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Global Characterization of Protein Secretion from Human Macrophages Following Non-canonical Caspase-4/5 Inflammasome Activation*[S]

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Gram-negative bacteria are associated with a wide spectrum of infectious diseases in humans. Inflammasomes are cytosolic protein complexes that are assembled when the cell encounters pathogens or other harmful agents. The non-canonical caspase-4/5 inflammasome is activated by Gram-negative bacteria-derived lipopolysaccharide (LPS) and by endogenous oxidized phospholipids. Protein secretion is a critical component of the innate immune response. Here, we have used label-free quantitative proteomics to characterize global protein secretion in response to non-canonical inflammasome activation upon intracellular LPS recognition in human primary macrophages. Before proteomics, the total secretome was separated into two fractions, enriched extracellular vesicle (EV) fraction and rest-secretome (RS) fraction using size-exclusion centrifugation. We identified 1048 proteins from the EV fraction and 1223 proteins from the RS fraction. From these, 640 were identified from both fractions suggesting that the non-canonical inflammasome activates multiple, partly overlapping protein secretion pathways. We identified several secreted proteins that have a critical role in host response against severe Gram-negative bacterial infection. The soluble secretome (RS fraction) was highly enriched with inflammation-associated proteins upon intracellular LPS recognition. Several ribosomal proteins were highly abundant in the EV fraction upon infection, and our data strongly suggest that secretion of translational machinery and concomitant inhibition of translation are important parts of host response against Gram-negative bacteria sensing caspase-4/5 inflammasome. Intracellular recognition of LPS resulted in the secretion of two metalloproteinases, a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) and MMP14, in the enriched EV fraction. ADAM10 release was associated with the secretion of TNF, a key inflammatory cytokine, and M-CSF, an important growth factor for myeloid cells probably through ADAM10-dependent membrane shedding of these cytokines. Caspase-4/5 inflammasome activation also resulted in secretion of danger-associated molecules S100A8 and prothymosin-α in the enriched EV fraction. Both S100A8 and prothymosin-α are ligands for toll-like receptor 4 recognizing extracellular LPS, and they may contribute to endotoxic shock during non-canonical inflammasome activation. *Molecular & Cellular Proteomics 16: 10.1074/mcp.M116.064840, S187–S199, 2017.*

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Gram-negative bacteria are associated with a wide spectrum of infectious diseases in humans, including pneumonia, bloodstream infections, wound infections, meningitis, as well as several sexually transmitted diseases (1). Innate immunity is the first defense response against pathogens. Macrophages are central effector cells of innate immunity detecting the presence of Gram-negative bacteria with their pattern recognition receptors (2). Gram-negative bacteria contain pathogen-associated molecular patterns, including the major cell wall component LPS, a potent activator of the innate immune response. Extracellular LPS is recognized by pattern recognition receptors called Toll-like receptor 4 (TLR4) (3), which activates transcription of genes encoding cytokines, chemokines, and co-stimulatory molecules in antigen-presenting cells (4). This results in the activation of the antimicrobial defense and adaptive immune response. Infection with Gram-negative bacteria may lead to the life-threatening condition called endotoxic shock, which is one of the major causes of death in intensive care units (5). This condition develops due to a dysregulation of the immune response, and the mechanisms initially recruited to fight the infection produce life-threatening tissue damage.

Inflammasomes are multimeric protein complexes of the innate immune system that are critical for both local and systemic inflammation (6). The most studied inflammasome

structure is the canonical NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasome, which is activated by several microbial stimuli as well as by endogenous danger signals, including ATP and monosodium urate (7). The NLRP3 inflammasome activates caspase-1, which in turn facilitates the proteolytic processing and secretion of pro-inflammatory cytokines IL-1β and IL-18. Recently, Kayagaki *et al.* (8) showed that mouse caspase-11 is involved in the recognition of infections with Gram-negative bacteria. This led to the discovery of the non-canonical caspase-11 inflammasome, which activates pyroptosis, an inflammatory form of cell death, in response to infections with Gram-negative bacteria. Subsequently, it was shown that caspase-11 recognizes intracellular LPS independently of TLR4 and mediates endotoxic shock in mice (9, 10). Very recently, also endogenous oxidized phospholipids were discovered to activate the caspase-11 inflammasome and IL-1 release without inducing pyroptosis (11). Human caspase-4 and caspase-5 are homologs of mouse caspase-11, and it was demonstrated that these inflammatory caspases also directly bind to intracellular LPS resulting in their activation (8–10). Subsequent studies have shown that the human non-canonical caspase-4/5 inflammasome can activate the canonical NLRP3 inflammasome resulting in secretion of IL-1β and IL-18 (12).

Protein secretion from cells is mediated through conventional and unconventional pathways. Conventionally secreted proteins have a signal peptide on their N terminus. The signal sequence directs them through the endoplasmic reticulum and Golgi apparatus to vesicles, which fuse with the plasma membrane and release their cargo into the extracellular space. Unconventionally secreted proteins lack the signal peptide and are secreted directly through the plasma membrane through vesicles, including exosomes derived from multivesicular bodies (13, 14). Recent system-level studies of protein secretion by activated immune cells, including macrophages, have highlighted the importance of different secretory pathways in innate immune response (15–18). We have shown that NLRP3 inflammasome activators ATP and crystallized monosodium urate induce robust protein secretion in human macrophages (19, 20), but the effect of non-canonical inflammasome activation on global protein secretion has remained uncharacterized. Here, we studied protein secretion in human macrophages in response to non-canonical caspase-4/5 inflammasome activation using label-free quantitative proteomics combined with bioinformatics. We show that non-canonical inflammasome activation triggers robust protein secretion through multiple secretion pathways and that the secreted proteins have important roles in host response against severe Gram-negative bacterial infection.

EXPERIMENTAL PROCEDURES

Ethics Statement—Primary human macrophages were derived from leukocyte-rich buffy coats from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). All human blood donors provided written informed consent.

Cell Culture and Stimulations—Monocytes from three donors per experiment were isolated and differentiated into macrophages as described previously (21). In total, 1.4×10^6 monocytes were seeded per well on 6-well plates. The monocytes were cultured in serum-free macrophage media (Macrophage-SFM, Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (ImmunoTools, Germany) and 50 units/ml penicillin/streptomycin (Lonza, Basel, Switzerland) at 37 °C and 5% CO_2 for 6 days to polarize the monocytes into macrophages of the pro-inflammatory M1-phenotype. On day 6, the cells were washed with PBS, supplied with fresh RPMI 1640 medium (Gibco) supplemented with L-glutamate and antibiotics, and subsequently mock-transfected with Lipofectamine or transfected with Ultrapure LPS (Invivogen, *Escherichia coli* 0111:B4 1 mg/ml) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) for the times indicated. Caspase-4 inhibitor (Z-YVAD-fmk,[1] 25 μM) was purchased from R&D Systems (Minneapolis, MN), and when used, it was added to media 1 h before LPS transfection.

Protein Identification and Quantification—Label-free quantitative proteomics was used to identify and quantify proteins secreted in response to LPS transfection. Total secretomes were fractionated into two fractions. The extracellular vesicles were enriched from equal supernatant volumes (40–60 ml of medium per condition) by size-exclusion filtration using Amicon 15-ml tubes with a 100-kDa cutoff. Subsequently, the flow-through was concentrated using Amicon 15-ml filters with a 10-kDa cutoff. The sample volumes were equalized using PBS, and equal volumes of the protein fractions were separated by SDS-PAGE and silver-stained (22). Then the gel lanes were cut into 5–6 pieces each, and the proteins were in-gel digested with trypsin (Promega) overnight in 37 °C and eluted as described previously (23). Peptides were desalted and concentrated before mass spectrometry by the STAGE-TIP method using a C18 resin disk (3 M Empore). The peptides were eluted twice with 0.1% TFA, 50% ACN, dried, and solubilized in 7 μl of 0.1% TFA for mass spectrometry analysis.

