Supplementary Information

Granulocyte Macrophage-Colony Stimulating Factor-induced Zn Sequestration Enhances Macrophage Superoxide and Limits Intracellular Pathogen Survival

Kavitha Subramanian Vignesh[1,2†], Julio A. Landero Figueroa[3†], Aleksey Porollo[4], Joseph A. Caruso[3] and George S. Deepe, Jr[2,5*]

[1]Department of Molecular Genetics, Biochemistry, Microbiology and Immunology, University of Cincinnati, OH 45267 USA

[2]Division of Infectious Diseases, College of Medicine, University of Cincinnati, Cincinnati, OH 45267 USA.

[3]University of Cincinnati / Agilent Technologies Metallomics Center of the Americas, Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221 USA

[4]Department of Environmental Health, University of Cincinnati, Cincinnati, OH 45267 USA

[5]Veterans Affairs Hospital, Cincinnati, OH 45220 USA.

Figure S1:

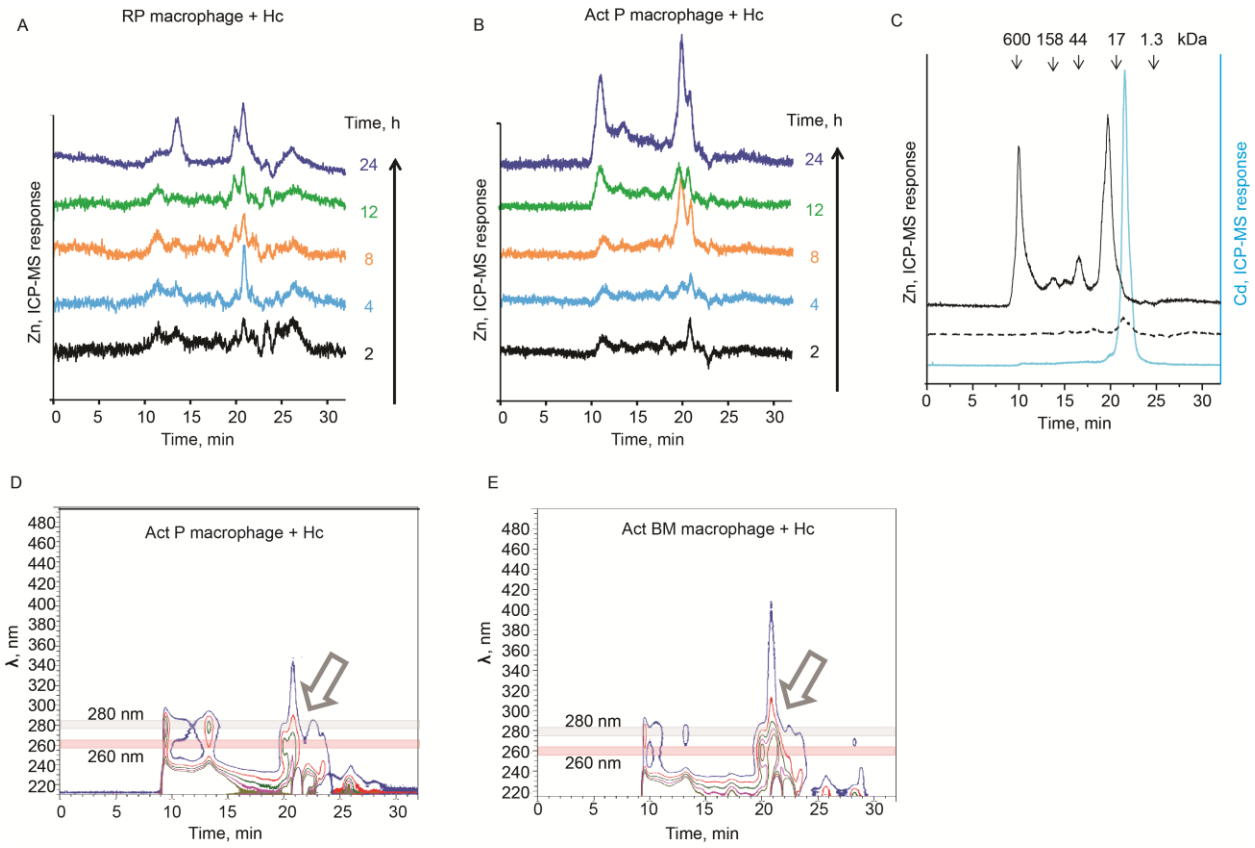

Figure S1: Chromatographic and spectral behavior of MT-Zn signal in macrophages. Related to Figure 1.

