Proteomic evaluation and validation of cathepsin D regulated proteins in macrophages exposed to *Streptococcus pneumoniae*

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Abbreviations

BMDM  Bone Marrow-Derived Macrophage
eEF2  Eukaryotic Elongation Factor 2
Hsp  Heat Shock Protein
MOI  Multiplicity of Infection
MOMP  Mitochondrial Outer Membrane Permeabilization
MDM  Monocyte-Derived Macrophage
PMA  Phorbol 12-myristate 13-acetate
ROS  Reactive Oxygen Species
SCX  Strong Cation Exchange
SOD  Superoxide Dismutase
Δψₘ  Mitochondrial Inner Transmembrane Potential
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**Summary:**

Macrophages are central effectors of innate immune responses to bacteria. We have investigated how activation of the abundant macrophage lysosomal protease, cathepsin D, regulates the macrophage proteome during killing of *Streptococcus pneumoniae*. Using the cathepsin D inhibitor pepstatin A, we demonstrate that cathepsin D differentially regulates multiple targets out of 679 proteins identified and quantified by 8-plex iTRAQ. Our statistical analysis identified 18 differentially expressed proteins that passed all paired t-tests ($\alpha = 0.05$). This dataset was enriched for proteins regulating the mitochondrial pathway of apoptosis or inhibiting competing death programs. Five proteins were selected for further analysis. Western blotting, following pharmacological inhibition or genetic manipulation of cathepsin D, verified cathepsin D-dependent regulation of these proteins, following exposure to *S. pneumoniae*. Superoxide dismutase-2 upregulation was temporally related to increased reactive oxygen species (ROS) generation. Gelsolin, a known regulator of mitochondrial outer membrane permeabilization, was downregulated in association with cytochrome c release from mitochondria. Eukaryotic elongation factor (eEF2), a regulator of protein translation, was also downregulated by cathepsin D. Using absence of the negative regulator of eEF2, eEF2 kinase, we confirm that eEF2 function is required to maintain expression of the anti-apoptotic protein Mcl-1, delaying macrophage apoptosis and confirm using a murine model that maintaining eEF2 function is associated with impaired macrophage apoptosis-associated killing of *Streptococcus pneumoniae*. These findings demonstrate that cathepsin D regulates multiple proteins controlling the mitochondrial pathway of macrophage apoptosis or competing death processes, facilitating intracellular bacterial killing.
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**Introduction:**

Tissue macrophages are central effectors of innate immunity against pathogenic micro-organisms, complementing the action of other components of the innate immune response (1). Transcriptomic and proteomic analysis confirms the broad range of genes that are activated in macrophages by micro-organisms (2-5). Phagocytosis and intracellular killing in phagolysosomes activates multiple signal transduction pathways whose concerted activity leads to a regulated host response to pathogens (6).

A central component of the macrophages capacity to handle ingested bacteria is its highly developed phagolysosomal system, which distinguishes the cell from other phagocytes (7, 8). Phagosomes fuse with lysosomes containing proteases, of which cathepsin D is the most abundant in macrophages (9). Cathepsin D expression in macrophages is highly differentiation dependent (10, 11). Cathepsin D is an aspartic protease, which possesses activity against a broad range of substrates (12). It plays a role in neurologic development, immune homeostasis, apoptosis initiation and tumor development (12). Although cathepsin D was formerly believed to act principally within the low pH environment of the lysosome, it has become apparent that it can have residual activity outside the lysosomal compartment (13). The role of cathepsin D in host defense is currently unclear, although it has been documented to cleave the cholesterol-dependent cytolysin listerolysin produced by *Listeria monocytogenes* (14). We have recently identified a critical role for cathepsin D in the host response of macrophages against *Streptococcus pneumoniae* (15). Cathepsin D activation was observed in macrophages following ingestion of *S. pneumoniae* into phagolysosomes and played a role in the induction of macrophage apoptosis, which contributed to
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microbial killing. Cathepsin D is well positioned to transduce signals from the phagolysosome during the intracellular killing of bacteria, but it is unclear how such an effect might be mediated.

In the current study, we applied an iTRAQ proteomic approach to identify proteins that were differentially expressed in macrophages during infection with *Streptococcus pneumoniae* (pneumococci) in the presence of an aspartic protease inhibitor, pepstatin A, as compared to vehicle control. We identified a number of differentially expressed proteins and confirmed their differential expression, following both pharmacological inhibition and genetic manipulation of cathepsin D, relating these to recognised features of pneumococcal infection in macrophages. We demonstrate that a number of these proteins regulate aspects of macrophage apoptosis, a process we have previously described as being critical to the successful control of pneumococcal infection in macrophages (16-18). In particular, we find that proteins known to regulate oxidant stress, endoplasmic reticulum (ER) stress, the expression of short-lived anti-apoptotic proteins and mitochondrial outer membrane permeabilization were differentially regulated in the presence of cathepsin D inhibition.
Experimental Procedures:

*Bacteria:* Type 2 *Streptococcus pneumoniae* (D39 strain, NCTC 7466) were grown in Brain Heart Infusion (BHI) media supplemented with 20% v/v FCS until an OD$_{610nm}$ of 0.6 was reached. Prior to infection, thawed aliquots were opsonized in RPMI (Sigma-Aldrich) containing 10% v/v anti-pneumococcal immune serum (16). For mouse experiments type 1 *Streptococcus pneumoniae* (WHO reference laboratory strain SSISP; Statens Serum Institut) were handled under identical conditions but were not opsonized prior to instillation. Bacterial numbers were assessed by the surface viable count method after inoculation on blood agar.

