SUPPORTING INFORMATION

Effective Method for Accurate and Sensitive Quantitation of Rapid Changes of Newly Synthesized Proteins

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SI 2. Methods

Cell Culture, THP-1 Cell Differentiation, Lipopolysaccharide (LPS) Treatment, and Time-Course O-Propargyl-Puromycin (OPP) Labeling

THP-1 human monocytes (ATCC) were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% fetal bovine serum (FBS, Corning) and 1% penicillin-streptomycin in an incubator with 5% CO_2 at 37 ˚C. Cells were equally split into eight flasks once the density reached ~7×10^5 cells/mL. Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) was added to the medium to the final concentration of 100 ng/mL to differentiate the monocytes into M0 macrophages for 48 hours as previously reported.[1] After that, the medium was removed, and the adherent macrophages were rested in the normal RPMI medium without PMA for 24 hours.[1] For the time-course OPP labeling samples, LPS (Sigma-Aldrich) was added to six flasks to the final concentration of 1 μg/mL. After 0, 0.5, 1, 2, 3, and 6 hours, respectively, the macrophages were treated with 30 μM OPP (Click Chemistry Tools) for 15 minutes. The medium was removed after the 15-min OPP treatment, and the cells were washed twice with ice-cold PBS before harvesting. The cells were centrifuged at 300 g for 5 minutes, and the supernatant was removed. For the control and background samples, 30 μM OPP was added to the medium without the treatment of LPS, and the other steps for cell harvest are the same.

Cell Lysis, Enrichment of Newly Synthesized Proteins, On-Bead Digestion, and Peptide Purification

The cells were lysed using a lysis buffer containing 100 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH=7.9; Sigma-Aldrich), 150 mM sodium chloride (NaCl), 1% sodium deoxycholate (SDC; Sigma-Aldrich), 10 units/mL benzonase nuclease (Sigma-Aldrich), and 1 tablet/10 mL EDTA-free cOmplete protease inhibitor cocktail (Roche) with end-over-end rotation at 4 ˚C for 1 hour. The lysate was centrifuged at 4696 g for 10 minutes to remove the cell debris. The OPP-labeled newly synthesized proteins were enriched through the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. For the time-course samples and the control sample, 20 μL azide agarose resin (Click Chemistry Tools), 1 mM $CuSO_4$, 5 mM Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA; Click Chemistry Tools), 5 % dimethyl sulfoxide (DMSO; Sigma-Aldrich), 15 mM sodium L-ascorbate (Sigma-Aldrich), and 15 mM aminoguanidine hydrochloride (Sigma-Aldrich) were sequentially added into each lysate. For the background sample, except without the catalytic reagents ($CuSO_4$ and THPTA), all the others are the same. The reaction lasted for 2 hours in the dark and was quenched by adding 10 mM dithiothreitol (DTT; Sigma-Aldrich). The enriched proteins were further reduced at 56 ˚C for 25 minutes and alkylated with 14 mM iodoacetamide at room temperature in the dark for 30 minutes.

The beads from all samples were stringently washed with 1 mL of the lysis buffer containing 2.5% sodium dodecyl sulfate (SDS) and 2.5% SDC four times at 80 ˚C, 1 mL of 8 M urea in 100 mM HEPES, pH=8.1 for four times, 1 mL of 50% isopropanol twice, and 1 mL of 50% acetonitrile (ACN) twice. Eventually, the beads were resuspended in the digestion buffer containing 50 mM HEPES, pH=8.1, 1.6 M urea, and 5% ACN. The

enriched newly synthesized proteins were digested with trypsin (Promega) at 37 ˚C overnight. The digestion was quenched with trifluoroacetic acid (Millipore) and pH was adjusted to ~2 before desalting. The supernatant containing eluted peptides was collected and peptides were purified using a tC18 Sep-Pak Vac Cartridge (Waters). The purified peptides were dried in a vacuum concentrator.