Each peptide mixture was analyzed on an Easy nLC1000 nano-LC system connected to a quadrupole Orbitrap mass spectrometer (QExactive, ThermoElectron, Bremen, Germany) equipped with a nanoelectrospray ion source (EasySpray/Thermo). For the liquid chromatography separation of the peptides, we employed an EasySpray column capillary of 25-cm bed length (C18, 2-μm beads, 100 Å, 75-μm inner diameter, Thermo). The flow rate was 300 nl/min, and the peptides were eluted with a 2–30% gradient of solvent B in 60 min. Solvent A was aqueous 0.1% formic acid, and solvent B was 100% acetonitrile, 0.1% formic acid. The data-dependent acquisition automatically switched between MS and MS/MS mode. Survey full scan MS spectra were acquired from a mass-to-charge ratio (m/z) of 400 to 1200 with the resolution $R = 70,000$ at m/z 200 after accumulation to a target of 3,000,000 ions in the quadruple. For MS/MS, the 10 most abundant multiple-charged ions were selected for fragmentation on the high energy collision dissociation (HCD) cell at a target value of 100,000 charges or maximum acquisition time of 100 ms. The MS/MS scans were collected at a resolution of 17,500. Target ions already selected for MS/MS were dynamically excluded for 30 s.

[1] The abbreviations used are: Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; DAMP, damage-associated molecular pattern; EV, enriched extracellular vesicle; IPA, Ingenuity Pathway Analysis; ITGAX, integrin α-X; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDH, lactate dehydrogenase; M-CSF, macrophage colony-stimulating factor; RS, Rest-secretome; VAS, V-type proton ATPase subunit S1; FDR, false discovery rate.

The resulting MS raw files were submitted to the MaxQuant software version 1.5.3.8 for protein identification using the Andromeda search engine. Carbamidomethyl (C) was set as a fixed modification, and protein *N*-acetylation and methionine oxidation were set as variable modifications. First search peptide tolerance of 20 ppm and main search error of 4.5 ppm were used. Trypsin without proline restriction enzyme option was used, with two allowed miscleavages. The minimal unique + razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. Label-free quantitation was employed with default settings. The SwissProt human database was used (August, 2016, with 154,660 entries) for the database searches. Known contaminants as provided by MaxQuant and identified in the samples were excluded from further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository with the dataset identifier PXD005083 and to MS-Viewer (45) with Search Keys: zrbigvruog and vvsyumthop.

Bioinformatic Analysis—The proteomic datasets were submitted to EnrichR (25). The output files of the enrichment analysis are tables that include *p* values, Benjamini-Hochberg adjusted *p* values, the *z* score of the deviation from the expected rank, as well as the "combined score," which is the combination of the *p* value with the *z* score by multiplying these two numbers as follows: $c = \ln(p) \cdot z$. In addition, the proteomic datasets were analyzed with Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Mountain View, CA, www.ingenuity.com) and STRING (http://string-db.org/ (26)).

LDH Assay—The LDH release of cells was measured using the cytotoxicity detection kit (LDH) (Roche Diagnostics, Switzerland) according to the manufacturer's instructions.

Luminex Assay—The human cytokine Luminex Bio-Plex Pro immunoassay kit designed to detect cytokines IL-1β, IL-18, and TNF were from Bio-Rad. The Luminex assay was performed according to the manufacturer's instructions.

Western Blotting—Protein samples were denatured at 95 °C for 10 min and separated on SDS-PAGE, transferred to PVDF transfer membranes (Trans-Blot Turbo Transfer System, Bio-Rad), blocked with 5% non-fat milk or 5% BSA in TBS/Tween (TBS-T), and incubated overnight at 4 °C with primary antibodies. The membranes were washed and incubated with appropriate HRP-conjugated secondary antibody for 1 h at room temperature, and unbound antibody was removed by washing with TBS-T. Proteins were visualized with Western Lightning ECL (PerkinElmer Life Sciences) on a ChemiDoc MP Imaging System (Bio-Rad). Antibodies against annexin-1 (sc-12740), galectin-3 (sc-56108), and Alix (sc-53540) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against P-eIF2α (catalog no. 9721S) was purchased from Cell Signaling Technologies (Danvers, MA). The antibody against CD11c (ITGAX, ab52632) was purchased from Abcam PLC (Cambridge, UK). Secondary antibodies were purchased from Dako (Dako Denmark A/S).

Experimental Design and Statistical Rationale—We analyzed three independent biological replicates with cells from two or three individual donors in each replicate with label-free quantitative proteomics. The allowed FDR was 0.01 (1%) for peptide and protein identification, and label-free quantitation was employed with default MaxQuant settings. The proteins with at least 2-fold increased secretion in all three biological replicates or in two out of three biological replicates with no opposing quantification values in the third replicate were considered to have increased secretion upon LPS transfection and were included for further bioinformatics analysis.

RESULTS AND DISCUSSION

Intracellular LPS Recognition Pathway Activates Both Conventional as well as Vesicle-mediated Protein Secretion in Human Macrophages—Protein secretion is a critical component of the innate immune response. Here, we have characterized the global protein release during intracellular LPS recognition in human macrophages. Intracellular LPS that activates the non-canonical inflammasome in macrophages triggers pyroptosis, which is associated with LDH release from the cells (27). We first measured the kinetics of LDH release from macrophages upon intracellular LPS recognition. Macrophages were mock-transfected or transfected with LPS after which cell culture supernatants were collected, and the LDH assay was performed. LDH release increased in a time-dependent manner from human macrophages starting at 3 h after LPS transfection (Fig. 1*A*). Triggering non-canonical caspase-4/5 inflammasome results in the activation of the NLRP3 inflammasome and leads to secretion of the biologically active form of IL-18. To study the kinetics of IL-18 secretion, macrophages were transfected with LPS for different time periods after which cell culture supernatants were collected, and IL-18 secretion was studied by Luminex assay.

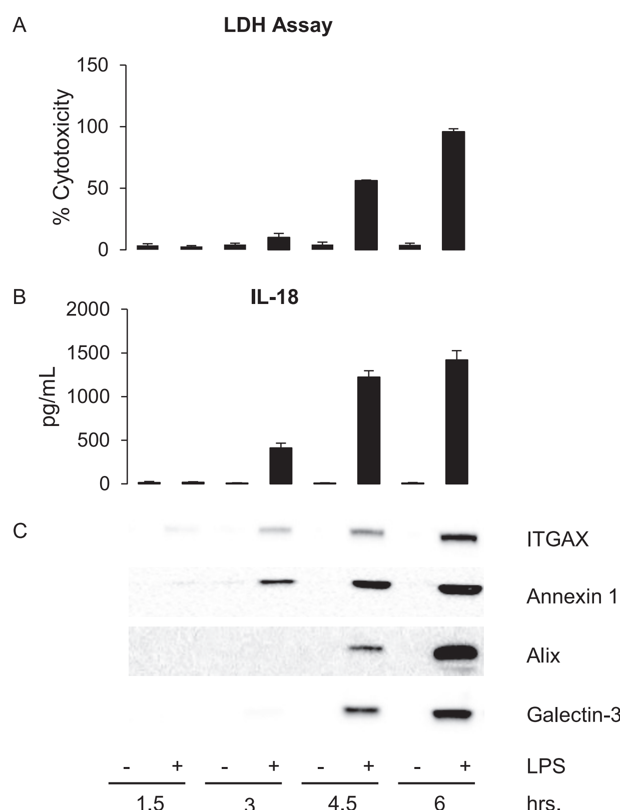

FIG. 1. **Intracellular LPS recognition pathway activates robust protein secretion in human macrophages.** Human macrophages were mock-transfected or transfected with LPS for different time periods after which cell culture supernatants were collected and lactate dehydrogenase release was analyzed (*A*) and release of IL-18 was measured by Luminex assay (*B*). Human macrophages were mock-transfected or transfected with LPS for different times. After this cell culture supernatants were collected, and enriched extracellular vesicle fraction was isolated. *C*, secretion of ITGAX, annexin-1, alix, and galectin-1 was analyzed by Western blotting.