*Cells and Infection:* THP-1 cells were cultured in RPMI plus 10% v/v FCS (complete media). THP-1 cells were differentiated to a macrophage phenotype by treating 0.4 x $10^6$ cell/ml with 200 nM PMA for 3 d, after which the PMA was removed, and the cells left to rest for a further 5 d after which cell numbers were determined. These cells have a phenotype similar to monocyte-derived macrophages (MDM), as evidenced by nuclear to cytoplasmic ratio, concentration of mitochondria and lysosomes, cell surface markers, phagocytic capacity, cytokine generation to Toll-like receptor agonists and susceptibility to apoptosis (19). Human MDMs were isolated from whole blood donated by healthy volunteers as previously described with informed consent as approved by the South Sheffield Regional Ethics Committee of Royal Hallamshire Hospital (Sheffield, United Kingdom) (16). After 14 d, representative wells were scraped to determine cell numbers. Murine bone-marrow derived macrophages (BMDM)s were isolated by culturing marrow from mice deficient in cathepsin D (20) or eukaryotic elongation factor 2 kinase (eEF2k) (21),
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or from the corresponding wild-type littermates. BMDMs were plated at 0.5 x 10^6 cells/ml for 14 d in DMEM containing 10% FCS and 10% conditioned L929 media (17). All cell types were infected with opsonized Streptococcus pneumoniae at a multiplicity of infection (MOI) of 10, or mock-infected (MI) as described elsewhere (16). Cells were incubated with 100 µM of the aspartic protease inhibitor pepstatin A or DMSO vehicle control.

SDS-PAGE and Western immunoblotting: Whole cell extracts and cytosolic fractions were isolated as previously described (18). Blots were incubated overnight at 4°C with antibodies against either gelsolin (rabbit polyclonal, 1:1000; Abcam), SOD-2 (rabbit polyclonal 1:1000; Abcam), heat shock protein (Hsp) A5 / glucose-regulated protein (Grp)78/BiP (rabbit polyclonal 1:200; Abcam), S100 calcium binding protein A6/calcyclin (rabbit polyclonal 1:1000; Abcam), human induced myeloid leukemia cell differentiation protein, murine Mcl-1, (rabbit polyclonal, 1:1000; Rockland), cytochrome c (mouse monoclonal, 1:1000; BD Biosciences), cathepsin D (goat polyclonal, 1:1000; R&D Systems), phospho-eukaryotic elongation factor (eEF) 2 (Thr56), eEF2 (both from Cell signaling, 1:1000), actin (rabbit polyclonal 1:5000; Sigma-Aldrich), or tubulin (mouse polyclonal 1:1000; Sigma-Aldrich). Protein detection was with horseradish peroxidase conjugated secondary antibodies (1:2000; Dako) and ECL (Amersham Pharmacia). Bands were quantified using Image J 1.32 software (NIH) and fold change from mock-infected, calculated and normalized to the fold change in tubulin or actin (18).
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**Cathepsin D activity assay:** Cathepsin D activity was measured in cell lysates using a fluorometric cathepsin D activity assay kit (Abcam) in accordance with the manufacturer’s instructions. Fluorescence was measured on a Packard Bioscience Fusion™ microplate analyzer. Cathepsin D activity in each lysed sample was expressed as a percentage of a comparative sample that had been treated with an excess (500 µM) of pepstatin A to act as a negative control.

**Sample preparation and iTRAQ labelling:** Protein samples were precipitated using ice-cold acetone at -20°C overnight, harvested by centrifugation at 21,000 × g at 4°C for 20 min (22) and resuspended in 1 M Triethyloammoniumbicarbonate (TEAB) at pH 8.5. Total protein quantification involved the Rc-Dc Quantification Assay (Bio-Rad; UK) according to the manufacturer’s instructions. 100 µg of each sample was used for the 8-plex iTRAQ technique (Applied Biosystems; CA, USA). These samples were reduced, alkylated, digested and labelled with iTRAQ reagents according to the manufacturer’s protocol (Applied Biosystems; CA, USA), as previously described (22). The labelling of samples was carried out with 15 sets for data analysis. Two independent biological triplicates (D39 labelled with reagents 115, 116, 117, and D39 with pepstatin A labelled with reagents 118, 119, 121) and one biological duplicate (MI, labelled with reagents 113 and 114) were applied (Figure 1). After incubation at room temperature, labelled samples were combined before being dried in a vacuum concentrator. Fractionation of samples using SCX (Strong Cation Exchange) on a BioLC HPLC system (Dionex, UK) was used to clean the samples, as well as to reduce their complexity (23). The SCX fractionation was carried out using a PolySulfoethyl A Column (PolyLC, USA) with a 5 µm particle size, 20 cm length × 2.1 mm diameter, and 200 Å pore size. The system was operated at a flow rate of 0.2
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ml.min\(^{-1}\) with an injection volume of 120 µl. The mobile phase comprised buffers A and B. Buffer A contained 10 mM KH\(_2\)PO\(_4\), 25% acetonitrile at pH 3, and buffer B consisted of 10 mM KH\(_2\)PO\(_4\), 25% acetonitrile and 500 mM KCl, at pH 3. A 60 min gradient was used, which was 5 min at 100% buffer A, followed by ramping from 5% to 30% buffer B over 40 min, then 30% to 100% B over 5 min, and finally holding at 100% A for 5 min. A UV detector UVD170U and Chromeleon Software (Dionex, The Netherlands) were used to record the chromatogram. Labelled peptide fractions were collected every minute, and then each fraction was dried in a vacuum concentrator. These dried labelled-peptides were then cleaned up using C\(_{18}\) Discovery DSC-18 SPE column (100 µg capacity, Supelco, Sigma) as detailed by Chong and Wright (24) before submission to the mass spectrometry instrument.