TMT Labeling of Peptides and the Fractionation Using High Performance Liquid Chromatography (HPLC)

The dried peptides from 8 samples were resuspended in 100 μL of 100 mM HEPES, pH = 8.5, and 30 μL ACN. The TMT10plex reagents were dissolved in 41 μL anhydrous ACN, and 5 μL were added to each sample (127N-0 h; 127C-0.5 h; 128N-1 h; 128C-2 h; 129N-3 h; 129C-6 h; 130N-control; 130C-background), respectively. The reaction was performed for 1 hour at room temperature with shaking and subsequently quenched with 10 μL of 5% hydroxylamine hydrochloride. The labeled peptides were combined, purified, and dried before fractionation. High-pH reversed-phase HPLC was employed to fractionate the combined sample using an XBridge C18 3.5 μm, 4.6×250 mm column (Waters) with an 80-min gradient of 5-60% ACN containing 10 mM ammonium formate (pH=10). The peptides were consolidated into 20 samples and each sample was purified using the StageTip method described previously.[2]

LC-MS/MS Analysis

The dried peptides were resuspended in a solution containing 5% ACN and 4% formic acid (FA) and loaded onto a microcapillary column packed with C18 beads (Magic C18AQ, 3 μm, 200 Å, 75 μm × 16 cm) by a Dionex UltiMate 3000 Wellplate Sampler. The peptides were separated by reversed-phase HPLC using an UltiMate 3000 binary pump with a 112-minute gradient of 3-20% ACN containing 0.125% FA. Data were acquired with a hybrid dual-cell quadrupole linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Elite, Thermo Scientific, with Xcalibur 3.0.63 software) using a data-dependent Top15 method. Each cycle included one full MS scan at the resolution of 60,000 in the Orbitrap with the automatic gain control (AGC) target of 10^6, followed by up to 15 MS/MS for the most intense ions. Selected ions were excluded from further analysis for 90 s. Ions with a single or unassigned charge were not sequenced. A width of 1.2 *m/z* was used for isolating the precursor ions, which were fragmented by HCD at 40% normalized collision energy. Fragments were detected in the Orbitrap cell with the resolution of 30,000 and the AGC target of 2×10^5. Maximum ion accumulation times were 1000 or 50 ms for one full MS scan or an MS^2 scan, respectively.

Database Searching, Data Filtering, and Relative Synthesis Rate Quantification

The raw files were converted into the mzXML format. The mass spectra were searched using the SEQUEST algorithm (version 28) [3] against the human proteome (*Homo sapiens*) database encompassing sequences of all proteins downloaded from UniProt (https://www.uniprot.org). The following parameters were used for the search: 20 ppm

precursor mass tolerance; 0.025 Da product ion mass tolerance; fully digested with trypsin; up to 3 missed cleavages; variable modifications: oxidation of methionine (+15.9949); fixed modifications: carbamidomethylation of cysteine (+57.0214) and the TMT labeling of lysine and the N-terminus (+229.1630).

The target-decoy method was employed to evaluate and control the false discovery rates (FDRs) of peptide and protein identifications.[4] Each protein sequence was listed in both forward and reversed orientations to estimate the FDR. Linear discriminant analysis (LDA) was used to distinguish correct and incorrect peptide identifications using parameters such as Xcorr, ΔCn, and precursor mass accuracy.[5] Peptides containing fewer than seven amino acids in length were discarded. The peptide spectral match was filtered to <1% FDR based on the number of decoy sequences in the final data set. Furthermore, the FDR was controlled to <1% at the protein level.

The ion intensities for the eight TMT channels were recorded and corrected using the isotopic information provided by Thermo. The abundance of each newly synthesized protein in every sample was calculated from the median TMT intensity of all peptides from this protein. The experiment was performed in technical triplicates. In each replicate, the abundances of proteins in the background sample were subtracted from the abundances of proteins in the other seven samples to eliminate the possible influence from nonspecific binding. The protein abundance was further normalized using the abundances of newly synthesized β-tubulin, β-actin, and GAPDH. The synthesis of these proteins is not affected by LPS. For every protein, the relative synthesis rate at each point in each replicate was calculated between the LPS-treated and the control samples. Eventually, the final relative

synthesis rate and the standard deviation were calculated from the average of the three replicates.