(A and B) Resting peritoneal (RP) macrophage and activated peritoneal (Act P) macrophages were infected with *H. capsulatum (*Hc), and SEC-ICP-MS analysis was performed on cell lysates collected at 2 - 24 h time intervals after infection, data are from one experiment; **(C)** SEC-ICP-MS of activated infected bone marrow macrophages (black solid line), the fraction from 19-23 min was collected, denatured and analyzed showing a decrease in Zn signal (black dotted line); the collected fraction was treated with Cd and analyzed again, showing a sharp Cd signal (blue line). The left axis corresponds to the Zn-related signal, and the right axis to the Cd-related signal. The 19-23 min peak corresponding to MTs undergoes a shift in retention time to the low molecular weight region upon treatment with Cd. The Cd signal represents de-oligomerized state

of MTs bound to Cd. The molecular weight in this region corresponds to the MTs in their monomeric state (6-7kDa), 2 independent experiments; **(D and E)** Contour map of UV-Vis absorbance (isoplots) of activated infected peritoneal and bone marrow (BM) macrophages from 210 to 500 nm (Y axis) and 0 to 30 min (X axis), at a response time of 5 spectra per second with a step of 2 nm, during the SEC-DAD-ICP-MS analysis of cell lysates. X axis, time in min; Y axis, wave length (λ), and each progressive line represents an increment of 5 mAu. The maximum of absorbance in each fraction can be seen as a round shape contour pattern, giving information of the composition of species eluted in the fractions. The chromatograms shows maxima at two wave lengths, 280 nm, characteristic of proteins containing aromatic amino acids; and 260 nm, characteristic of nucleic acids (at HMW, first part of the chromatogram) and –S-S-, -S-metal- complexes. The ~20 min region clearly indicates that the absorbance maximum is at 260 nm in all cases, while in the high molecular weight region of the chromatogram both, 280 and 260 nm maxima are observed; representative of 3 independent experiments.

Figure S2:

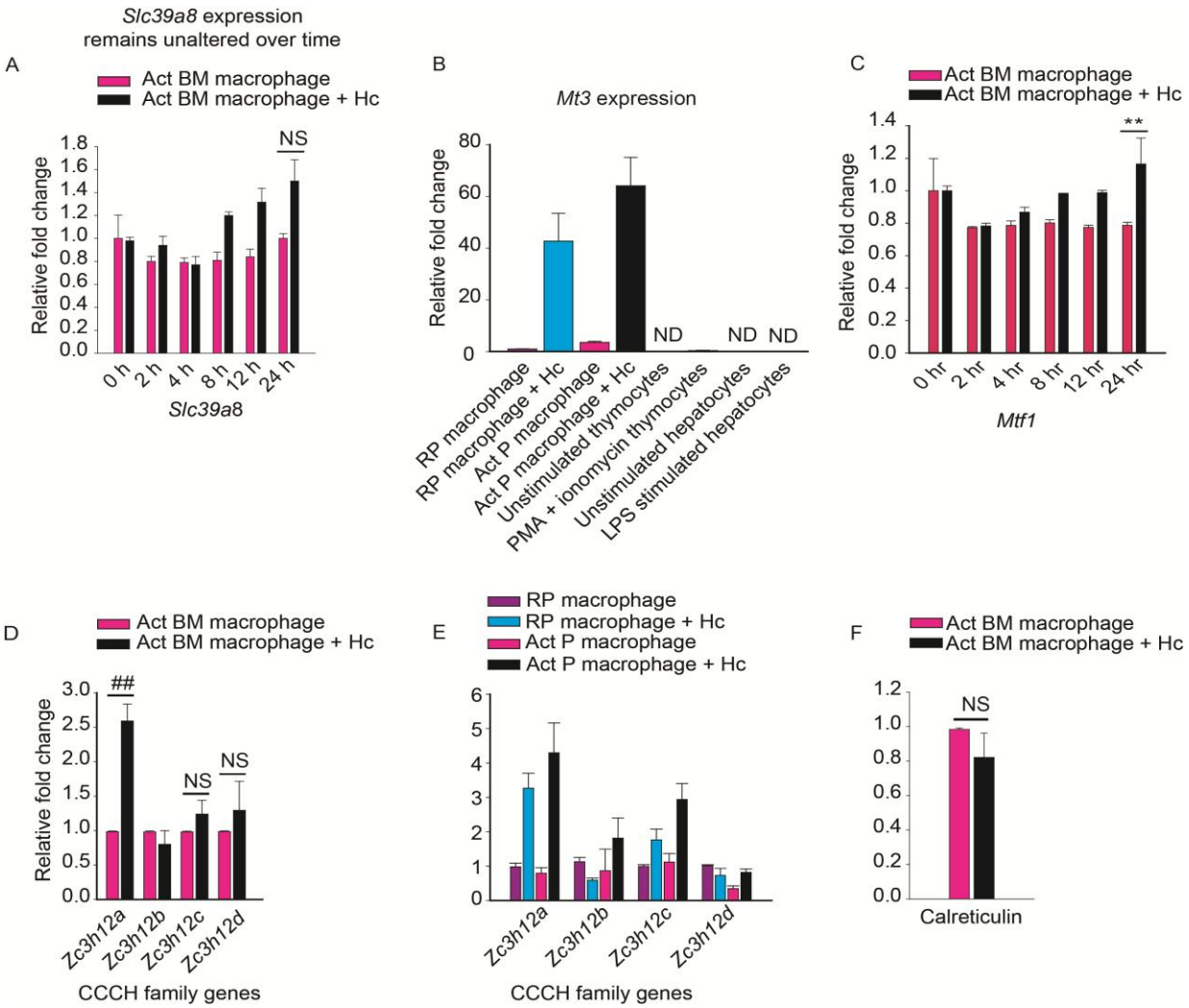

Figure S2: GM-CSF specifically alters Zn transporter and MT regulation. Related to Figure 2.

(A) GM-CSF specifically targets the expression of *Slc39a2*, *Slc30a4* and *Slc30a7*, because *H. capsulatum* (Hc) infected activated bone marrow macrophages (Act BM) do not alter expression of another importer, *Slc39a8* over time. qRT-PCR of activated infected macrophages normalized to activated macrophages . Samples were collected at timed intervals post infection, data are mean ± SEM; NS, not significant, from 2 independent experiments; **(B)** qRT-PCR analysis of *Mt3* expression in thymocytes and hepatocytes of 6-8 week old male C57BL/6 mice, left

untreated or treated with 50 ng/ml PMA + 500 ng/ml ionomycin or 10ng/ml LPS *E.coli* 055: B5 were compared to resting peritoneal (RP) and activated peritoneal (Act PM) macrophages. Values are normalized to resting peritoneal macrophage control, ND, not detected, data are mean ± SEM, n= 3 independent experiments; qRT-PCR on peritoneal and bone marrow macrophages reveals that GM-CSF either modestly or does not significantly alter the expression of **(C)** *Mtf1*; **(D, E)** CCCH-zinc finger family members, *Zc3h12a-d,* (note that the change in *Zc3h12a* occurs irrespective of GM-CSF activation in peritoneal macrophages) and **(F)** calreticulin, data are mean ± SEM, NS, not significant, n=3 independent experiments.

Figure S3:

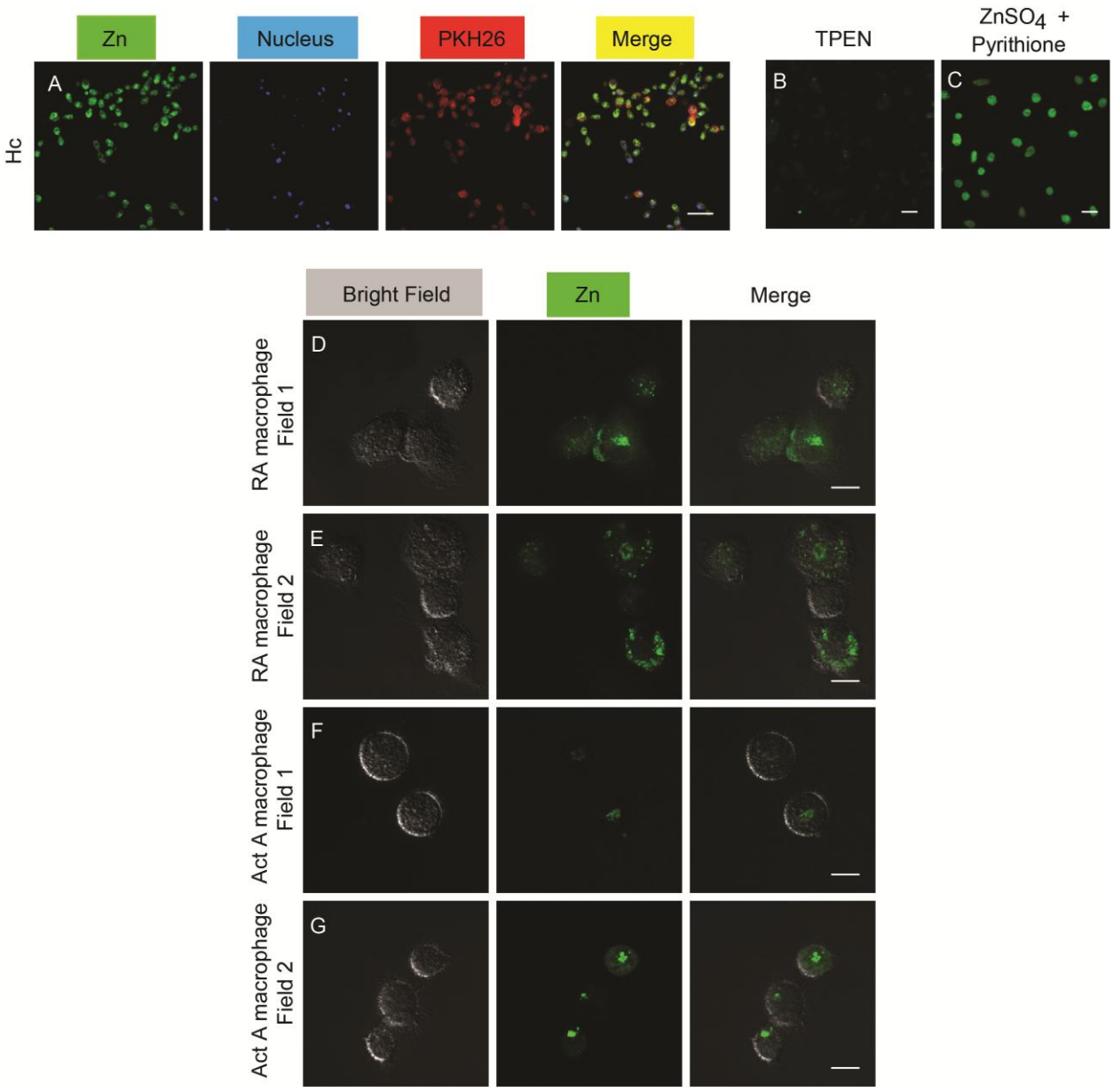

Figure S3: Sensitivity of Zinpyr-1 and GM-CSF driven Zn localization in alveolar macrophages. Related to Figure 3.

(A) *H. capsulatum* (Hc) cultured in Ham's F12 was stained as described in supplementary experimental procedures and imaged; merge, overlay of Zn, nucleus, and *H. capsulatum*, scale bar, 20 µm; Bone marrow macrophages were treated with **(B)** 2 µM TPEN overnight or **(C)** 100

μM $ZnSO_4$ and 20 μM pyrithione for 30 min, stained with Zinpyr-1, data represent images obtained from 3 different fields; scale bar, 20 μm; Labile-Zn staining of **(D, E)** resting alveolar (RA) macrophages and **(F, G)** activated alveolar (Act A) macrophages; bright field (left panel), Zn (middle panel), merge (right panel); zoom images, scale bar, 20 μm; representative of 2 independent experiments.