**LC-MS/MS UHR-TOF analysis:** Tandem mass spectrometry of LC-MS iTRAQ labelled samples was carried out on a maXis hybrid ultra-high resolution quadrupole time-of-flight system (Bruker Daltonics, Coventry UK) coupled to an Ultimate 3000 nano-flow HPLC (Dionex, Surrey, UK) (25). All LC-MS iTRAQ samples were first desalted online using a 5 mm, 300 µm ID LC-Packings C\(_{18}\) PepMap trap cartridge under 0.1% TFA and 3% ACN for 15 min, and eluted to a 15 cm, 75 µm ID LC-Packings C\(_{18}\) PepMap analytical column in 0.1% formic acid (FA), with an ACN gradient extending from 3% to 95%. Elution was performed on a predefined 70 min gradient program (3%-35% ACN) with a 20% wash step (35%-95% ACN) as described (26). TOF-MS screening measurements were performed on a predefined 50-2200 m/z acquisition window at 2500 ToF summations (approx. 2 Hz) at R (resolution) =40000 @ 622 m/z. Collision-induced dissociation (CID) MS/MS acquisition were performed over the same 50-2200 m/z window at R=40000 @ 622
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m/z with three intensity binned precursors of charge +2 to +4 with at least 2500 counts between 250-1400 m/z, deriving from the TOF-MS screening experiment. Accumulation times for MS/MS were also intensity binned at a maximum of 5000 summations (approx. 1 Hz, if precursor ≤ 2.5×10^3 ion counts) and a minimum of 2000 summations (approx. 2.5 Hz, if precursor ≥ 2×10^4 ion counts). An optimised set of isolation windows was used based on the precursor m/z to achieve at least 90% precursor recovery; 1.5 m/z for < 300 m/z, 3 m/z for < 500 m/z, 4 m/z for < 1000 m/z and 5.5 m/z for < 1400 m/z. Selected precursors after fragmentation twice were actively excluded for 90 seconds from further analysis. To overcome the increased stability due to the iTRAQ labels, collision energies were increased by 10% to optimise for peptide fragmentation.

**MS/MS Data Analysis:** Data processing of LC-MS/MS samples were first parsed using proprietary vendor analysis software, microTOF Control v 2.3 Service Pack 1, and processing module, Data Analysis v 4.0 Service Pack 2 (all from: Bruker Daltonik GmbH, Bremen Germany). MS/MS data recovery to MGF was processed via an embedded daMGF script. Data were then searched against the IPI human database (downloaded from http://www.ebi.ac.uk, on September 2009, with 84,032 protein entries) using a local Phenyx v2.5 (Genebio, Geneva Switzerland) processing cluster at the ChELSI Institute. The search parameters were set as follows: MS tolerance was 0.4 Da and MS/MS tolerances were set at: peptide tolerance 0.2 Da, charge +1, +2, +3 and +4. Minimum peptide length, z-score, maximum p-value and AC score were 6, 5.5, 10^-6 and 6, respectively. The enzyme mode for searching was trypsin, permitting up to two missed cleavages. Modifications were performed as follows: 8-plex iTRAQ mass shifts (+304 Da, K and N-term) as fixed modification,
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cys CAM (+57 Da) as fixed modification on the C residue, and oxidation of methionine (+16 Da) as variable modification on the M residue. These data were then searched for within the reversed IPI human database to estimate the false positive peptide discovery rate (FDR) using the formula, FDR = 2 decoy_hits / (decoy_hits + true_hits), as detailed elsewhere (27). Processed data were exported to Excel (Microsoft 2008, USA) for further analyses via Phenyx local export to retrieve peptide identification data and reporter intensity values for each separately identified peptide. Peptide data and intensity values were parsed locally to Mathematica v7.0 (Wolfram Research, Oxfordshire UK) with more details found in Pham et al. (28). From this, only peptides with ≥ 2 peptide identifications were used for both identification and quantitation. Functional classification of the identified proteins was generated through use of Ingenuity Pathway Analysis (Ingenuity ® Systems, www.ingenuity.com).

Measurement of reactive oxygen species (ROS): Production of intracellular ROS was measured using the cell permeable molecule 2′, 7′-dichloro-dihydrofluorescein diacetate (DCF; Sigma-Aldrich) (29). Macrophages were pre-incubated with 10 µM DCF for 30 min before infection for the indicated time periods. Cells were washed in PBS then analyzed by flow cytometry.

Measurement of mitochondrial inner transmembrane potential (Δψm). To detect loss of Δψm at the required time-points, cells were incubated with 10 µM 5,5′, 6,6′-tetrachloro-1, 1′, 3,3′ tetraethylbenzimidazolocarbocyanine iodide (JC-1; Sigma-Aldrich) for 15 min and analyzed by flow cytometry. Loss of Δψm was demonstrated by a loss of fluorescence on the FL-2 channel as previously described (18).
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**Mouse infection model:** eEF2 kinase knock-out or littermate control, female C57Bl6 mice at 8-14 weeks of age, received $5 \times 10^5$ colony forming units of type 1 *Streptococcus pneumoniae* via intratracheal instillation and 24 h lungs were harvested, homogenized and bacterial colony counts obtained by the surface viable count method (17). All experiments were performed in accordance with the UK Animals Act, authorised under a UK Home Office Licence, and approved by the animal project review committee of the University of Sheffield.

**Statistical analysis:** Macrophage data was recorded as mean ± standard error of the mean (SE) unless otherwise stated. Statistical testing was performed using Prism® 5.02 software (GraphPad Software Inc.) with relevant statistical tests described in the figure legends. Significance was defined as $p < 0.05$.

Proteomic values were recorded as median values. For the proteomic analysis we used two biological triplicates (for D39 and D39+PepstatinA). In order to identify differentially expressed proteins we carried out $t$-test comparisons between the iTRAQ reporter ions’ intensities (considering all MS/MS spectrum for a given protein). Therefore significance increased with the number of MS/MS spectra obtained for a protein. To be significant all $t$-tests of each pairwise comparison were less than $\alpha = 0.05$, with a false discovery rate of $\leq 1\%$ (data not shown).
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Results:

Macrophage ingestion of *S. pneumoniae* is associated with cathepsin D activation

Following phagocytosis of *S. pneumoniae*, the fusion of lysosomes with the phagosome, generates a phagolysosome where bacteria are killed (30). Of the proteolytic enzymes present, cathepsin D is the most abundant of the cathepsin proteases (9). To confirm that *S. pneumoniae* infection activated cathepsin D in MDM, a fluorogenic substrate of cathepsin D was used as a marker of activation. D39 infected cells showed significant activation of cathepsin D at 16 h post-infection, compared to mock-infected cells (Figure 2A). This result was corroborated by Western blot on differentiated THP-1 cells 16 h post infection (Figure 2B). Infection caused an increase in the active 35 kDa form of cathepsin D (31, 32).