Statistical Analysis

Gene Ontology (GO)-based enrichment analysis was performed based on cellular component, molecular function, and biological process using the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system.[6] P-values were calculated using Fisher's exact test. Proteins that changed the relative synthesis rate by at least 1.5-fold were considered to be significantly affected by the treatment.

Data Availability

The raw files are publicly available on:

http://www.peptideatlas.org/PASS/PASS01547. The password is DD5554qg.

SI 3. Analysis of Nonspecific Binding Proteins on Azide Agarose Resins

Newly synthesized proteins labeled with OPP are enriched through the click reaction using the azide agarose resins. Despite the high efficiency of the click reaction and the stringent washes using high-salt solutions, strong detergents, and organic solvents, nonspecific binding proteins cannot be completely eliminated, and they may potentially affect the quantification of the relative synthesis rates. The negative control experiment is commonly used in the studies using affinity purification to distinguish *bona fide* interacting proteins from the nonspecific binding background.[7-11] Therefore, in this work, we included a background sample where the click reaction-based enrichment was performed without the catalysts. The background sample was quantified simultaneously with other samples collected at different time points using multiplexed proteomics. Eventually, the abundances of proteins from the treatment were corrected by the abundances of the corresponding ones from the background sample.

Overall, the ratios of the abundances between nonspecific binding proteins and the enriched ones are in a range of 0 to 0.43, and the median ratio is 0.01, indicating that nonspecific binding proteins were largely removed by the stringent washes (Table S3 and Figure S1A). However, a considerable amount of some proteins was found to nonspecifically bind to the resins, which may affect the quantification results of these proteins if their abundances were not corrected. For example, a couple of histones, i.e., histone H2B type 1-J (H2BC11; 0.24 ± 0.04) and histone H2A type 1 (H2AC11; 0.22 ± 0.03), were found to nonspecifically bind to the resins. In addition, the ratios of heat-shock proteins (HSPE1; 0.43 ± 0.12), cytoskeletal proteins (TUBB4A, 0.17 ± 0.05), and

ribosomal proteins (RPS21; 0.22 ± 0.04) were higher than those of other proteins. The results are consistent with the previous studies using affinity purification methods.[8]

We further compared the properties of proteins with the highest or lowest 25% nonspecific binding ratios, including protein structure, hydrophobicity, isoelectric point, and protein-protein interactions (Table S3 and Figures S1B-E). Proteins with a significantly higher percentage of amino acids in coils ($P < 0.001$) and a lower percentage of amino acids in α-helices ($P < 0.001$) have a higher ratio of nonspecific binding (Figures S1B and S2A) because proteins with more flexible structures have a higher chance to interact with the resins. Proteins with higher ratios of nonspecific binding are more hydrophilic than those with lower ones ($P < 0.001$) (Figure S1C). The linkage between the azido group and the agarose resin is polyethylene glycol (PEG), which is hydrophilic and promotes the interactions between the resins and hydrophilic proteins or the hydrophilic component of proteins. Consistent with the hydrophobicity, the percentages of hydrophobic amino acid residues (phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), and Leucine (Leu)) in proteins with higher ratios of nonspecific binding are significantly lower ($P < 0.001$), while those of charged amino acids (lysine (Lys), aspartic acid (Asp), and glutamic acid (Glu)) in proteins with higher ratios of nonspecific binding are significantly higher ($P < 0.001$) (Figure S2B). In addition, the isoelectric points (pIs) of proteins with the higher ratios of nonspecific binding are markedly lower ($P < 0.001$), meaning that the pIs may also affect the interactions between the resins and proteins (Figure S1D). Furthermore, the nonspecific binding may happen on the enriched proteins. As shown in Figures S1E and S2C, proteins with more protein-protein interactions or existed in more complexes have higher ratios of nonspecific binding.