Figure S4:

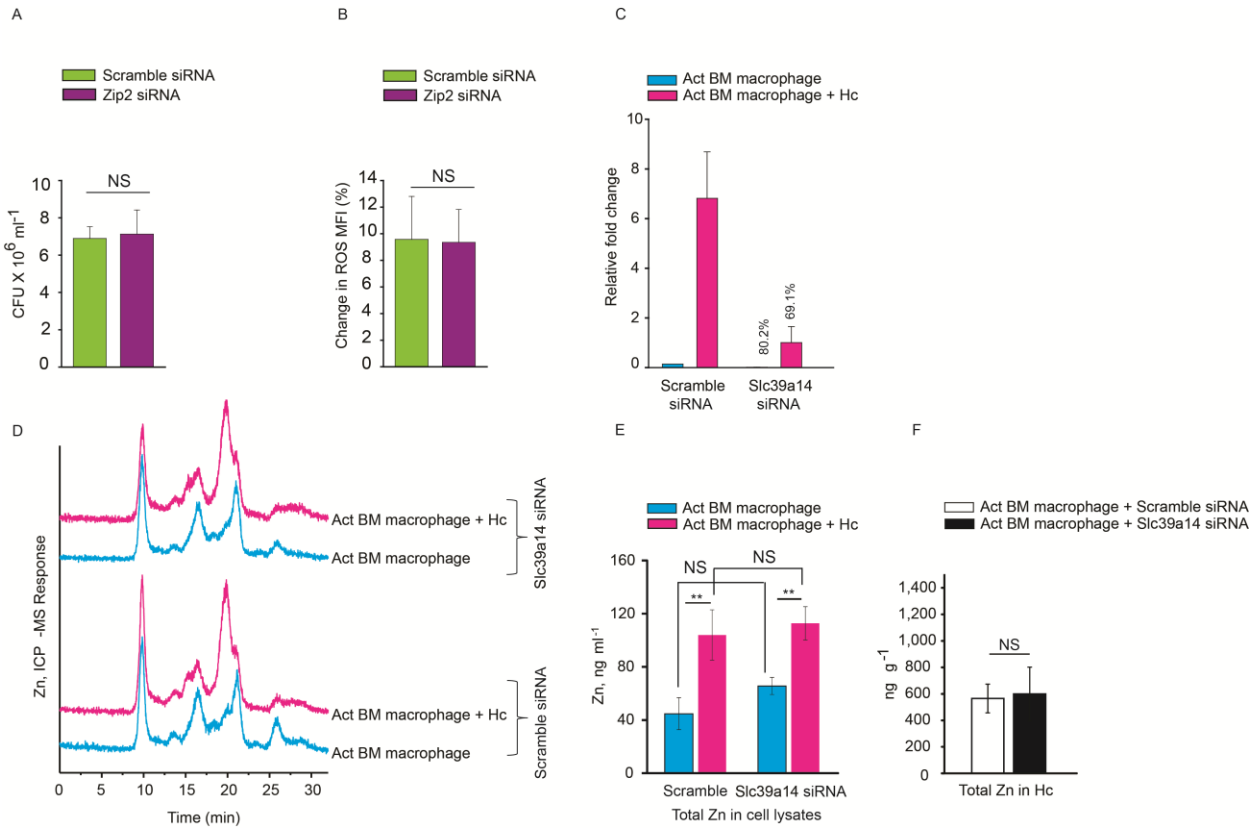

Figure S4: Role of *Slc39a2* and *Slc39a14* in GM-CSF function. Related to Figure 5.

(A) Growth inhibition of *H. capsulatum* (Hc) in scramble siRNA or Slc39a2 siRNA treated activated bone marrow (Act BM) macrophages compared to scramble-treated control; **(B)** % change in ROS MFI in scramble and Slc39a2 siRNA treated activated infected macrophages compared to scramble siRNA treated activated macrophages; **(C)** qRT-PCR analysis of *Slc39a14* in activated infected macrophages treated with scramble or Slc39a14 siRNA, values are normalized to scramble treated activated macrophages, percent decrease in expression compared to scramble siRNA controls, 2 independent experiments; **(D)** SEC-ICP-MS analysis of Slc39a14 silenced activated and infected macrophages compared to scramble siRNA control, Y axis, Offset Zn signal, 3 independent experiments; **(E)** Total Zn analysis by ICP-MS in cell lysates from scramble and Slc39a14 silenced activated and infected macrophages; **(F)** Total Zn analysis

by ICP-MS in yeasts from scramble and Slc39a14 silenced activated macrophages, data are mean ± SEM; 3 independent experiments.