*A quantitative proteomic approach demonstrates a wide range of proteins are regulated by cathepsin D following exposure to *S. pneumoniae*. *

Cathepsin D activates multiple cellular pathways (33-35). In view of the broad substrate range of cathepsin D (36), we opted to perform analysis using iTRAQ to allow an unbiased quantitative proteomic approach to examine how cathepsin D activation regulated the macrophage proteome during *S. pneumoniae* infection.

Macrophages were exposed to *S. pneumoniae* (D39) in the presence or absence of the cathepsin D inhibitor pepstatin A (PepA). Cells were analyzed 16 h post infection, in the late stages of the antimicrobial response, a time at which a program of apoptosis is initiated and provides a late increment to bacterial killing in this model (18). All MS/MS data were submitted to our Phenyx server and as a result, 15,734 peptides corresponding to 679 proteins were identified (Supplemental tables S1 and S2).
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false positive peptide discovery rate of 1.1 % was detected. The numbers of peptides contributing to each detected protein are also presented (Supplemental table S1). Example spectra for iTRAQ labelled peptide fragmentation of high and low intensities are also shown (Supplemental figure 1).

In order to better assess cathepsin D regulated proteins involved in apoptosis, we used a statistical method based on peptide-level intensities of iTRAQ reporters to determine which proteins show differential regulation to statistically significant levels during infection in the presence or absence of cathepsins D activation. The application of median-corrected intensities at the peptide level, rather than protein level, provides more accuracy for distinguishing smaller changes in protein expression (28). All proteins quantified here are presented showing the relationship between relative abundance ratios and p-values (from t-test) to give an indication of statistical significance.

We focused our attention on the comparison of biological triplicates of *S. pneumoniae* infection in the presence or absence of pepstatin A (D39+pepstatin A vs. D39) to identify cathepsin D regulated proteins. We assumed that most cathepsin D regulated proteins would in fact also be differentially regulated by infection, since our prior data showed there was little cathepsin D activation in unstimulated macrophages in the absence of exposure to *S. pneumoniae* (15). Although we planned to use subsequent Western blotting to validate this our experimental protocol using an 8-plex iTRAQ technique allowed us to include a biological duplicate of mock-infected cells for comparison with the biological triplicate of *S. pneumoniae* challenged cells incubated without pepstatin A (MI vs. D39), see Figure 1. If we considered proteins identified
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by at least two distinct peptides and ratios with p-values ≤ 0.05, eighteen proteins with
differential expression regulated by cathepsin D were identified (Table 1 and
Supplementary table S3). Using these criteria, we identified 26 proteins that were
differentially expressed following exposure to S. pneumoniae (Supplemental table
S4).

Of the 18 proteins (2.7 % of the 679) that showed cathepsin D mediated regulation
(Table 1), ten proteins were elevated and eight proteins reduced in D39+ Pepstatin A
samples vs. D39. Functional classification of these 18 proteins using Ingenuity
Pathway Analysis showed that they could be grouped into a number of different
functional categories, including cellular assembly and organization, and cell
morphology (Table 2). However, the most significant classification grouping was that
of cell death. Indeed, twelve (66.7%) of the 18 differentially expressed proteins were
identified as being directly or indirectly involved in cell death processes (Table 2).

Given that cathepsin D has previously been implicated in the regulation of apoptosis
pathways in myeloid cells (13), and we have shown macrophage apoptosis in
response to S. pneumoniae is necessary for bactericidal clearance in this model (17,
18), several of these proteins were investigated further to determine how cathepsin D
activation might influence pathways involved in the regulation of macrophage
apoptosis during S. pneumoniae exposure.

Validation of proteomic data

Differentiated THP-1 cells, under the same conditions as used for the iTRAQ
analysis, were lysed and Western blots performed to verify the proteomics findings. In
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total, five proteins were examined further, all known to have links to cell death processes. In every case, Western blot analysis with densitometry performed on four biological replicates confirmed the findings of the iTRAQ analysis, providing evidence, not only that each protein was differentially expressed in the presence of pepstatin A, but also that each protein was differentially regulated by infection. Superoxide dismutase (SOD)-2 (decreased 1.68 fold, p=0.005 in D39+pepstatin A vs. D39 by iTRAQ analysis) and glucose sensitive heat shock protein (Hsp) A5, also termed glucose-regulated protein of 78 kDa (Grp78) or BiP, (decreased 1.15 fold, p=0.006 in D39+pepstatin A vs. D39 by iTRAQ analysis), were both elevated in infection, but upregulation was reversed by pepstatin A indicating a role for cathepsin D in protein upregulation (Figure 3A and B). In contrast, the actin regulatory protein gelsolin (upregulated 1.22 fold, p=0.0005 in D39+pepstatin A vs. D39 by iTRAQ analysis) and the translation factor eukaryotic elongation factor (eEF)2 (upregulated 2.30 fold, p=0.00005 in D39+pepstatin A vs. D39 by iTRAQ analysis) were downregulated by Western blot following S. pneumoniae exposure, a reduction that was blocked by pepstatin, implicating cathepsin D in the down-regulation observed (Figure 4A and B). A fifth protein, the calcium binding protein, which enhances the transcriptional activity of the tumor suppressor p53 (37) and can increase transcription of caspase 3 (38), S100A6 or calcyclin (upregulated 1.86 fold, p=0.0000003 in D39+pepstatin A vs. D39 by iTRAQ analysis), was upregulated during infection, but in this case pepstatin A treatment resulted in further upregulation (Figure 4C). These results indicate that the iTRAQ analysis appeared to have identified potential cathepsin D targets with known roles in the regulation of cell survival.
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Pharmacological inhibition of cathepsin D could however in theory have off-target effects. To confirm our findings further we also repeated Western blots and densitometry on BMDM derived from cathepsin D deficient infant mice or their wild-type littermates. This confirmed cathepsin D dependent upregulation of SOD-2 and HspA5 (Figure 5A-B) and downregulation of gelsolin and eEF2 (Figure 6A-B) following infection, with an increase in S100A6 following infection that was further increased in the absence of cathepsin D (Figure 6C).