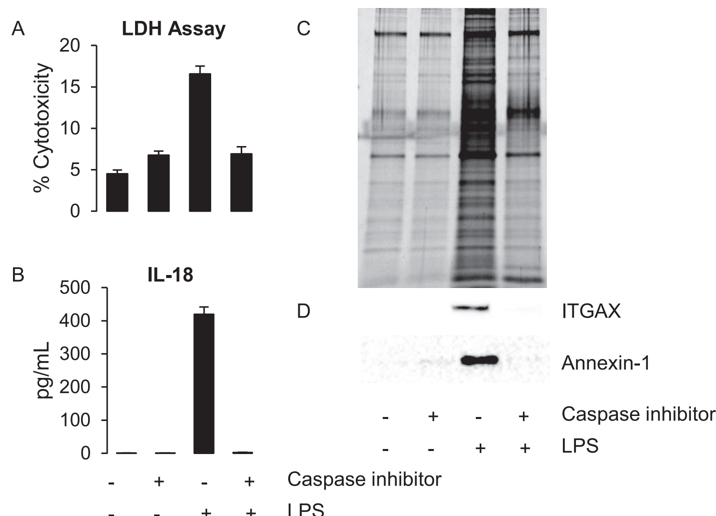

FIG. 2. **Non-canonical inflammasome activates vesicle-mediated protein secretion in human macrophages.** Macrophages were left untreated or pre-treated with caspase-4 inhibitor Z-YVAD-fmk (25 μM) for 1 h after which they were mock-transfected or transfected with LPS for 3 h. After that, the cell culture supernatants were collected and lactate dehydrogenase release was analyzed (*A*) and release of IL-18 was measured by Luminex assay (*B*). Macrophages were left untreated or pre-treated with caspase-4 inhibitor Z-YVAD-fmk (25 μM) for 1 h after which they were mock-transfected or transfected with LPS for 3 h. After this the cell culture supernatants were collected, and the enriched extracellular vesicle fraction was isolated. *C,* proteins were separated with SDS-PAGE and visualized with silver staining. *D,* secretion of ITGAX and annexin-1 was analyzed by Western blotting.

IL-18 secretion started at 3 h after LPS transfection correlating with LDH release (Fig. 1*B*).

Then, we examined how the intracellular LPS recognition pathway affects global protein secretion. For this, macrophages were mock-transfected or transfected with LPS for different time periods, and the cell culture supernatants were collected and concentrated. The total secretome was separated into two fractions as follows: enriched extracellular vesicle (EV) fraction and rest-secretome (RS) fraction using size-exclusion centrifugation as described previously (16). After this, the proteins in EV and RS fractions were separated by SDS-PAGE and visualized with silver staining. Enhanced protein secretion was seen in both EV and RS fractions already at 1.5 h after LPS transfection, and protein secretion clearly increased time-dependently after stimulation (supplemental Fig. 1). Protein secretion dramatically increased at 6 h after LPS transfection (supplemental Fig. 1), probably due to cell death, which was seen as high secretion of LDH at 6 h post-stimulation (Fig. 1*A*). This showed that intracellular LPS recognition pathway is a potent activator of protein secretion. To further characterize activation of EV secretion in human macrophages in response to LPS transfection, we studied the secretion kinetics of known EV marker proteins Alix, annexin-1, galectin-3, and ITGAX. In accordance with silver staining results, secretion of these proteins increased time-dependently in human macrophages in response to LPS transfection (Fig. 1*C*). We have previously shown that TLR4 activation by extracellular LPS does not induce EV-mediated protein secretion in human macrophages (16). In contrast to this, the current results strongly suggest that intracellular LPS is a potent activator of EV-mediated protein secretion.

Non-canonical Inflammasome Activates EV-mediated Protein Secretion in Human Macrophages—Next, we wanted to verify that LDH release and IL-18 secretion induced by LPS transfection is dependent on caspase activity. For this, macrophages were pre-treated with caspase-4 inhibitor (Z-YVAD-fmk) before stimulation with intracellular LPS. Both LDH release (Fig. 2*A*) and IL-18 secretion (Fig. 2*B*) were completely suppressed by the inhibitor demonstrating that LPS transfection-induced LDH and IL-18 response is dependent on the non-canonical inflammasome.

To demonstrate that intracellular LPS-induced EV-mediated protein secretion is dependent on the non-canonical inflammasome, macrophages were transfected with LPS for 3 h in the absence and presence of caspase-4 inhibitor. After this, the EV fraction was enriched, and the proteins were separated by SDS-PAGE and visualized with silver staining. The inhibitor clearly decreased EV-mediated protein secretion in response to LPS transfection (Fig. 2*C*). In accordance with this result, Western blotting analysis showed that caspase-4 inhibitor completely blocks secretion of EV marker proteins ITGAX and annexin-1 induced by LPS transfection (Fig. 2*D*). In conclusion, these results show that EV-mediated protein secretion following intracellular LPS recognition is dependent on non-canonical inflammasome activation.

Global Protein Secretion Analysis upon Non-canonical Caspase-4/5 Inflammasome Activation—To characterize the effect of intracellular LPS stimulation to total protein secretion, we performed LC-MS/MS analysis using label-free quantification (Fig. 3). Based on the kinetic experiments, we isolated EV and RS fractions from mock-transfected and LPS-transfected macrophages at 1.5 h after stimulation for

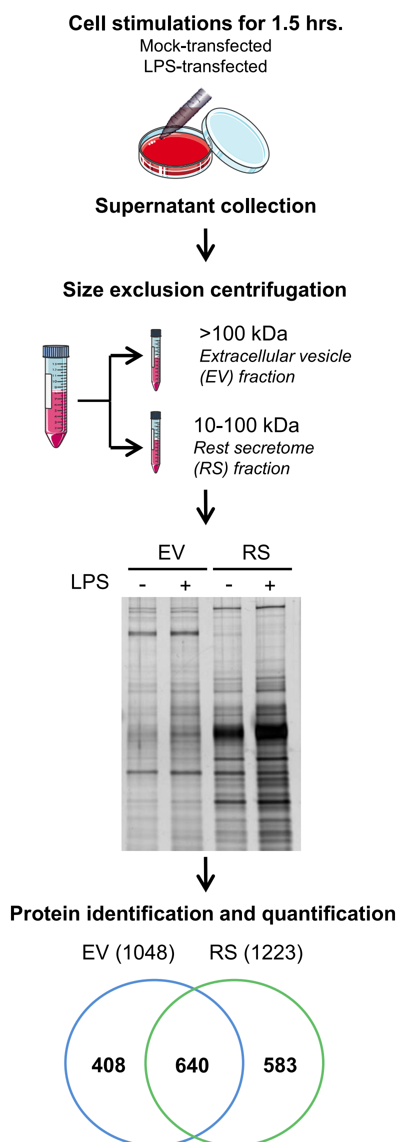

FIG. 3. **Global protein secretion analysis.** Human macrophages were either mock-transfected or transfected with LPS. The supernatants were collected, and the secreted proteins were separated into two fractions using size-exclusion centrifugation. The proteins from EV and RS fractions were separated with SDS-PAGE, visualized with silver-staining, and in-gel-digested into peptides. The resulting peptides were analyzed by label-free quantitative proteomics followed by extensive bioinformatics analysis of the proteins whose secretion was increased upon bacterial infection.