SI 4. Supporting Figures

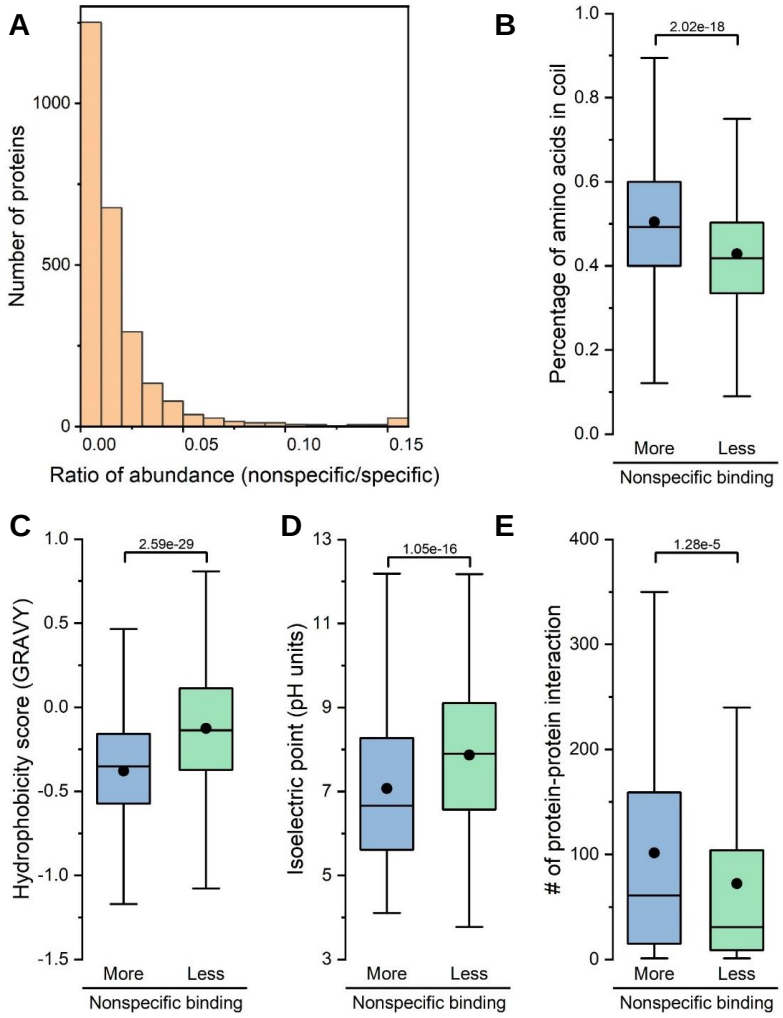

Figure S1. Analysis of nonspecific binding proteins from the enrichment using the azide agarose resins. (A) Distribution of the ratios of the abundances between nonspecific binding proteins and enriched ones. (B-E) Comparison of (B) protein structure, (C) hydrophobicity, and (D) isoelectric point, and (E) number of protein-protein interactions between proteins with higher and lower ratios of nonspecific binding.