Superoxide dismutase (SOD)-2 is upregulated in macrophages during S. pneumoniae infection in a cathepsin D dependent manner.

The first protein of interest to be investigated further was SOD-2. SOD-2 is a protein which protects mitochondria against oxidative stress (39). The iTRAQ analysis, verified by Western blots, documented cathepsin D-dependent SOD-2 upregulation. To validate these results in primary cells, and to elucidate the potential kinetics of this upregulation, a timecourse was performed using MDM. Western blot analysis demonstrated that S. pneumoniae infection dramatically increased SOD-2 levels from 8 h post-infection (Figure 7A). S. pneumoniae exposed cells treated with pepstatin A showed reduced SOD-2 expression compared to non-treated cells. Enhanced expression of SOD-2 is considered one of the first lines of defense against excess levels of ROS, which are produced by activated macrophages (40, 41), and SOD-2 is known to be differentially expressed following bacterial infection (42, 43). In order to confirm enhanced ROS production in our model and relate its kinetics to SOD-2 upregulation, MDM production of ROS was measured using a fluorescent reporter for ROS, DCF. ROS production increased from 8 h after exposure to S. pneumoniae.
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(Figure 7B). The kinetics of ROS production mirrored the upregulation of mitochondrial SOD-2.

**Gelsolin is downregulated by cathepsin D in macrophages following S. pneumoniae infection**

The next protein of interest to be studied further was the actin regulatory protein gelsolin (44). Gelsolin reduces apoptosis both at the level of mitochondrial outer membrane permeabilization (45) and caspase activation (46, 47). A time course of gelsolin expression in MDM revealed downregulation in a cathepsin D-dependent fashion from 12 h post-infection, with maximal downregulation observed at 16 h (Figure 8A). The maximal downregulation coincided with cytochrome c translocation to the cytosol (Figure 8B), a hallmark of mitochondrial outer membrane permeabilization (MOMP), which is widely recognized as being the point of no return in an apoptotic programme of death (48). These data suggest a model in which cathepsin D acts to downregulate gelsolin, thus contributing to the destabilization of the mitochondria, leading to apoptosis.

**Cathepsin D induces eukaryotic elongation factor 2 (eEF2) downregulation enhancing apoptosis and bacterial killing.**

The eukaryotic elongation factor 2 (eEF2) was also identified as a factor downregulated by cathepsin D. eEF2 was reduced 16-20 h post-infection in a cathepsin D-dependent fashion (Figure 9A). Proteins with short half-lives, such as Mcl-1, a key regulator of macrophage susceptibility to apoptosis, including during S. pneumoniae infection, are exquisitely sensitive to alterations in protein translation (18, 49, 50). eEF2 activity is negatively regulated by eEF2 kinase, which prevents
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protein translation via the phosphorylation of eEF2 (51). During cellular stress, eEF2 kinase is activated with the purpose of reducing protein synthesis and conserving cellular energy sources (21, 52). We found an increase in eEF2 kinase activity 12-16 h post-infection (Figure 9B), but there was no evidence that this was regulated by cathepsin D activation (data not shown). By studying macrophages deficient in eEF2 kinase we could examine the role of eEF2 using a system where eEF2 activity is inappropriately prolonged in the absence of the normal inhibitory effect of phosphorylation. As compared to wild-type bone marrow-derived macrophages (BMDM), BMDM from eEF2 kinase−/− mice (53), maintained Mcl-1 levels (Figure 9C) and had delayed dissipation of Δψm after S. pneumoniae infection (Figure 9D-E).

We conclude from these results that inactivation of eEF2-dependent protein translation is required for Mcl-1 downregulation after S. pneumoniae infection and that cathepsin D induced downregulation of eEF2 can drive apoptosis by reducing the translation of Mcl-1.

One of the consequences of macrophage apoptosis during S. pneumoniae infection is to increase bacterial killing (18). To test whether our proteomic screen was identifying targets that could link macrophage apoptosis with bacterial killing, we measured bacterial clearance in the eEF2 kinase−/− mice that had preservation of Mcl-1 and delayed macrophage apoptosis. Pneumococcal clearance was reduced by approximately 0.5 log in eEF2 kinase−/− mouse lungs (Figure 10). This difference reflects the relatively modest effect on apoptosis of maintaining active eEF2 by inhibition of eEF2 phosphorylation, since cathepsin D activation still induced eEF2 downregulation and also the fact that cathepsin D can exert its effects on macrophage survival at several points, as this screen has shown. However the reduction in
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bacterial killing did establish that our screen identified proteins that not only influenced macrophage apoptosis but also bacterial clearance \textit{in vivo}. 
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Discussion:
To better understand how the co-ordination of antimicrobial host defense in the phagolysosome regulates macrophage survival we have examined how activation of one of the most abundant lysosomal proteases, cathepsin D, influences the macrophage proteome (9). We show that a range of proteins are differentially expressed in the presence of cathepsin D, with a high proportion predicted to impact the regulation of the mitochondrial pathway of apoptosis. Western blotting validated a number of the proteins identified by proteomic analysis and we found evidence that these changes fit well with the temporal sequence of molecular events that are associated with macrophage apoptosis during *S. pneumoniae* infection, a process we have recently demonstrated involves cathepsin D activation (15).