subsequent analysis. The proteomic analysis was performed for three independent biological replicates. Altogether, we identified 1048 proteins from the EV fraction and 1223 proteins from the RS fraction (Fig. 3 and supplemental Tables 1 and 2). From these, 640 were identified from both fractions. This overlap is in line with a recent study by Zhu *et al.* (28), who characterized exosomes and exosome-free secretome fractions from tumor-associated macrophages. They postulate that the secretion of macrophage proteins follows multiple pathways, including vesicles only, conventional secretion only, and dual pathways.

All the identified proteins were classified based on their cellular localization, biological processes, and molecular functions as well as canonical pathways to get an overview of the EV and RS proteomes (supplemental Tables 3 and 4). Classification based on cellular localization showed that extracellular vesicular exosome, cytosol, and focal adhesion are the main components in both fractions. The top canonical pathways in the EV fraction dataset include several pathways related to translational regulation (EIF2 signaling; protein regulation of eIF4 and p70S6K (ribosomal protein S6 kinase β-1) signaling; and mechanistic target of rapamycin signaling). In addition, protein ubiquitination and phagosome maturation were among the top scored pathways. All the top biological processes in EV dataset were related to antigen presentation and translation. These data show striking similarities to the extracellular vesicles released from human macrophages upon influenza A virus infection (18). In RS data different metabolic processes were highly dominating based on KEGG and Gene Ontology: Biological Processes. This was seen also with canonical pathways, where the most significant pathways include glycolysis and gluconeogenesis. In addition, leukocyte extravasation signaling was among the most enriched canonical pathways identified from the RS fraction. Leukocyte extravasation is the process by which leukocytes migrate from blood to tissue during inflammation, and it has an important role in innate immune response. Similarly to EV fraction, protein ubiquitination and phagosomal pathways were also enriched in the RS dataset reflecting their important functions in macrophages.

We used label-free quantification to get more detailed information how Gram-negative bacterial infection affects protein secretion. The proteins with at least 2-fold increased secretion in all three biological replicates or in two out of three biological replicates with no opposing quantification values in the third replicate were considered to have increased secretion upon LPS transfection. Altogether, 48 and 36 proteins met these criteria in EV and RS fractions, respectively (Tables I and II). We used monocyte-derived human macrophages in our experiments to gain biologically the most significant data. These cells are much more heterogeneous starting material than cell lines. We have seen this in our previous studies (19, 29), and here these differences manifest in quantification fold differences obtained from different biological replicates.

All the proteins with increased secretion upon LPS transfection were analyzed with multiple bioinformatics tools to elucidate their role in non-canonical inflammasome activation. The main biological processes, molecular functions, and pathways regulated by intracellular LPS stimulation in human macrophages are summarized in Fig. 4, and the full analysis details are included in supplemental Tables 5 and 6. Similarly to total EV identification data, the main canonical pathways up-regulated by LPS transfection in the EV fraction were

Table I
Proteins with increased secretion upon non-canonical inflammasome activation in the EV fraction
The fold-changes are calculated from LFQ values, and LPS only means that the protein was identified only from LPS-transfected samples.

Accession no.	Protein name	Exp. 1	Exp. 2	Exp. 3
P05109	Protein S100-A8	11,3	LPS only	5,0
P06454	Prothymosin α	5,1	LPS only	2,5
P09603	Macrophage colony-stimulating factor 1	8,3	LPS only	4,1
P27348	14-3-3 protein θ	3,8	3,2	2,0
P30273	High affinity immunoglobulin ε receptor subunit γ	LPS only	LPS only	LPS only
P39019	40S ribosomal protein S19	3,7	LPS only	39,1
P47756	F-actin-capping protein subunit β	7,9	LPS only	2,9
P50914	60S ribosomal protein L14	LPS only	LPS only	LPS only
P61247	40S ribosomal protein S3a	6,5	13,8	8,8
P62263	40S ribosomal protein S14	2,2	LPS only	5,4
P62280	40S ribosomal protein S11	2,6	LPS only	LPS only
P62750	60S ribosomal protein L23a	LPS only	LPS only	LPS only
P62753	40S ribosomal protein S6	LPS only	LPS only	LPS only
P62851	40S ribosomal protein S25	4,1	LPS only	23,0
P62917	60S ribosomal protein L8	4,2	19,5	3,6
O00626	C-C motif chemokine 22	0,0	LPS only	LPS only
O14672	Disintegrin and metalloproteinase domain-containing protein 10	0,0	LPS only	LPS only
O75937	DnaJ homolog subfamily C member 8	LPS only	0,0	LPS only
P00450	Ceruloplasmin	4,6	0,0	LPS only
P02649	Apolipoprotein E	5,6	2,6	1,2
P04839	Cytochrome *b*-245 heavy chain	1,0	LPS only	2,2
P05362	Intercellular adhesion molecule 1	LPS only	LPS only	1,5
P07195	L-Lactate dehydrogenase B chain	2,6	7,8	1,6
P08238	Heat shock protein HSP 90-β	2,4	3,6	1,3
P09525	Annexin A4	LPS only	LPS only	1,3
P22314	Ubiquitin-like modifier-activating enzyme 1	2,5	1,8	2,0
P23396	40S ribosomal protein S3	1,5	2,6	8,3
P41218	Myeloid cell nuclear differentiation antigen	1,6	3,2	3,7
P50281	Matrix metalloproteinase-14	0,0	LPS only	LPS only
P55058	Phospholipid transfer protein	6,4	0,0	LPS only
P55209	Nucleosome assembly protein 1-like 1	2,5	0,0	2,5
P61026	Ras-related protein Rab-10	2,5	LPS only	1,5
P62081	40S ribosomal protein S7	1,6	LPS only	LPS only
P62241	40S ribosomal protein S8	1,6	3,0	LPS only
P62244	40S ribosomal protein S15a	0,0	LPS only	3,3
P62277	40S ribosomal protein S13	1,6	LPS only	5,3
P62888	60S ribosomal protein L30	16,1	0,0	3,3
P67809	Nuclease-sensitive element-binding protein 1	0,0	LPS only	LPS only
P80723	Brain acid soluble protein 1	2,8	1,7	3,3
Q13093	Platelet-activating factor acetylhydrolase	1,6	2,3	LPS only
Q13740	CD166 antigen	3,2	0,0	3,7
Q16831	Uridine phosphorylase 1	0,0	LPS only	LPS only
Q6UWP8	Suprabasin	4,5	0,0	LPS only
Q92598	Heat shock protein 105 kDa	1,0	3,1	2,4
Q969P0	Immunoglobulin superfamily member 8	0,0	LPS only	LPS only
Q96AX1	Vacuolar protein sorting-associated protein 33A	0,0	LPS only	LPS only
Q96L50	Leucine-rich repeat protein 1	LPS only	0,0	LPS only
Q99460	26S proteasome non-ATPase regulatory subunit 1	1,0	LPS only	2,0

related to translation regulation. The main KEGG pathway and Gene Ontology: Molecular Component showed very high enrichment of the “ribosome” term in the EV fraction upon non-canonical inflammasome activation. In the RS data, the main biological processes and KEGG pathways point to the activation of several immune response pathways and antigen presentation upon inflammation.