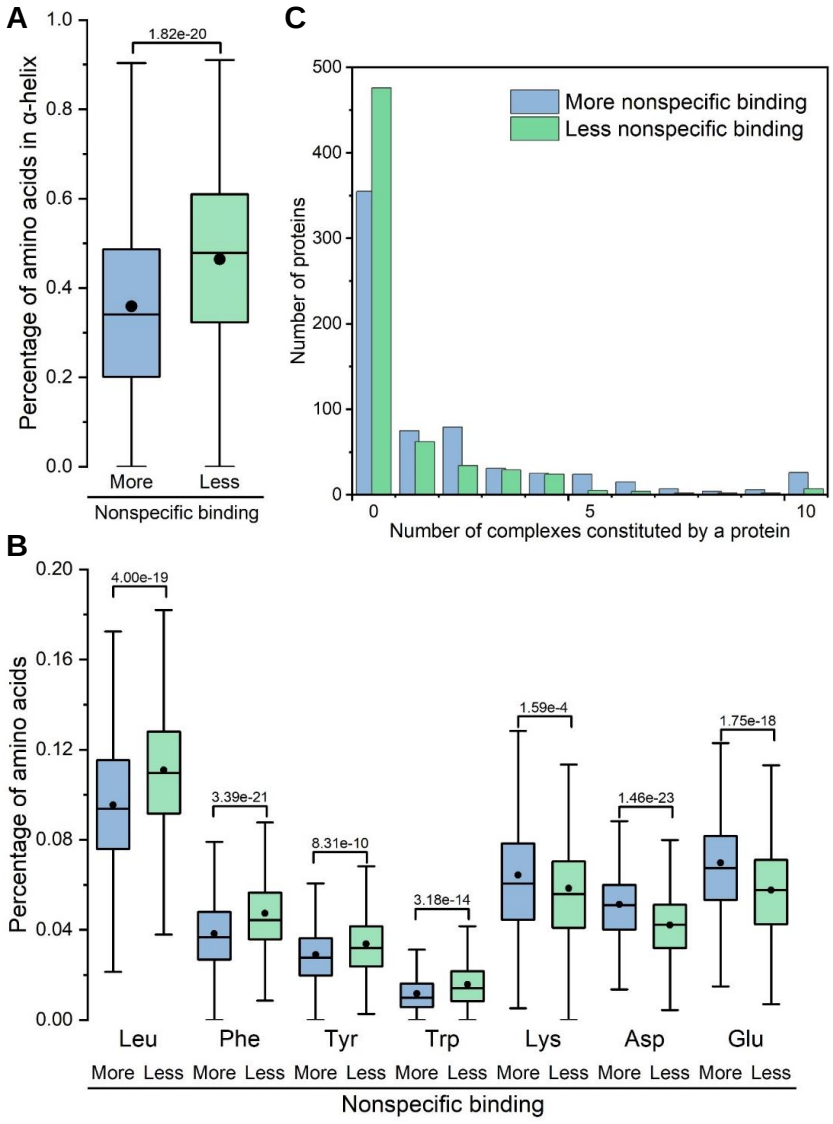

Figure S2. Analysis of nonspecific binding proteins on the azide agarose resin. Comparison of (A) protein structure, (B) amino acid composition, and (C) number of complexes constituted by the protein between proteins with higher and lower ratios of nonspecific binding.