To identify cathepsin D regulated proteins following the internalization of *S. pneumoniae* into macrophage phagolysosomes we have focused on comparison of biological triplicates of macrophages exposed to *S. pneumoniae* in the presence or absence of the cathepsin D inhibitor pepstatin A. We focused on this comparison since our prior data showed that there was limited activation of cathepsin D in unstimulated macrophages in the absence of exposure to *S. pneumoniae* (15). Our approach was not based on a fold cut-off since our prior results suggest this approach can have limitations (28). Instead our statistical approach involved recently described methodology for quantitative proteomic analysis (25, 54, 55). The use of median-corrected intensities and t-tests at the peptide level rather than at the protein level offer higher significance levels and allow the detection of smaller fold changes (28). We believe this was an appropriate approach in the current study. Small fold changes in regulators of apoptosis can have significant effects on phenotype. For example we
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previously found that a 1.3 fold change in the anti-apoptotic protein Mcl-1 was associated with a pro-survival effect in the early stages of microbial killing by macrophages and helped the cell withstand the pro-apoptotic effects of increased cellular stress (18). We were therefore keen not to exclude proteins with low levels of differential fold regulation in our model. In addition we have shown iTRAQ can suppress fold change (25, 56) so we elected to identify targets with the iTRAQ approach, without excluding those with lower fold changes, but to validate these vigorously by Western blotting.

The validation approach was also critical to proving that the differentially regulated proteins we identified in cells exposed to *S. pneumoniae*, in the presence or absence of pepstatin A, were also differentially regulated by infection itself. Although we believed there was not significant cathepsin D activity in unstimulated mock-infected cells our comparison of the mock-infected biological replicate versus the *S. pneumoniae* exposed triplicate only identified 2 of our 5 targets (with a third having a p value that almost reached statistical significance p=0.058, data not shown) but this appeared to reflect the stringency of our statistical approach since all five proteins were differentially regulated by Western blotting. Thus our approach showed it could identify relevant targets when biological triplicates were compared but its sensitivity was reduced when a biological duplicate was used. On the other hand the variation between the biological duplicates or triplicates was much less than the variation between experimental conditions suggesting that those differentially regulated proteins represented were unlikely to be false positives (Supplemental figure S2).
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Studies of the macrophage transcriptome in response to *Streptococcus* spp. reveal early upregulation of multiple genes involved in immune responses, including those involved in the regulation of apoptosis (57, 58). At our late time point after bacterial challenge our analysis did not find identify many proteins with primary host defense functions, but confirmed that many proteins regulating cell death pathways were prominently regulated. Similar findings have been found following challenge of macrophages with viruses such as Influenza A virus and epithelial cells with respiratory viruses, with several regulators of apoptosis, that we identified, also showing differential regulation in these studies (59, 60).

Cathepsin D activity is maximal at low pH with a broad substrate range but the majority of the proteins we identified as being regulated by cathepsin D are not localized to the phagolysosomal compartment (36, 61). The proteomic approach we employed does not identify cleavage events and therefore does not characterize the cell degradome, unlike some recently described approaches (62). The changes to the cell proteome are therefore likely to reflect a broad range of indirect effects. Many of the proteins were upregulated, emphasizing that direct enzymatic degradation of targets was not the sole mechanism. The findings are in line with recent data which emphasize that key cell proteases active during apoptosis, such as caspases, can induce extensive changes to the cell proteome not just through the degradation of protein targets but also indirectly by altering gene transcription and protein-protein interactions (62).

The data set of proteins regulated by cathepsin D is enriched for proteins predicted to influence a variety of cell death pathways. We have previously shown ROS are not
Cathepsin D regulated proteins

required for effective killing of *S. pneumoniae* (63). SOD-2 converts superoxide anions into hydrogen peroxide (41), but will not influence bacterial killing. Converting superoxide to hydrogen peroxide does not prevent apoptosis since both species can induce apoptosis (64, 65). However by limiting the concentration of superoxide SOD-2 may enable preferential permeabilization of the outer mitochondrial membrane and therefore apoptosis development, rather than permeabilization of the inner mitochondrial membrane (66), which would trigger an alternative non-apoptotic caspase-independent programmed cell death (67-69). The induction of SOD-2 fitted well with the kinetics of ROS generation. SOD-2 generation did not result in a decrease in overall ROS, since it regulates mitochondrial ROS (41) and since ROS was measured by DCF, which measures a variety of ROS species (70) but in this case it is likely to be the kind of ROS and its specific location rather than the overall quantity which influences the death program. Another of the proteins that was upregulated in response to cathepsin D activation, HspA5, is a marker of ER stress (71). It is induced by oxidant stress and protects against certain forms of ROS-induced cell death so potentially could also dampen down the effects of excessive mitochondrial injury and necroptosis Although HspA5 can limit induction of apoptosis in certain circumstances (71), in the context of a model with prominent apoptotic cell death, these observations suggest that cathepsin D may regulate the tension between different cell death pathways during the cell stress associated with innate immune responses in macrophages.

Interestingly, our study revealed another protein, S100A6 or calcyclin, was upregulated in infection, but in this case pepstatin A treatment did not reverse the change but further increased upregulation. S100A6 enhances the transcriptional
Cathepsin D regulated proteins

activity of the tumor suppressor p53 (37) and elevated levels of S100A6 enhance apoptosis by inducing transcriptional upregulation of caspase 3 (38). It could therefore increase the susceptibility to apoptosis after infection. It therefore might seem counter-intuitive that cathepsin D inhibition further increased S100A6 expression but cathepsin D might be acting to slow down the increase of S100A6, putting a brake on the onset of apoptosis and allowing the bactericidal function of the macrophage to continue.

