Legend to supplementary figures:

Supplementary Fig. S1: Flow cytometry analysis and clone showing the highest fluorescence levels selected, amplified and used.

(A) Kb heavy chain of the MHC class I molecule is expressed at the cell surface of Raw-Kb murine macrophages. (B) Kb efficiently binds the Kb-restricted epitope SSIEFARL (derived from gB), as revealed by the strong 2E2 activation when exogenous peptide was added to the culture medium for 30 minutes before fixation and addition of the CD8+ T cells. 2E2 activation is measured by quantifying beta-galactosidase produced by the hybridoma.

Supplementary Fig. S2: Fractionation efficiency using immunoblots for several cellular markers

Western blot analysis of 10 µg of protein of total membrane (TM) and total cell lysate (TCL) preparations of RAW 267.4 mouse macrophages. Western blots confirm the high purity of our TM preparations. The following antibodies were used: gamma-actin (actin, cytoplasm), nucleoporin p62 (nucleus), Na+/K+ ATPase (Plasmamembrane), Annexin II (Plasmamembrane), lysosome-associated membrane glycoprotein 1 (LAMP1, lysosomal membrane), mitochondrial import receptor subunit Tom20 (Tom20, mitochondrial membrane).

Supplementary Fig. S3: Reproducibility of peptide intensities across replicates

Scatter plots of abundance measurements for peptide ions identified using mRP-C18, SCX and GELFREE as first dimension of separation. While SCX and GELFREE display quite wide distributions of peptide intensities, mRP-C18 displays a very narrow distribution. mRP-C18 also has the lowest CV of 27% attesting its reproducibility. 91% of quantified peptides using mRP-C18 have been detected in 3 replicates, while in GELFREE only 46% have been detected in 3 replicates respectively.

Supplementary Fig. S4: Comparison of three different fractionation techniques for quantitative membrane proteomics of RAW264.7 macrophages

(A) Venn diagram representation of protein identification obtained using strong cation exchange (SCX), GELFREE and macroporous reversed phase (mRP) fractionation of macrophage membrane proteins. Different fractionation techniques complement each other for comprehensive large-scale proteomics. (B) Distribution of sequence coverage for separation techniques. A higher sequence coverage of identified proteins is typically obtained using mRP fractionation compared to SCX and GELFREE.

Supplementary Fig S5: MS/MS spectra of phosphopeptides.

Annotated MS/MS spectra of phosphopeptides identified in experiments from fractionation of membrane proteins using SCX, GELFREE and mRP (separate pdf file).

Supplementary Fig. S6: Scatter plots of abundance measurements for peptide ions identified in control and TNF-α stimulated extracts

Peptide ion intensity distributions for 3 replicates are very narrow in control and TNF-α stimulated macrophages. 95 % of all ions showed RSD values less than 58 % across all three biological replicates, attesting of the reproducibility of the method.

Supplementary Fig. S7: Fold change measurements for citrate synthase and ATP synthase subunit b

The consistency of fold change measurements is shown for citrate synthase and ATP synthase subunit b, each identified with 9 peptides. The extracted ion chromatogram shows the decrease in peptide ion abundance for a selected peptide. Our workflow enabled accurate detection of protein with high reproducibility as shown by consistent fold changes for all peptides assigned to a specific protein.

Supplementary Fig. S8: MS/MS spectra of ubiquitinated peptides

Annotated MS/MS spectra of ubiquitinated peptides identified in TNF-α activated macrophages (separate pdf file).

Supplementary Fig. S9: Abundance of fluorescently-labeled Annexin A5 at the plasma membrane and 7-amino actinomycin D using flow cytometry

Flow cytometry analyses revealed that apoptotic and dead cells represented approximately 15 % of the cell population in both control and TNF-α activated macrophages. Similar results were also obtained when cells were stained with 7-amino actinomycin D.

