. 6). Class I contained proteins with evidence for cleavage of extracellular regions, evidenced by the detection of peptides along the complete protein sequence, while peptides exclusively derived from the extracellular part of the protein were found in the secretome samples. 27 of the 30 proteins in this class have been shown or proposed before to be proteolytically cleaved, indicating the quality of the data and the utility of this classification (Fig. 6, supplemental Table S3). Class II proteins were defined by the detection solely of peptides of the external protein part (Fig. 6). Cleavage evidence has been documented for 17 of the 30 proteins belonging to this class. Class III had the same criteria as class I, but with the restriction that a maximum of two peptides were detected in the secretome samples. For 23 out of the 50 proteins in this class, proteolytic cleavage has been proposed. Finally, class IV contained 24 proteins for which peptides were found in the secretome originating from both intracellular and extracellular regions, suggesting that they may be secreted as intact protein rather than shed via proteolytic cleavage. In this class, only two proteins (Clcl1 and Abcb11) have been suggested to be cleaved, and soluble forms exist for two others (Fgfr4 and Mca32). Interestingly, especially in class I and II we found a high number of proteins (34) whose peptides were mainly detected after LPS stimulation (Fig. 6), indicating that cleavage is promoted in activated macrophages. This includes 10 proteins that were significantly induced exclusively on the secretome level (Ldr, Atp6ap1, H2-L, H2-K1, Hacr2, Ilta4, App, Gpnmb, Alcam, Mpeg1) (category L in Fig. 3). In addition, H-2T23 and Bsg served as well as four proteins assigned to class III (H-D1, Adam8, Itgb2, Il1rap), showed the same trend without reaching significance. These patterns indicate LPS-induced ectodomain shedding without a change in intracellular protein synthesis.

Ectodomain shedding in response to stimulation has been described for Ldr (80), which binds low-density lipoprotein and mediates its endocytosis. The fast release of Ldr (already detected at 1 h) may prevent the neutralization of LPS by lipoproteins that would inhibit LPS-induced antibacterial programs (81). LPS-induced secretion of Gpnmb has been shown to act as a feedback regulator of the pro-inflammatory response (82), and its shedding in melanocytes has been demonstrated to be mediated by matrix metalloproteinases (83). Furthermore, the described profile is seen for all detected H-2 class I histocompatibility proteins, which have been proposed to undergo extracellular cleavage mediated by α-secretases (84).

A second regulation pattern, combining induced secretion with reduced intracellular protein synthesis, was identified for proteins in class I (Pvr11, Tnfsf1a, Gig1, Ptprs, Pnxn2b2, Csf1r), class II (Sema4d), and class III (Il6ra). For all these proteins, extracellular cleavage either is annotated in UniProt or has been reported previously (Fig. 6, supplemental Table S3). Notably, the soluble form of semaphorin-4D (Sema4d) is the ligand of plexin-B2 (Pnxn2b2).

As ectodomain shedding is known or proposed for most of the transmembrane proteins in class I and III, this may be a universal mechanism for the release of these proteins to the extracellular space. Our experimental data validate the proposed shedding mechanisms for H-2L, H-2-K1, H-2-T23, H-2-D1, Hacr2, Itg4a4, and Sema4a, and we detected novel proteins released by means of proteolytic cleavage (Alcam, Mpeg1, Itgb2, Il1rap).

Proteolytic Processing of Secretory Proteins—Protein secretion depends in most cases on protein translocation to the endoplasmic reticulum through the presence of an N-terminal signal peptide, which is then removed proteolytically. To detect potential signal peptide cleavage sites, we created a database of secreted proteins identified in this study and used this to reanalyze our mass spectrometric data, but without specifying a proteolytic enzyme. In a two-step database search approach, 34 proteins were detected and validated containing a nontryptic peptide close to the N-terminal...
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end of the protein (Fig. 7A, supplemental Dataset S1, supplemental Table S4). Next, we compared the detected potential signal peptide cleavage sites to the annotations in UniProt (37) and to the predicted cleavage site in SignalP4.0 (85). Full agreement among annotation, prediction, and our results was found for 20 proteins. In contrast, for six proteins our experimentally determined cleavage sites were in discordance with the UniProt annotation but confirmed the predictions in SignalP. Notably, for Ctsh we found two nontryptic peptides, each of which confirmed either the UniProt annotation or the SignalP prediction (differing by one amino acid), showing that both forms coexist. In five cases our data disagreed with both UniProt and SignalP. Here, for Ccl5, Cst3, and Cstb the detected nontryptic peptide suggested a shorter

**Fig. 6.** Plasma membrane proteins detected in the secretome. Transmembrane proteins are represented by black bars, with the first transmembrane domain relative to the extracellular space aligned at zero. Exclusive detection of extracellular peptides in the secretome samples suggests the release of class I to III proteins by ectodomain shedding. Green stars: the intracellular peptide was detected in the secretome with just one MS/MS spectrum. Purple stars: proteins with no annotation in UniProt defining the direction of protein insertion into the membrane. Pink stars: proteins that start and end with a cytoplasmic domain and thus do not fit into our illustration scheme.