Although cathepsin D inhibited competing death pathways, we found evidence that cathepsin D contributed to regulation of the mitochondrial apoptosis pathway at two key points. We have previously shown that Mcl-1 levels determine the onset of MOMP in this model (18, 72). Gelsolin regulates caspase activation (46, 47) and prevents MOMP and translocation of cytochrome c to the cytosol, reducing activation of caspase 9 (45). We documented that gelsolin levels declined immediately prior to cytochrome c translocation. Moreover, we also observed that cathepsin D downregulated eEF2, which catalyzes a key translocation step during protein translation, and is therefore an important regulator of protein synthesis (73, 74). Mcl-1, which has a short half-life of only 20-30 mins is critically dependent on translation to maintain intracellular levels (50). We showed using BMDM lacking eEF2 kinase, a negative regulator of eEF2, that eEF2 activity allows maintenance of Mcl-1 expression, delays the mitochondrial apoptosis pathway and also delays apoptosis-associated macrophage killing of *S. pneumoniae*, in a murine model in which apoptosis contributes to bacterial clearance (17). eEF2 is also downregulated following human metapneumovirus, respiratory syncytial virus and HIV-1 infection, with increased apoptosis susceptibility (59, 75).
Cathepsin D regulated proteins

Modulation of protein translation via eEF2 is not a recognized point of regulation for innate host responses so this is a novel finding of our study. Microbial factors such as diphtheria toxin and the exotoxin A from *Pseudomonas aeruginosa* exploit manipulation of protein translation by targeting eEF2 for ADP-ribosylation (74), emphasizing the importance of this cell function to host responses. Protein translation is highly efficient in alveolar macrophages and maintenance of factors regulating protein translation, in their optimal phosphorylation state, is one mechanism accounting for the longevity of differentiated tissue macrophages (76). Modulation of protein translation is a potent point of regulation for macrophage commitment to apoptosis, which is manifested by alteration in molecules with very short half-lives, such as FLIP (73), or in our model Mcl-1 (18).

In conclusion, our results suggest that cathepsin D activation during host defense has multiple effects on the macrophage proteome with convergence on those pathways regulating cell death. These alterations stimulate the mitochondrial pathway of apoptosis but inhibit competing cell death pathways. This emphasizes the complexity of regulation of cell death during innate responses and emphasizes that regulation of cell death involves appropriate control of competing pathways to allow the coordinated induction of cell death. These results emphasize the potential of proteomics to identify novel points of regulation of death processes, as exemplified by our novel finding that regulation of protein translation is a key molecular switch controlling the commitment to apoptosis during the execution of antimicrobial host defense.
Cathepsin D regulated proteins

**Acknowledgments:**

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References:

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cathepsin D from normal and malignant human breast cell lines. *Breast Cancer Res Treat* 35,211-220


Cathepsin D regulated proteins


Cathepsin D regulated proteins

**Figure Legends:**

**Figure 1: Experimental schematic.** A biological duplicate of mock-infected (MI) cells and two biological triplicates of *Streptococcus pneumoniae* exposed cells incubated with vehicle control (D39) or with pepstatin A (D39 + Pepstatin A) were used for analysis.

**Figure 2: Infection with *Streptococcus pneumoniae* is associated with activation of cathepsin D in macrophages.** (A) Cathepsin D activity was measured in whole cell lysates at 16 h in mock-infected (Spn-), or *Streptococcus pneumoniae* exposed (Spn+) monocyte-derived macrophages, n=4, ***= p<0.001, Student’s t-test. (B) Western blot of differentiated THP-1 cells 16 h after mock-infection (Spn-) or exposure to *Streptococcus pneumoniae* (Spn+). The blot is representative of three independent infections.

**Figure 3: Validation of iTRAQ analysis for SOD-2 and HspA5 in differentiated THP-1 macrophages.** Representative Western blots of total protein from mock-infected (Spn-) or *Streptococcus pneumoniae* exposed (Spn+) differentiated THP-1 macrophages, 16 h after infection, cultured in the presence (+) or absence (-) of pepstatin A (PepA), probed for (A) superoxide dismutase-2 (SOD-2) or (B) heat shock protein A5 (HspA5). Densitometry was carried out on each Western blot and each protein’s fold change was compared relative to the mock-infected (MI) level after
Cathepsin D regulated proteins

adjustment for any fold change in tubulin, n=4 *= p< 0.05, 1-way ANOVA with Bonferroni post-test.

**Figure 4: Validation of iTRAQ analysis for gelsolin, eukaryotic elongation factor 2 (eEF2) and calcyclin (S100A6) in differentiated THP-1 macrophages.**
Representative Western blots of total protein from mock-infected (Spn-) or *Streptococcus pneumoniae* exposed (Spn+) differentiated THP-1 macrophages in the presence (+) or absence (-) of pepstatin A (PepA) probed for (A) gelsolin, (B) eukaryotic elongation factor 2 (eEF2) or (C) calcyclin (S100A6) 16 h post-infection. Densitometry was carried out and each protein’s fold change was compared relative to the mock-infected (MI) level after adjustment for any fold change in tubulin, n=4 *= p< 0.05, 1-way ANOVA with Bonferroni’s post-test.

**Figure 5: Cathepsin D deficient macrophages validate iTRAQ findings for SOD-2 and HspA5.** Representative Western blots for (A) SOD-2 and (B) HspA5 from wild-type (WT) and Cathepsin D knock-out (KO) BMDMs 16 h after mock- infection (Spn-) or exposure to *Streptococcus pneumoniae* (Spn+), in the presence (+) or absence (-) of pepstatin A (PepA). Blots are representative of three independent experiments. Densitometry was carried out and each protein’s fold change was calculated relative to the mock-infected (MI) level after adjustment for any fold change in tubulin, n=3 *= p< 0.05, 2-way ANOVA with Bonferroni post-test.

**Figure 6: Cathepsin D deficient macrophages validate iTRAQ findings for gelsolin, eEF2 and S100A6.** Representative Western blots for (A) gelsolin (B) eukaryotic elongation factor 2 (eEF2) and (C) S100A6 from wild-type (WT) and
Cathepsin D regulated proteins

Cathepsin D knock-out (KO) BMDMs 16 h after mock-infection (Spn-) or *Streptococcus pneumoniae* exposure (Spn+), in the presence (+) or absence (-) of pepstatin A. Blots are representative of three independent experiments. Densitometry was carried out and each protein’s fold change was calculated relative to the mock-infected (MI) levels after adjustment for any fold change in tubulin, n=3 *= p< 0.05, 2-way ANOVA with Bonferroni post-test.