Supplementary Fig. S10: Lysosomal degradation activities when stained with Lysosensor

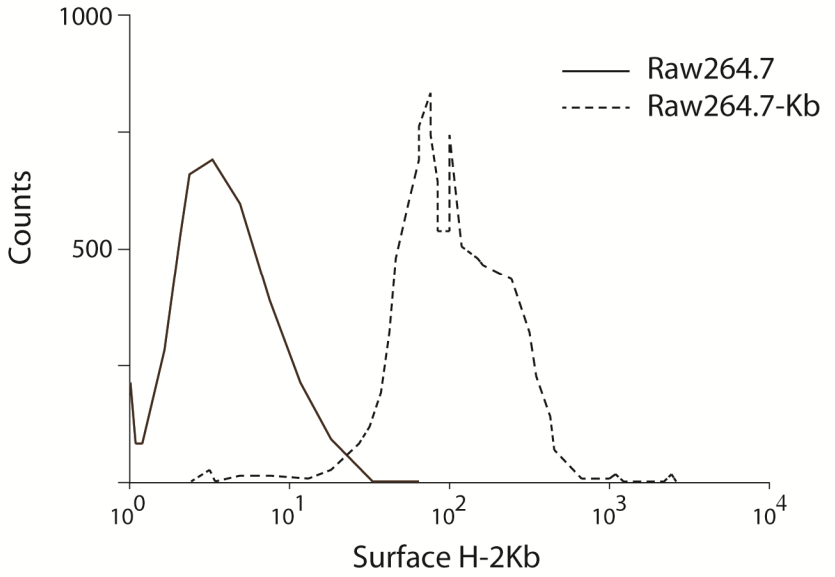
Macrophages stimulated with TNF-α displayed increased lysosomal degradation activities when stained with LysoSensor, a pH-sensitive fluorescent probe that accumulates in acidic organelles.

Supplementary Fig. S11: Integrated model of the TNF-α modulated functions favoring antigen MHC class I presentation

TNF-α can mediate the induction of mitophagy in murine macrophages through the activation of $cPLA_2$. This activation leads to the induction of lipid mediators such as arachidonic acid, which promotes the formation of reactive oxygen species. Increased ROS levels can impair mitochondrial functions resulting in a decrease in their transmembrane potential, $\Delta\Psi_{mt}$. Impaired mitochondrial proteins are engulfed in a double membrane organelle called the autophagosome that later fuses with lysosomes to form autophagolysosomes where they are degraded. Mitochondrial peptides/antigens engulfed in the autophagolysosome can be retrotranslocated into the cytosol where they can be further degraded by the proteasome and processed by the conventional MHC class I machinery. Alternatively, they can remain within the vacuolar compartment where they are degraded by lysosomal proteases, and the resulting peptides are cross-presented to MHC class I molecules.

Supplementary Figure S1.

A)