Soluble Secretome Is Highly Enriched with Inflammation-associated Proteins upon Bacterial Infection—We identified altogether 36 proteins from the RS fraction, which show increased secretion upon intracellular LPS stimulation (Table II). The following four proteins were identified from all three biological replicates: β2-microglobulin; Ig κ chain C region; translation machinery-associated protein 7 (TMA7); and

Table II

Proteins with increased secretion upon non-canonical inflammasome activation in the RS fraction

The fold changes are calculated from label-free quantitation values, and LPS only means that the protein was identified only from LPS-transfected samples.

Accession no.	Protein name	Exp. 1	Exp. 2	Exp. 3
P01834	Ig κ chain C region	LPS only	LPS only	LPS only
P61769	β2-Microglobulin	6,3	LPS only	LPS only
Q15904	V-type proton ATPase subunit S1	4,3	LPS only	LPS only
Q9Y2S6	Translation machinery-associated protein 7	4,2	LPS only	LPS only
O00175	C-C motif chemokine 24	1,7	10,1	LPS only
O00391	Sulfhydryl oxidase 1	2,6	2,0	1,1
O00410	Importin-5	2,2	LPS only	1,4
O15305	Phosphomannomutase 2	13,4	LPS only	1,5
O43493	Trans-Golgi network integral membrane protein 2	LPS only	LPS only	1,8
P00492	Hypoxanthine-guanine phosphoribosyltransferase	2,2	2,1	1,4
P01008	Antithrombin-III	LPS only	LPS only	0,0
P01911	HLA class II histocompatibility antigen, DRB1–15 β chain	LPS only	0,0	LPS only
P07900	Heat shock protein HSP 90-α	1,8	2,2	3,3
P07998	Ribonuclease pancreatic	55,5	0,0	LPS only
P08238	Heat shock protein HSP 90-β	1,8	2,1	3,1
P08571	Monocyte differentiation antigen CD14	3,0	2,7	1,6
P08637	Low affinity immunoglobulin γ Fc region receptor III-A	6,1	1,1	2,2
P09211	Glutathione *S*-transferase P	2,8	4,5	1,9
P13284	γ-Interferon-inducible lysosomal thiol reductase	35,0	3,9	1,0
P15090	Fatty acid-binding protein, adipocyte	1,1	7,5	2,1
P30041	Peroxiredoxin-6	1,6	3,4	2,4
P31944	Caspase-14	LPS only	LPS only	0,0
P33316	Deoxyuridine 5′-triphosphate nucleotidohydrolase, mitochondrial	2,5	0,0	2,1
P60983	Glia maturation factor β	2,1	LPS only	0,0
P68402	Platelet-activating factor acetylhydrolase IB subunit β	2,5	0,0	LPS only
P84077	ADP-ribosylation factor 1	1,3	LPS only	LPS only
Q01813	ATP-dependent 6-phosphofructokinase, platelet type	LPS only	LPS only	0,0
Q07812	Apoptosis regulator BAX	1,6	LPS only	LPS only
Q16610	Extracellular matrix protein 1	2,0	2,6	1,3
Q58FF8	Putative heat shock protein HSP 90-β2	1,5	LPS only	LPS only
Q86VP6	Cullin-associated NEDD8-dissociated protein 1	2,2	LPS only	1,2
Q8NFC6	Bi-orientation of chromosomes in cell division protein 1-like 1	1,2	LPS only	LPS only
Q96AT9	Ribulose-phosphate 3-epimerase	24,5	LPS only	1,0
Q9BVM4	γ-Glutamylaminecyclotransferase	LPS only	0,0	LPS only
Q9H5X1	MIP18 family protein FAM96A	LPS only	0,0	LPS only
Q9Y547	Intraflagellar transport protein 25 homolog	LPS only	0,0	LPS only

V-type proton ATPase subunit S1 (VAS). From these, β2-microglobulin is involved in the presentation of peptide antigens to the immune system, Ig κ chain C region is part of the Fc-γ receptor signaling pathway involved in phagocytosis and innate immune response, and VAS has a role in phagosome maturation. C-C motif chemokine 24 (CCL24) was identified as highly up-regulated in RS fraction upon LPS transfection in two biological experiments. CCL24, formerly known as eotaxin-2, is a highly chemotactic cytokine for resting T cells and eosinophils. It has an important role in eosinophil trafficking in allergy and asthma (30).

The most enriched biological processes and KEGG pathways from the RS fraction were linked to activation of immune and inflammatory responses upon non-canonical inflammasome activation (Fig. 4). The main IPA network from this dataset was "Hematological disease, Dermatological diseases and conditions, Immunological disease" (Fig. 5). Most of the proteins in this network are related to inflammatory response. These inflammatory proteins included cluster of differentiation 14 (CD14), heat shock protein 90α (HSP90A), and heat shock protein 90β (HSP90B). Interestingly, both CD14 and HSP90A bind LPS and are involved in mediating its pro-inflammatory effects. HSP90B is a molecular chaperone that supports proper protein folding and maintains protein stability in response to cellular stress. Non-canonical inflammasome activation also enhanced secretion of peroxiredoxin-6 and fatty acid-binding protein 4 (FABP4). The function of peroxiredoxin-6 during Gram-negative bacteria infection is likely to protect host cells against oxidative stress. FABP4 in turn binds phospholipids, and it modulates inflammatory and metabolic response (31). In addition to these proteins, we detected enhanced secretion of Bax in LPS-transfected macrophages in two biological replicates. Bax functions as a key mediator in apoptosis and inflammation in