**Fig. 7.** Proteolytic cleavage site detection in secreted proteins. A, experimentally detected signal peptides, indicating all proteins with a nontryptic peptide found in the N-terminal part. B, potential proteolytic cleavage sites, indicating proteins with additional nontryptic peptides.
processed sequence than the annotated or predicted cleavage sites, possibly explained by secondary processing by additional proteases. This could also cause the detection of multiple nontryptic peptides, as seen for Ccl4 and Sdc4. In all, our dataset experimentally confirmed 20 UniProt annotations for signal peptides and proposed the reannotation of an additional six proteins.

This approach can also be used to detect alternative cleavage sites within proteins. For Tnf we detected the intramembrane cleavage site, resulting in the C1-domain as annotated in UniProt, as well as a nontrytic peptide close to the annotated start of the soluble form. Given that the annotation of mouse Tnf is derived from the human orthologue by similarity, and as the sequence similarity in this region is rather low, the detected nontryptic peptide could represent the N-terminal end of the soluble form of mouse Tnf (Fig. 7B, supplemental Fig. S9). In cathepsinB we detected a nontryptic peptide corresponding to the N terminus of the mature protein, produced by cleavage between the activation peptide and the mature chain at residue 80 (Fig. 7B), again demonstrating the suitability of our approach for detecting proteolytic processing sites possibly explaining similar observations in various other proteins (Fig. 7B).

**DISCUSSION**

One of the main challenges in quantitative proteomics is the detection of small proteome changes that are the result of a specific perturbation and that often operate on a rapid time scale. In this study, we combined pulsed SILAC and the selective enrichment of newly synthesized proteins via click-chemistry to address this question. We have demonstrated, using LPS-stimulated macrophages as a model system, that this allows the detection and quantification of proteins that are specifically induced upon the treatment and whose change in abundance remained undetected using full or pulsed SILAC only. This reflects the unique aspect of the approach, which biochemically enriches the newly synthesized fraction of the proteome while depleting the large body of preexisting proteins, thus improving the capability of quantifying proteome changes in perturbed cells. This allows the detection of proteome responses on a rapid time scale, demonstrated by the identification and quantification of close to 5000 proteins that had incorporated the SILAC label after a 20-min pulse.

To obtain a systems view on proteomic events during the first 3 h of macrophage activation, we studied newly synthesized proteins in intracellular and extracellular fractions and combined this with the analysis of overall transcript and protein levels in a time course manner. This led to the identification of a larger number of changing proteins in this model system than in previous studies (11, 15–18, 39). One exception is a study by Du et al. (39), who identified >700 differentially expressed proteins after 10 min of LPS stimulation among 2144 detected proteins. However, they applied extremely relaxed cutoff criteria to qualify a protein as differentially expressed (fold change of >1.2 or <0.8, based on an experiment without replicates). In addition, our approach has allowed us to directly correlate transcriptional response to protein synthesis and to sequester proteins that are then externalized. Importantly, this has identified regulatory mechanisms of individual genes/proteins while giving insight into functionalities of proteins with shared temporal expression profiles. This has revealed that not only are many canonical pro-inflammatory proteins (NF-κB, Tnf) rapidly induced, but this is accompanied by the induction of proteins that modulate the immune response (e.g. Ier3, Ctnnb, Socs3, Zfp36, Gpnmb), suggesting that both processes are carefully balanced from the earliest onset of macrophage activation.

Although the synthesis of many proteins followed the expression of their corresponding transcripts, there were multiple exceptions to this rule (Fig. 3). For instance, several proteins involved in the cell cycle showed reduced RNA levels in response to stimulation without a change in protein level. Conversely, minor differences in RNA abundance still resulted in significant changes in protein synthesis for several proteins. This indicates that regulation at the post-transcriptional level is an important and widespread mechanism for adjusting the immune response. This has highlighted cases of translation lagging behind transcription, increased protein turnover, and negative feedback regulation. Among the striking observations was the group of proteins in the secretome that did not show a concomitant change in transcript or intracellular protein levels (Fig. 3, category L), indicating enhanced secretion via vesicles and exosomes specifically induced by LPS. In this respect, an intriguing protein is Gpnmb, which has been implicated in the repression of pro-inflammatory cytokines (82) and is a marker protein for melanosomes (86). This might imply that melanosome formation and secretion are an integral part of the innate immune response.

An important asset resulting from the quantitative nature of the study and the depth of protein coverage was the ability to investigate ectodomain shedding and proteolytic processing of membrane-embedded and secreted proteins. Both are important processes in the regulation of secretome composition and fine-tuning of protein functionality (87). We have identified multiple instances of proteins being shed or processed as a response to LPS activation, emphasizing the diversity and complexity of events that are engaged in immune response.

As a whole, this study has significantly broadened the view on the proteins and regulatory mechanisms underlying macrophage activation. Specifically, whereas most of the recent insights on this topic are based on RNA expression measurements, here we distinguished between regulated RNA species that did or did not result in a change in protein abundance. Thus, the approach used here to combine pAHA and pSILAC labeling should be amenable to a variety of other systems. The ability to detect changes on a rapid time scale should be extremely useful in attempts to identify the proteins...
that are direct effectors of a cellular perturbation, rather than indirect effects observed at later time points.

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