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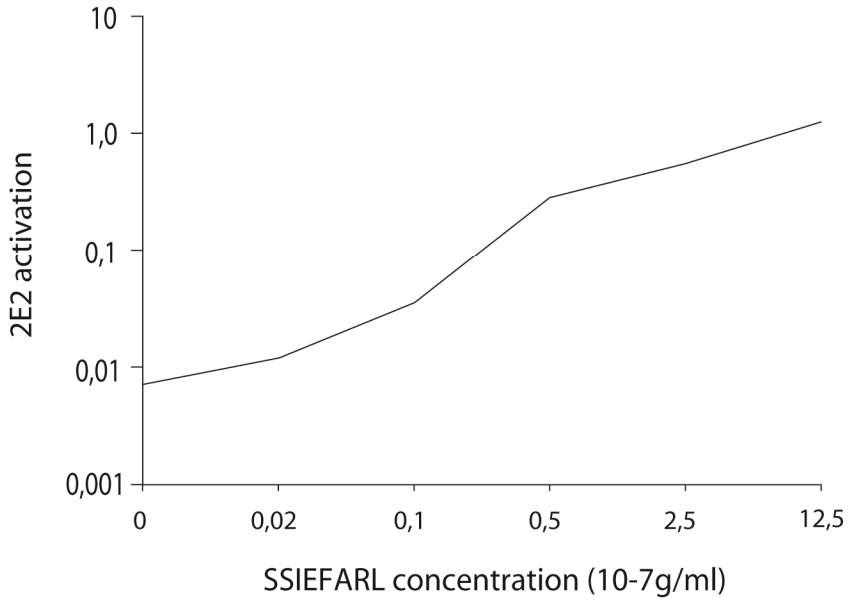