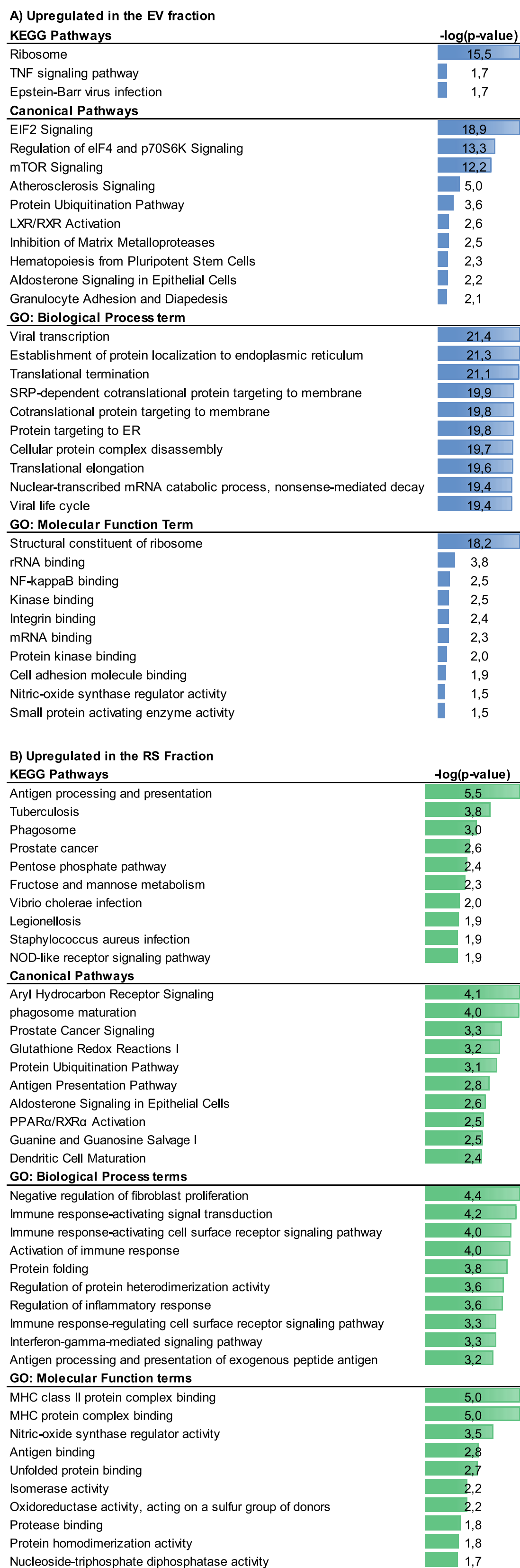

FIG. 4. **Gene ontology and pathway analysis for proteins with increased secretion upon non-canonical inflammasome activation.** Proteins that had at least 2-fold increased secretion upon non-canonical inflammasome activation in at least 2 out of 3 experiments were analyzed using Ingenuity Pathway Analysis and EnrichR. Upregulated KEGG and canonical pathways as well as gene ontology terms in the EV-enriched fraction (*A*) and RS fraction (*B*).

a cell type-specific manner, and it is involved in the activation of the mitochondrial apoptotic pathway. It induces destabilization of the mitochondrial outer membrane resulting in caspase-3 activation and apoptosis. However, we did not detect caspase-3 activation, a hallmark of apoptosis in LPS-transfected macrophages (data not shown). Therefore, it is tempting to speculate that Bax would have a yet uncharacterized role in pyroptosis, and future studies are needed to elucidate the possible role of Bax in the activation of pyroptosis.

Intracellular LPS Stimulation Activates Strong Vesicle-mediated Secretion of Translation-related Proteins—Several ribosomal proteins were highly abundant in the EV fraction upon LPS transfection (Table I). Ribosomal proteins are core components of the translational machinery, and Gene Ontology: Molecular Component and pathway analyses showed very high enrichment in “ribosome” and “translation regulation” (Fig. 4). Protein interaction analysis also showed a major cluster of ribosomal proteins in the EV fraction upon non-canonical inflammasome activation (Fig. 6*A*). Inhibition of translation is a central component of the host’s innate immune response against viral infections (32). However, it is less obvious in the case of bacterial infections because bacteria have their own translational machineries. It has been recently suggested that translation inhibition is involved also in the host response to bacterial pathogens (33). More specifically, Chakrabarti *et al.* (34) showed that Gram-negative bacteria *Pseudomonas entomophila* induces global suppression of translation in the *Drosophila melanogaster* gut. They also demonstrated that the inhibition of translation by *P. entomophila* was due to phosphorylation of eukaryotic initiation factor 2α (eIF2α) by stress kinase GCN2. For this reason, we wanted to study whether non-canonical inflammasome inhibits translation by phosphorylating eIF2α. Macrophages were mock-transfected or transfected with LPS for different time periods. After this, cell lysates were prepared, and phosphorylating eIF2α was studied by Western blotting. Transfection of LPS induced phosphorylation of eIF2α at 1.5 h after stimulation in macrophages and stayed detectable up to 4.5 h (Fig 6*B*). In conclusion, our data suggest that activation of the caspase-4/5 inflammasome results in the secretion of translational machinery and concomitant inhibition of translation.

Non-canonical Caspase-4/5 Inflammasome Activates EV-mediated Secretion of ADAM10 and MMP14, Which Is Associated with M-CSF and TNF Release—Another cluster in the EV protein interaction network (Fig. 6*A*) was centered around apolipoprotein E. It has direct connections to two interesting

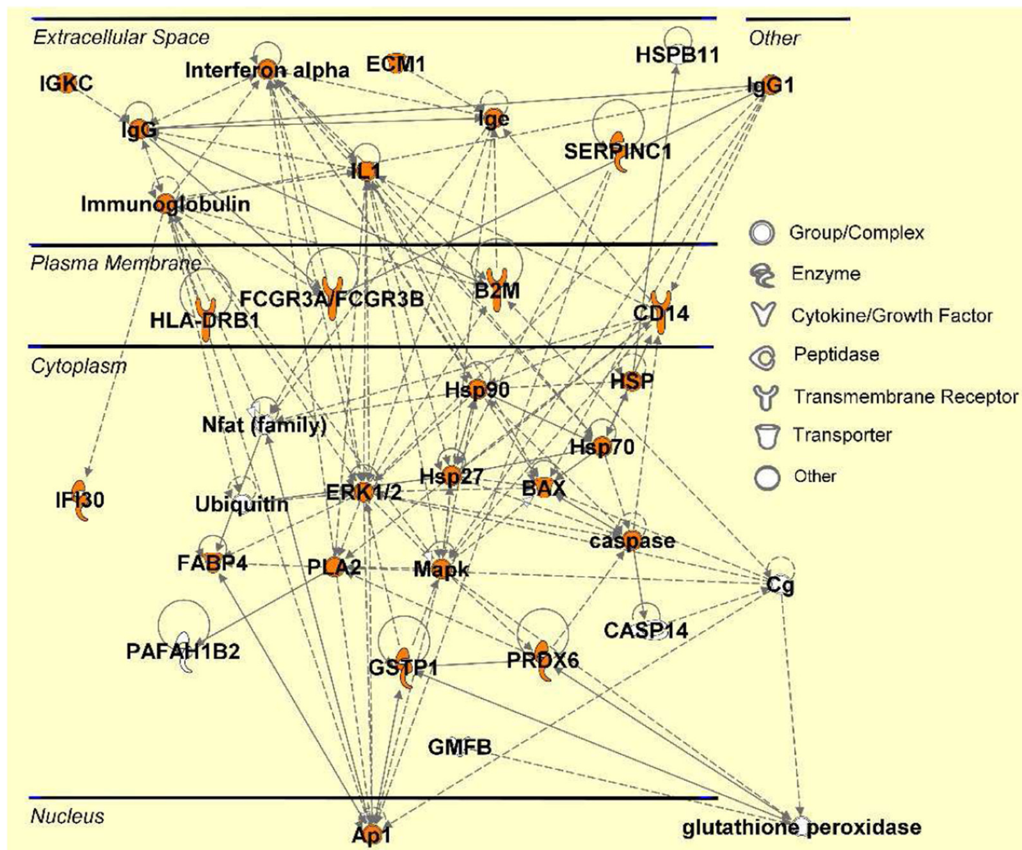

FIG. 5. **Network analysis for the RS fraction proteins with increased secretion upon intracellular LPS stimulation.** The main Ingenuity Pathway Analysis network from proteins with at least 2-fold increased secretion in at least 2 out of 3 experiments upon non-canonical inflammasome activation in the RS fraction is "Hematological disease, Dermatological diseases and conditions, Immunological disease." This network has score 39 and 16 focus molecules. The network has been overlaid with main "Diseases and Functions" found in IPA analysis, and the proteins highlighted with *orange* belong to "Inflammatory response," "Immunological disease," and "Inflammatory disease."

metalloproteinases, matrix metalloproteinase 14 (MMP14) and a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), which we identified from enriched EV fraction in response to LPS transfection (Table I). MMP14 and ADAM10 are known to convert transmembrane molecules into a soluble form through a proteolytic process called ectodomain shedding, and they also share common substrates (35, 36). In addition, it has also been shown that a close relative and functionally similar protease to ADAM10, ADAM17 (37), can induce proteolytic processing and shedding of macrophage colony-stimulating factor (M-CSF) (38). M-CSF is a cytokine that promotes the release of proinflammatory chemokines and has an important role in innate immunity and in inflammatory processes. Interestingly, we identified M-CSF in EV-enriched fraction in all three biological replicates (Table I). The presence of ADAM10 and M-CSF in EV fraction isolated from LPS-transfected macrophage supernatants suggests that caspase-4/5 inflammasome induces shedding of M-CSF through the ADAM-dependent pathway. However, genetic approaches like gene silencing of caspase-4 and/or caspase-5 are required to confirm the role of non-canonical inflammasome in membrane shedding of M-CSF following intracellular LPS recognition.