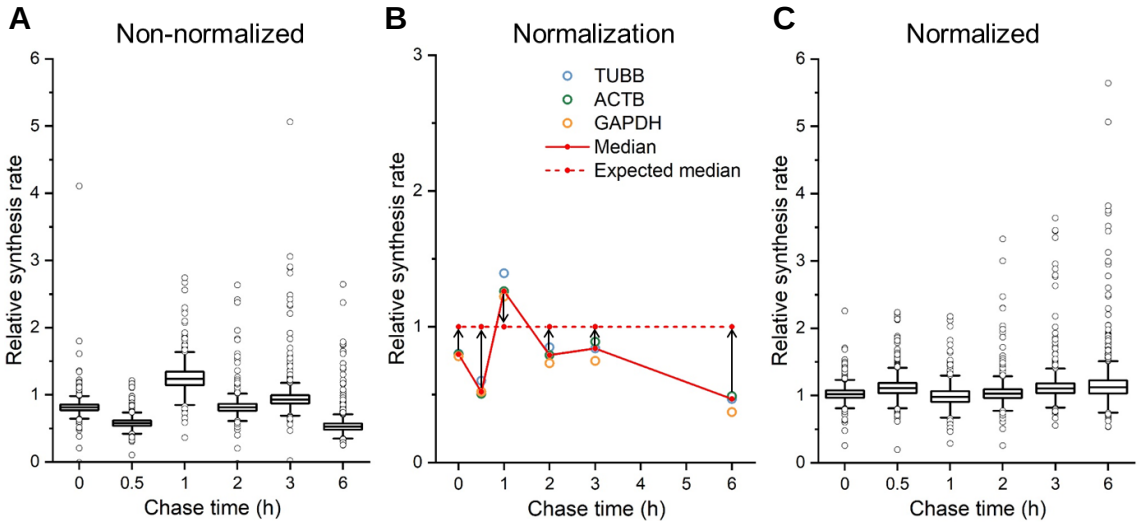

Figure S3. Normalization of relative protein synthesis rates. (A) Distribution of non-normalized relative synthesis rates at each time point for one replicate. (B) Example of protein abundance normalization. The median relative synthesis rates at each time point were calculated from three proteins (TUBB, ACTB, and GAPDH). The relative synthesis rates for other proteins were normalized based on the median ratio of these three proteins at each time point. (C) Distribution of normalized relative synthesis rates at each time point.

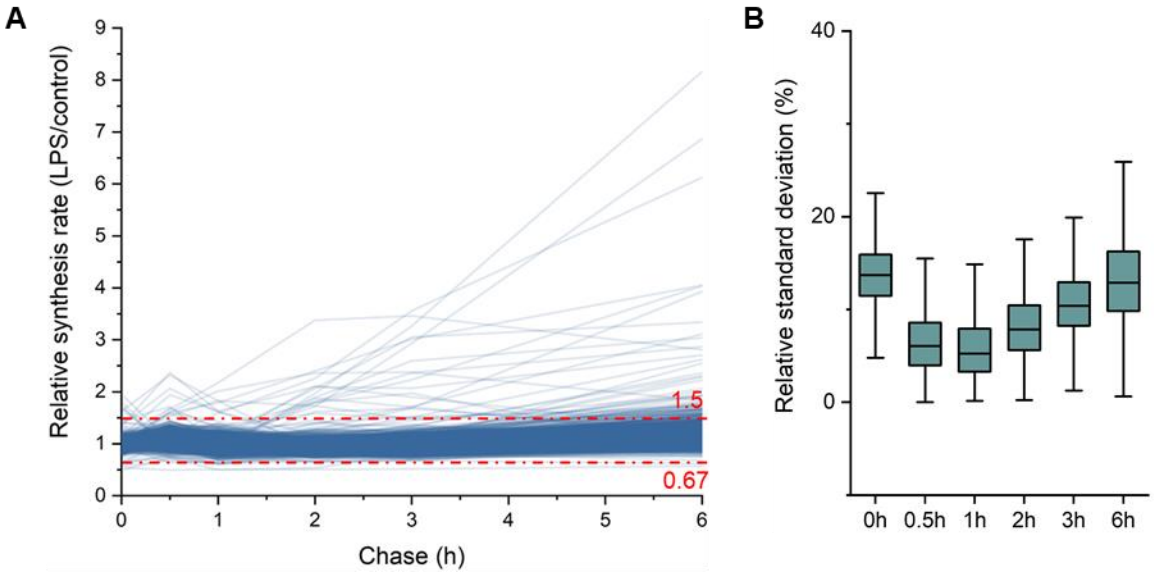

Figure S4. The relative synthesis rates and relative standard deviation for all quantified proteins.

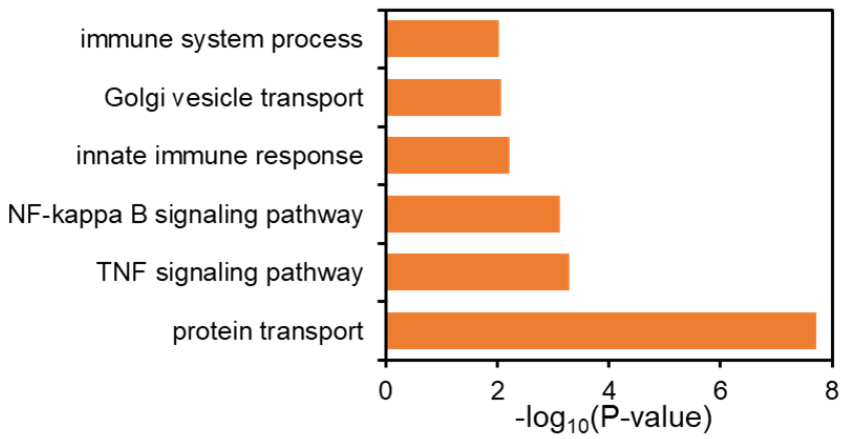

Figure S5. Clustering of 238 newly synthesized proteins with significantly affected synthesis rates in the LPS-induced THP-1 macrophages.