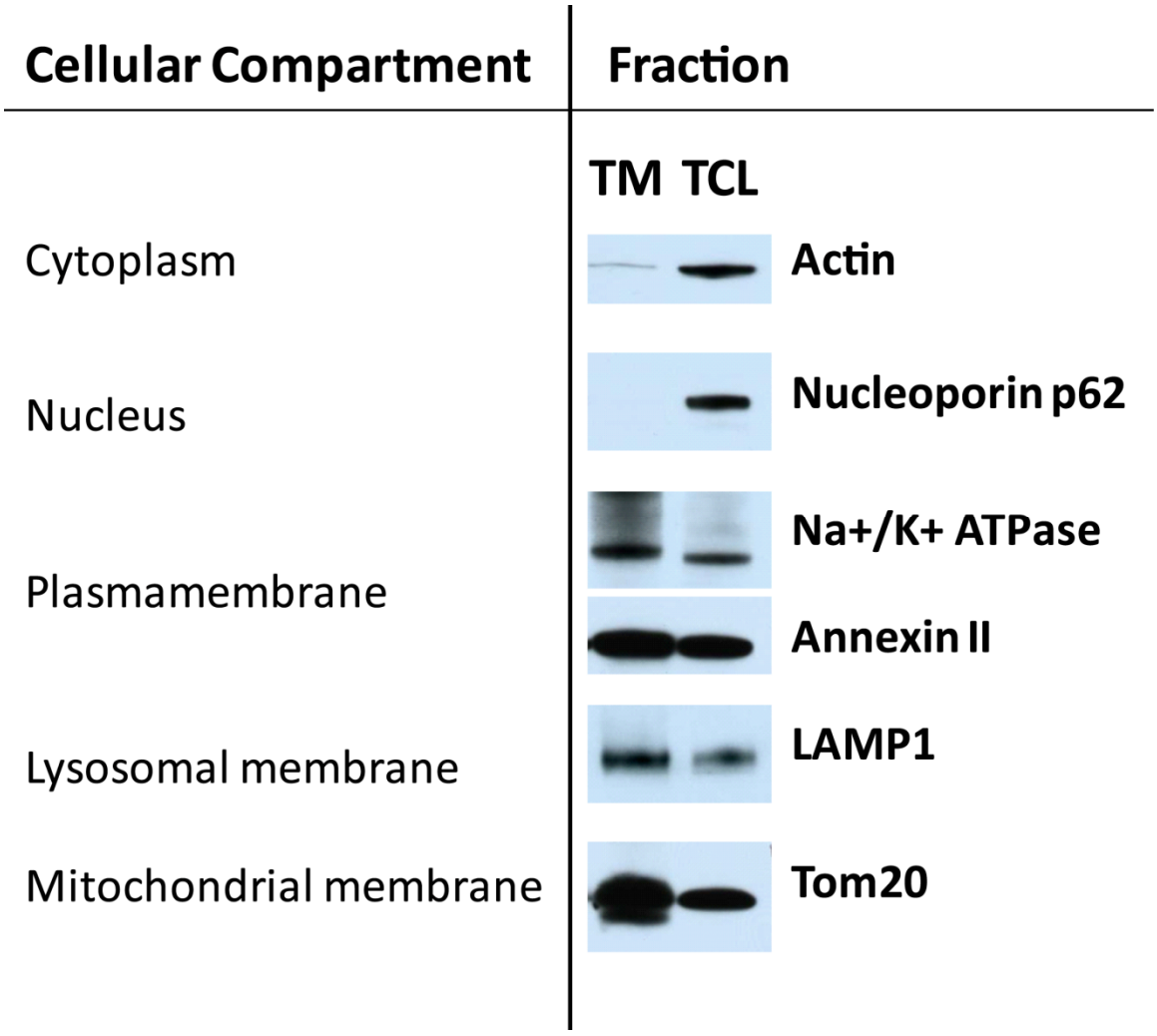
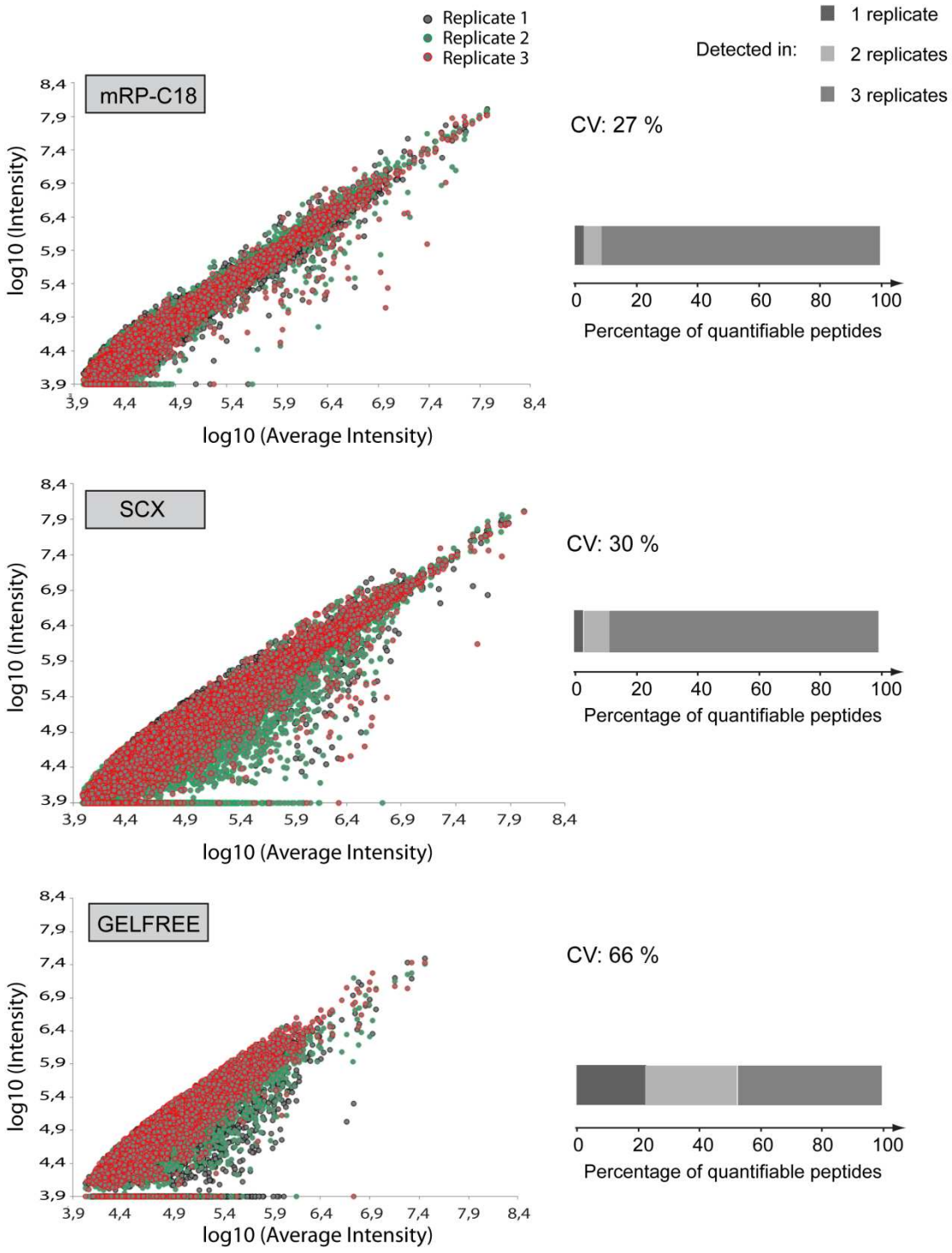