Tumor necrosis factor (TNF) is a key pro-inflammatory cytokine. Ingenuity Pathway Analysis showed that TNF is among the most significant upstream regulator activated upon bacterial infection (Fig. 7*A*), and it was also seen as one of the enriched KEGG pathways in EVs (Fig. 4). In addition to M-CSF release, both ADAM10 and ADAM17 are known to induce proteolytic processing and shedding of TNF. STRING interaction analysis showed high confidence interactions between TNF, ADAM10, and ADAM17 (Fig. 7*B*). Moreover, both ADAMs and TNF have previously been identified from EVs (39, 40). Because the non-canonical inflammasome activated EV-mediated secretion of M-CSF and ADAM10, we next analyzed whether LPS transfection also activates the release of TNF. Macrophages were mock-transfected or transfected with LPS after which cell culture supernatants were collected, and TNF secretion was analyzed by Luminex assay. LPS transfection enhanced TNF secretion at 3 h post-stimulation, and after this TNF release increased time-dependently (Fig. 7*C*). In conclusion, our results suggest that intracellular LPS activates mem-

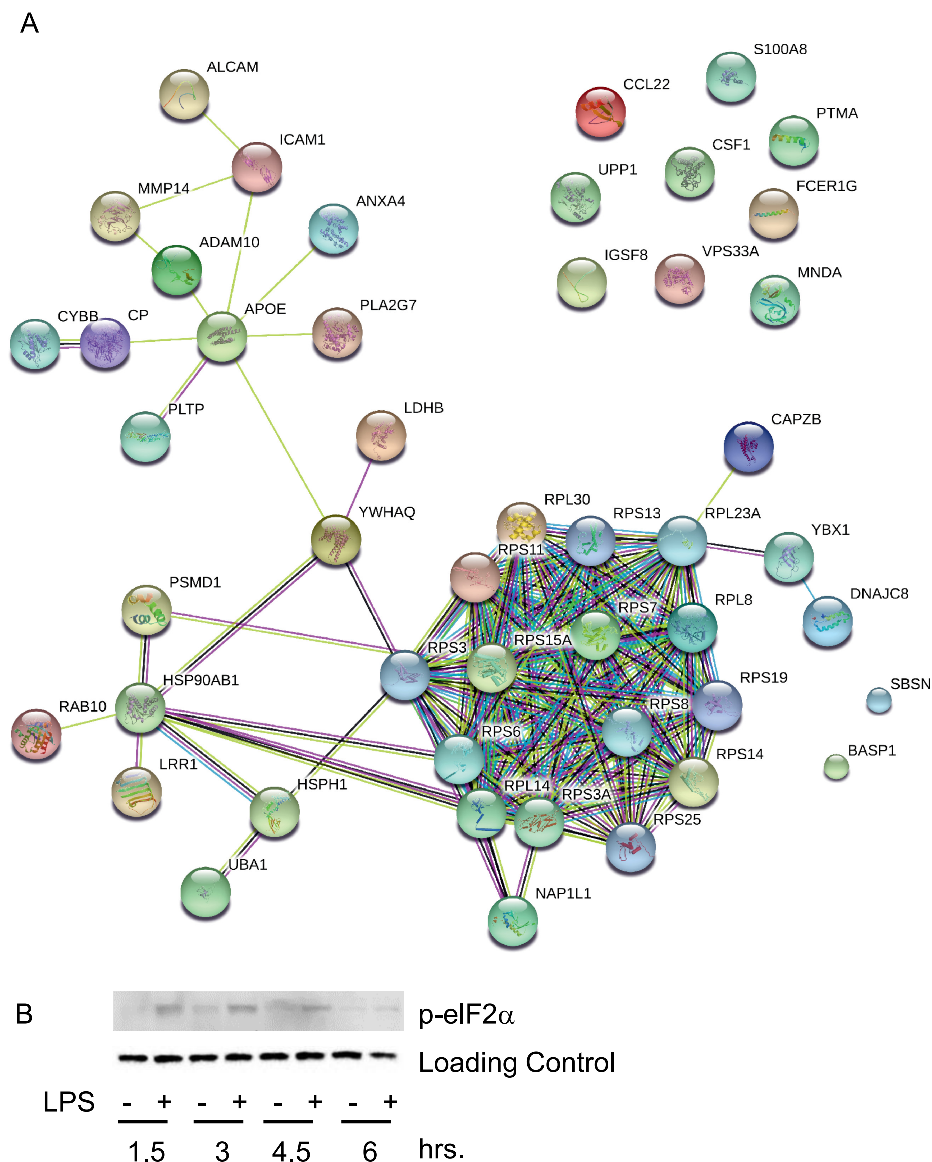

FIG. 6. **Ribosomal proteins are highly abundant in EV fraction, and intracellular eukaryotic initiation factor 2α is phosphorylated upon non-canonical inflammasome activation.** *A,* STRING analysis was used to visualize connections within the proteins that had at least 2-fold increase in secretion upon non-canonical inflammasome activation in at least 2 out of 3 experiments. STRING analysis was done with minimum required interaction score set to "medium confidence 0.400," and only query proteins were included. *B,* human macrophages were either mock-transfected or transfected with LPS for different times. After this, the cells were collected, and cell lysates were prepared. Phosphorylation of eIF2α was analyzed with Western blotting with anti-phospho-eIF2α antibodies. Silver-stained gel was used as the loading control (supplemental Fig. 3).

brane shedding of M-CSF and TNF probably through an ADAM-dependent pathway. To study whether the secretion of TNF in response to intracellular LPS is dependent on caspase activity, macrophages were pre-treated with caspase-4 inhibitor before transfection with LPS. After 3 h of stimulation, cell culture supernatants were collected, and TNF secretion was analyzed with Luminex assay. Surprisingly, the inhibitor clearly increased intracellular LPS-induced secretion of TNF (supplemental Fig. 2). The data demonstrate that intracellular LPS-induced TNF secretion is independent of the non-canonical inflammasome. Further studies are needed to elucidate the intracellular receptors and signaling pathways that mediate TNF release following cytosolic LPS recognition.