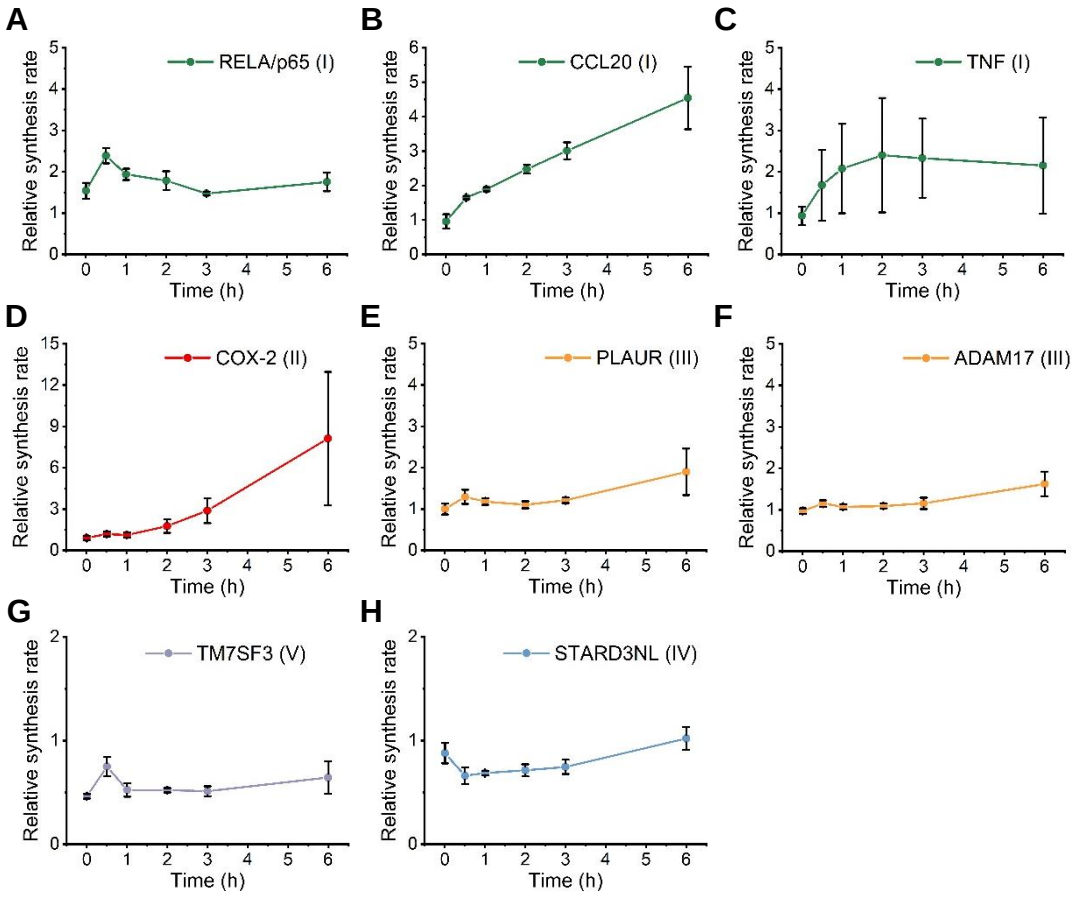

Figure S6. Relative synthesis rates of proteins in THP-1 macrophages treated with LPS. The error bars represent one standard deviation at each time point.

SI 5. REFERENCES

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