Figure 7: *Streptococcus pneumoniae* infection induces cathepsin D dependent upregulation of superoxide dismutase-2 (SOD-2). (A) Representative Western blots probed for superoxide dismutase-2 (SOD-2) from mock-infected (Spn-) or *Streptococcus pneumoniae* exposed (Spn+) monocyte-derived macrophages (MDM) cultured in the presence (+) or absence (-) of pepstatin A (PepA) at the designated time points after challenge. Densitometry was carried out and fold change was calculated relative to the mock-infected (MI) level after adjustment for any fold change in tubulin, n=3. (B) Intracellular ROS was measured at the indicated timepoints in MDM, mock-infected (MI) or *Streptococcus pneumoniae* exposed (D39), in the presence of vehicle control or pepstatin A (+P). Data are from six separate donors. * p< 0.05, *** p< 0.001, 2-way ANOVA with Bonferroni post-test, comparing MI vs D39.

Figure 8: *Streptococcus pneumoniae* infection induces cathepsin D dependent downregulation of gelsolin. (A) Representative Western blots probed for gelsolin from mock-infected (Spn-) or *Streptococcus pneumoniae* exposed (Spn+) monocyte-derived macrophages (MDM) cultured in the presence (+) or absence (-) of pepstatin A
Cathepsin D regulated proteins (PepA) for the indicated time periods. Densitometry was carried out and fold change was calculated using mock-infected (MI) levels after adjustment for any fold change in tubulin, n=3, ** p< 0.01, 2-way ANOVA with Bonferroni post-test. (B) Cytosolic fractions were obtained from mock-infected (Spn-) or Streptococcus pneumoniae exposed (Spn+) monocyte-derived macrophages (MDM) at the designated time points. Western blots were probed for cytochrome c, and actin was used as a cytosolic loading control. The blots are representative of three independent experiments.

**Figure 9: Cathepsin D-mediated downregulation of eukaryotic elongation factor 2 (eEF2) has functional consequences for the regulation of the mitochondrial pathway of apoptosis.** (A) Western blots of total protein from mock-infected (Spn-) or Streptococcus pneumoniae exposed (Spn+) differentiated THP-1 cells at the designated time points in the presence (+) or absence (-) of pepstatin A (PepA) were probed for eukaryotic elongation factor 2 (eEF2). The blots depicted are representative of three independent experiments Densitometry was carried out and fold change was calculated using the mock-infected (MI) level after adjustment for any fold change in actin, n=3, * p< 0.05, 2-way ANOVA with Bonferroni post-test. (B) Western blots probed for phospho-eEF2 (peEF2) from Spn- or Spn+ differentiated THP-1 cells at the indicated time points after bacterial challenge. The blots are representative of three independent experiments. (C) Western blot of protein probed for myeloid cell leukemia (Mcl)-1 from wild-type (WT) or eEF2 kinase knock-out (KO) bone marrow-derived macrophages (BMDM), mock-infected (Spn-) or challenged with Streptococcus pneumoniae (Spn+), in the presence (+) or absence (-) of pepstatin A (PepA) and cultured for 16 h. The blots are representative of three independent experiments Densitometry was carried out and fold change was
Cathepsin D regulated proteins calculated using mock-infected (MI) levels after adjustment for any fold change in actin, n=3, * p< 0.05, 1-way ANOVA with Dunnet’s post-test vs. MI (D). Representative histograms and (E) pooled data from JC-1 staining of BMDMs expressing (WT) or deficient (KO) in eEF2 kinase. In the histograms grey fill represents Spn-, red fills Spn+. The pooled data shows the percentage of cells showing loss of inner mitochondrial transmembrane potential (∆ψₘ), n=3, *= p< 0.05, 2-way ANOVA with Bonferroni post-test.

**Figure 10: Eukaryotic elongation factor 2 (eEF2) kinase deficient mice have reduced bacterial clearance.** Bacterial counts were estimated in lungs from wild-type (WT) or eukaryotic elongation factor 2 kinase deficient (KO) mice infected with 5x10⁵ colony forming units of type one *Streptococcus pneumoniae* for 24 h, n=11 per group, * p< 0.05, Mann-Whitney U test. Two WT mice and one eEF2k KO mouse cleared the bacteria completely.
Table 1: Proteins showing significant differential expression in D39 + pepstatin A samples vs. D39. A list of identifying peptide sequences can be found in supplemental table S1 in the supplemental data.
Table 2: Top five functional classifications (by p-value) of the 18 proteins regulated by cathepsin D in infection (D39 + pepstatin A vs D39), as analysed by Ingenuity Pathway Analysis.

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Cathepsin D regulated proteins

Figure 1
Figure 2

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Cathepsin D regulated proteins

[Graph showing Cathepsin D activity (% of control) with Spn conditions: -, +]

[Image showing Western blot for Active Cat D and Actin with Spn conditions: -, +]
Cathepsin D regulated proteins

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Cathepsin D regulated proteins

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Fold change from MI

- 0.00
- 0.25
- 0.50
- 0.75
- 1.00

- 1.25
- 1.50

* statistically significant
Cathepsin D regulated proteins

Figure 5

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Fold change from Mi

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Fold change from MI

- WT
- KO
Figure 7

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SOD-2

Tubulin

B

Fold change from MI

Time post-infection (h)

Molecular & Cellular Proteomics
Cathepsin D regulated proteins

Figure 8

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Gelsolin
Tubulin

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Gelsolin
Tubulin

![Graph showing fold change from MI over time post-infection (h) for Spn and PepA conditions.]

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Cytochrome c
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Cytochrome c
Actin

Legend:
- Pepstatin A
+ Pepstatin A
Cathepsin D regulated proteins

Figure 9

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Cathepsin D regulated proteins

Figure 10