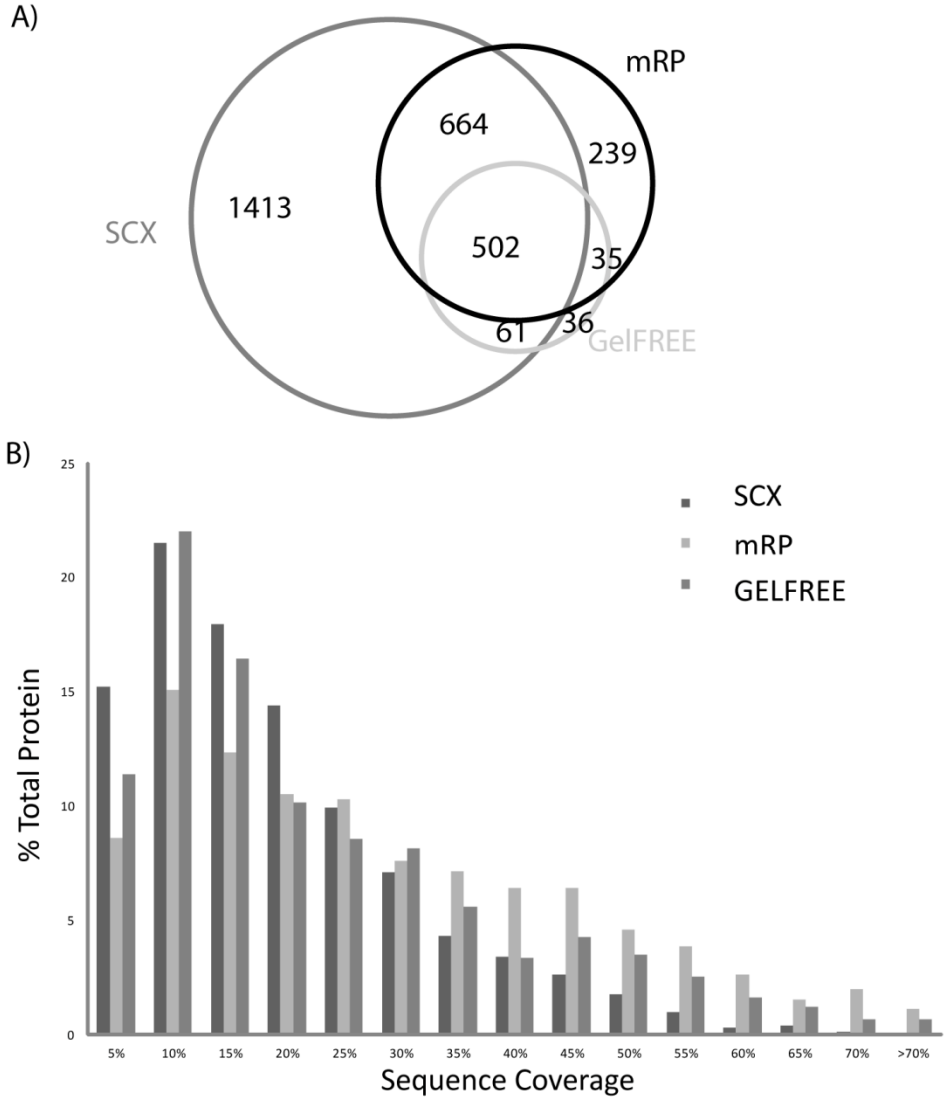
Supplementary Figure S2



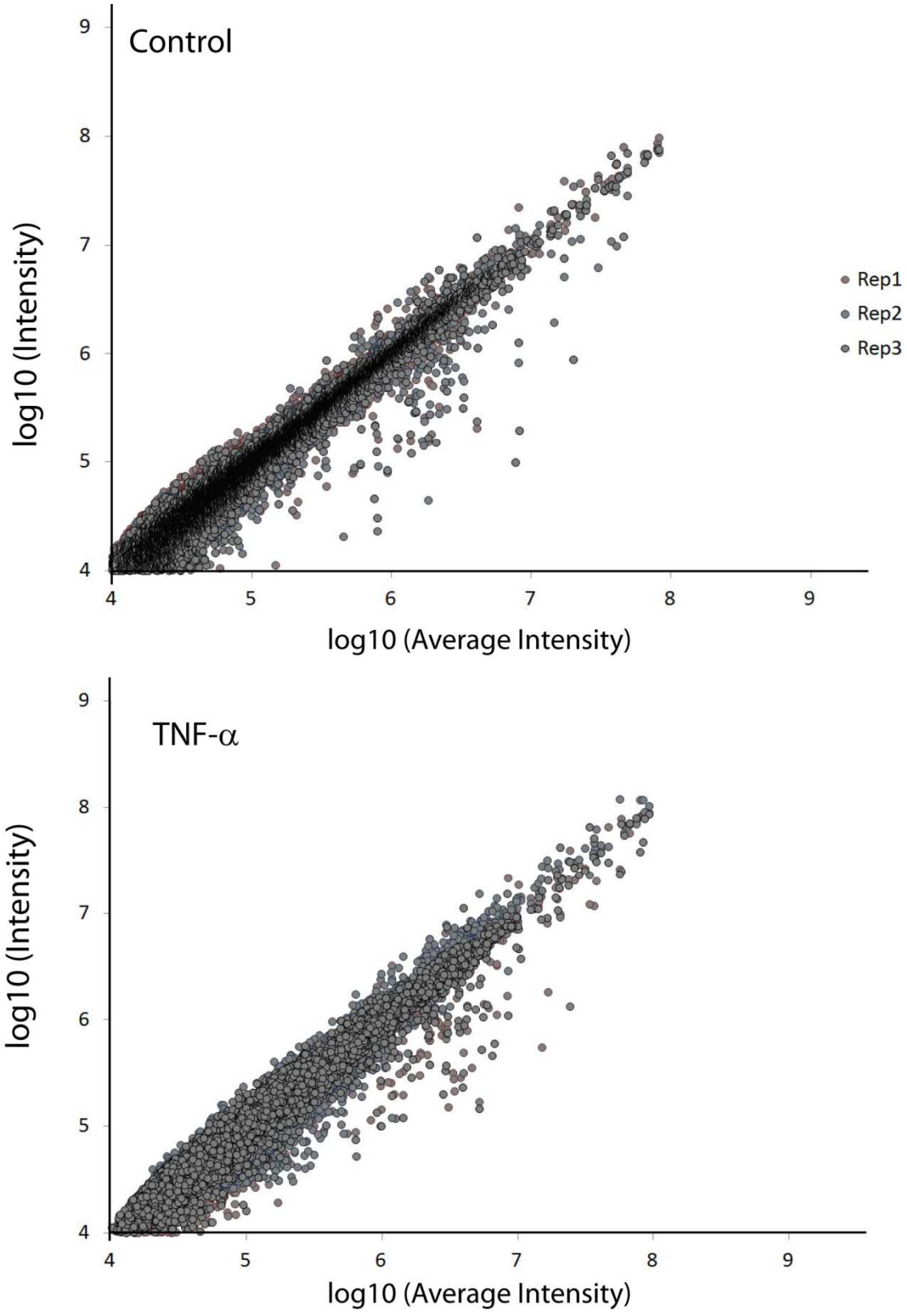
Supplementary Figure S3



Supplementary Figure S4

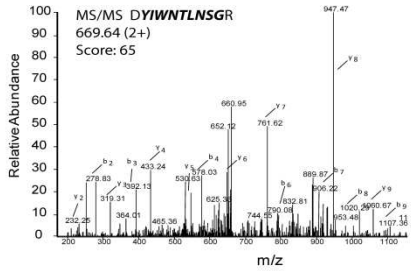


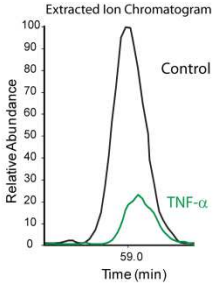
Supplementary Figure S6

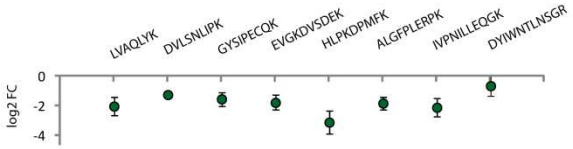


Supplementary Figure S7

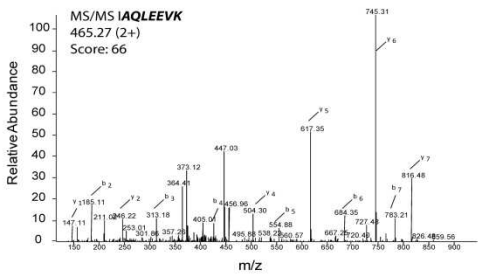
Citrate Synthase
9 peptides
Sequence Coverage: 33%
Proteinfoldchange: -2.2