EV-mediated Secretion of Endogenous TLR4 Ligands Is Activated Upon Intracellular LPS Stimulation—Damage-associated molecular patterns (DAMPs) are host biomolecules that can activate or enhance a noninfectious inflammatory response. In our experiments, LPS transfection enhanced secretion of DAMP protein S100A8 in enriched EV fraction in all three biological replicates (Table I). S100A8 protein is predominantly found as calprotectin, S100A8/A9, which has many intra- and extracellular functions. S100A8/A9 stimulates

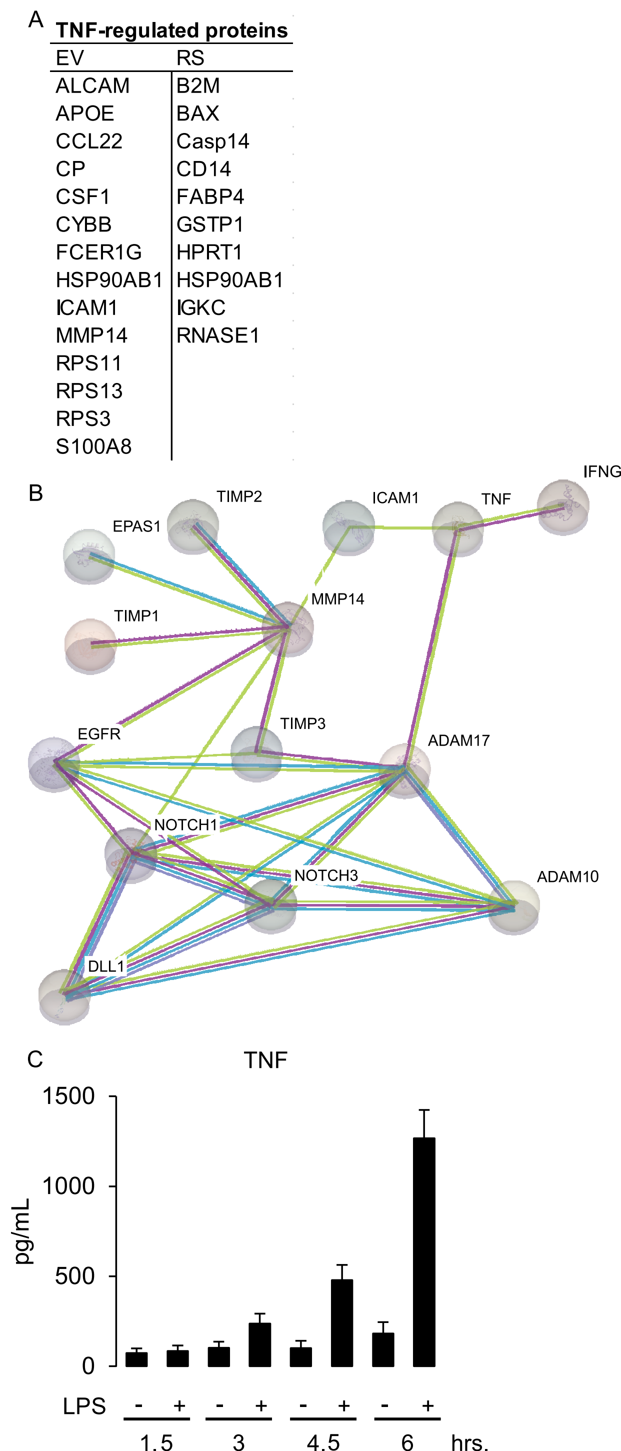

FIG. 7. **Intracellular LPS stimulation activates TNF secretion.** *A,* IPA analysis shows that TNF is an upstream regulator for several proteins that had increased secretion upon LPS transfection. *B,* STRING network analysis with input ADAM10, ADAM17, TNF, and MMP14; the minimum required interaction score was set to "high confidence 0.700," and maximum of 10 proteins were added to the network. *C,* human macrophages were mock-transfected or transfected with LPS for different times. After this, cell culture supernatants were collected, and TNF secretion was measured with Luminex assay.

innate immune cells by binding to TLR4 and receptor for advanced glycation end products (41). S100A8/A9 has many pro-inflammatory functions, and it even promotes lethal endotoxin-induced shock (42). It also has antimicrobial activity toward bacteria and fungi and exerts its antimicrobial activity probably via chelation of Zn^{2+}, which is essential for microbial growth. Additionally, S100A8/A9 is an interesting biomarker for different inflammatory disorders, including inflammatory bowel disease and rheumatoid arthritis (41). Similar to S100A8, LPS transfection enhanced EV-mediated secretion of prothymosin-α in all three biological replicates (Table I). Prothymosin-α is a ubiquitous polypeptide that activates dendritic cells to produce IL-12 through a TLR-dependent pathway and directs adaptive immunity toward Th1 response (43). Furthermore, prothymosin-α is similar to S100A8/A9, a DAMP that triggers TLR4 signaling (44). In conclusion, it is likely that caspase-4/5 inflammasome-induced EV-mediated secretion of S100A8 and prothymosin-α proteins amplifies TLR4 response and contributes to endotoxic shock during overwhelmed activation of the non-canonical inflammasome.

Concluding Remarks—Protein secretion is one of the most important ways how cells communicate with each other. In immunology, the studies in protein secretion have so far focused mostly on analyzing the secretion of small soluble signaling proteins, cytokines, and chemokines that have well characterized roles in immune response. During microbial infection, cytokines activate inflammation, and chemokines recruit immune cells to the site of infection. Recent system level characterizations using modern mass spectrometry-based proteomics approaches have provided important novel information how innate immune cells, including macrophages, activate much more global protein secretion than just secreting cytokines and chemokines and that this global protein secretion is an important part of the innate immune response to different activation stimuli. We have previously shown that activators of canonical NLRP3 inflammasome, including monosodium urate and ATP, induce robust unconventional vesicle-mediated protein secretion in human macrophages (19, 20). Our present results show that also non-canonical caspase-4/5 inflammasome activates EV-mediated protein secretion in human macrophages. In addition, both canonical and non-canonical inflammasomes activated the secretion of many danger signal proteins (Table I) (19, 20).

Inflammasomes are protein complexes that are critical for both local and systemic inflammation. Here, we have characterized global protein secretion in human macrophages in response to non-canonical caspase-4/5 inflammasome activation. Activation of human macrophages with intracellular LPS induced strong protein secretion using multiple, partly overlapping protein secretion pathways already after 1.5 h of stimulation. In-depth quantitative proteomic analysis of soluble secretome and enriched extracellular vesicle fraction showed that the soluble secretome was highly enriched with

inflammation-associated proteins upon intracellular LPS stimulation. Several ribosomal proteins were highly abundant in EV fraction upon LPS transfection, and our results strongly suggest that caspase-4/5 inflammasome activation results in the secretion of translational machinery and concomitant inhibition of translation. Translation inhibition is well characterized as part of the host's innate immune response against viral infections, and our results provide important new information how bacteria can elicit similar host response mechanisms. Non-canonical inflammasome activation also activated membrane shedding of TNF, a key inflammatory cytokine, and M-CSF, which is an important growth factor for myeloid cells. In addition, intracellular LPS recognition by the caspase-4/5 inflammasome resulted in the secretion of CD14 and HSP90A, which bind LPS and are involved in mediating its pro-inflammatory effects. Furthermore, non-canonical inflammasome also activated EV-mediated secretion of TLR4 ligands S100A8 and prothymosin-α that may contribute to endotoxic shock during overwhelmed activation of non-canonical inflammasome.

In conclusion, we provide the first comprehensive characterization of protein secretion activated by non-canonical inflammasome activation, and we show that the secreted proteins have critical roles in host response against severe Gram-negative bacterial infection.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository with the dataset identifier PXD005083 and annotated spectra are available at MS-Viewer (45) (http://prospector2.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer) with search Keys: zrbigvruog and vvsyumthop.

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S This article contains supplemental material.

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