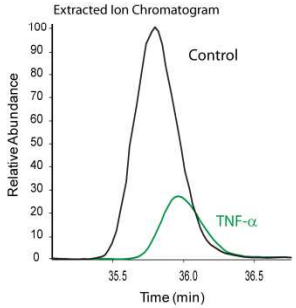


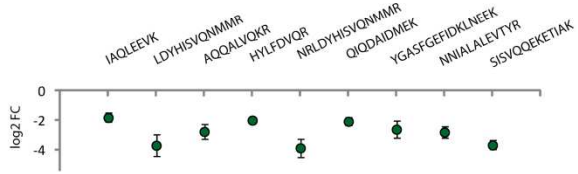




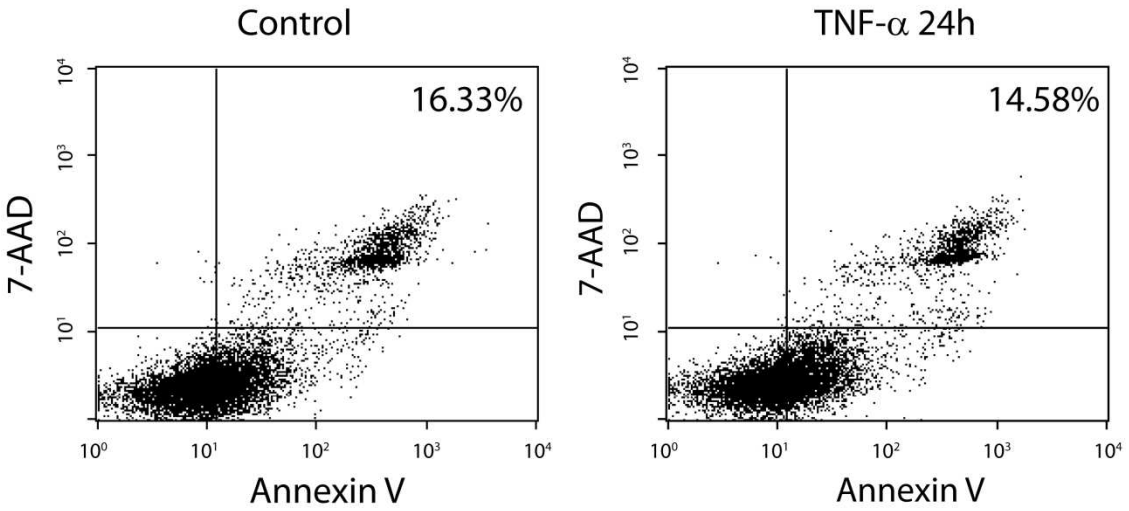
ATP Synthase subunit b
9 peptides
Sequence Coverage: 35%
Proteinfoldchange: - 6.15



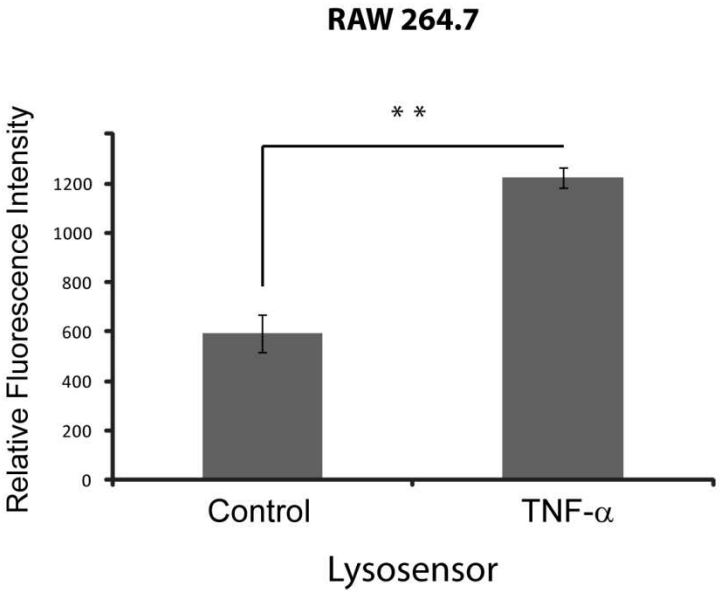




Supplementary Figure S9



Supplementary Figure S10



Supplementary Figure S11