Figure S5:

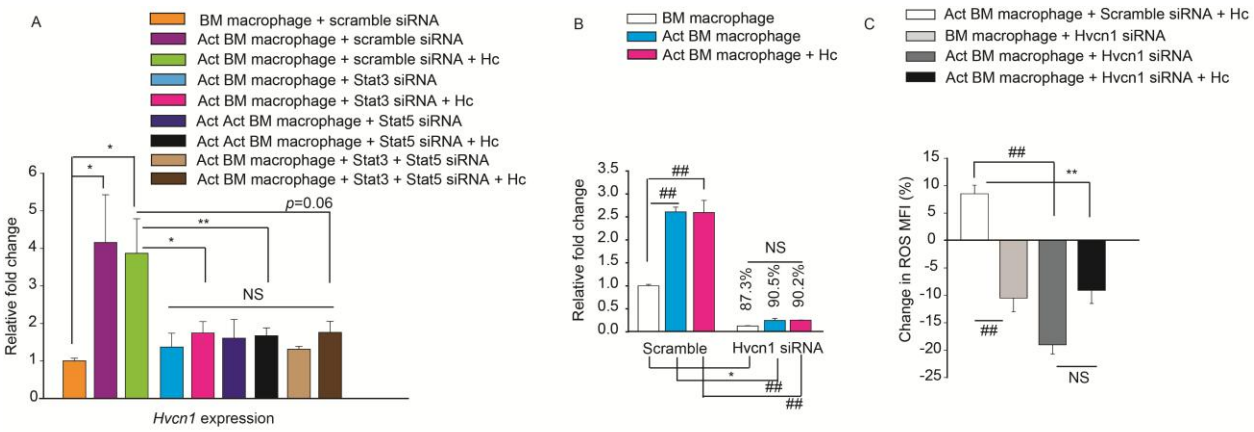

Figure S5: Regulation of *Hvcn1* expression and its role in superoxide burst. Related to Figure 6.

(A) qRT-PCR of *Hvcn1* in *Stat3* and *Stat5* silenced activated and infected macrophages compared with scramble siRNA treated control, data are mean ± SEM, 2 independent experiments; **(B)** qRT-PCR of *Hvcn1* in activated bone marrow (Act BM) macrophages treated with scramble or Hvcn1 siRNA and infected with *H. capsulatum* (Hc), % decrease in expression compared to scramble siRNA treated controls, 2 independent experiments; **(C)** % change in ROS MFI in *Hvcn1* silenced activated and infected macrophages compared to scramble siRNA treated activated macrophages, data are mean ± SEM, 3 independent experiments.

Figure S6:

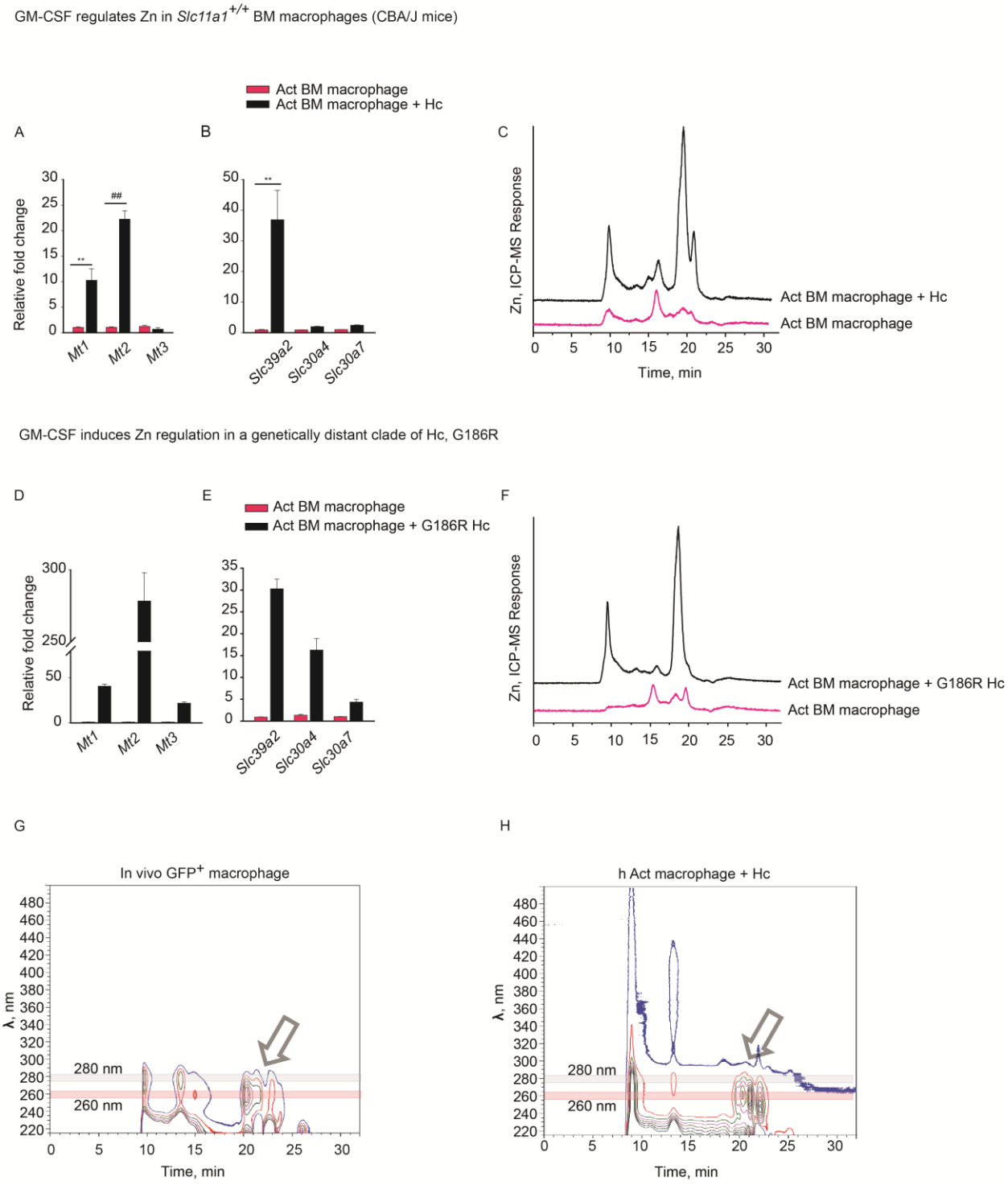

Figure S6: Zn regulation by GM-CSF occurs across different strains of mice and clade of *H. capsulatum*. Related to Figure 7. (A, B) qRT-PCR analysis of *Mt1*, *Mt2*, *Mt3*, *Slc39a2*,

Slc30a4 and Slc30a7 in Slc11a1$^{+/+}$ activated bone marrow (Act BM) macrophages derived from CBA/J mice and infected with *H. capsulatum* (Hc), data are mean ± SEM, 4 experiments; **(C)** SEC-ICP-MS analysis of activated and infected macrophages from CBA/J mice, Y axis, Offset Zn signal, data are mean ± SEM, 4 independent experiments; **(D, E)** qRT-PCR analysis of *Mt1, Mt2, Mt3, Slc39a2, Slc30a4 and Slc30a7* in activated macrophages infected with G186R *H. capsulatum*, data are mean ± SEM, 2 independent experiments; **(F)** SEC-ICP-MS analysis of activated macrophages infected with G186R *H. capsulatum*, Y axis, Offset Zn signal, 4 independent experiments; **(G and H)** UV-Vis isoplot from the SEC-DAD-ICP-MS analysis of cell lysates from GFP^{+} macrophages obtained by sorting lung leukocytes from *in vivo* infection studies and from human activated (h Act) infected macrophages. The ~20 min region as pointed by the arrow indicates that the absorbance maximum is at 260 nm in both cases, while in the high molecular weight region of the chromatogram both, 280 and 260 nm maxima are observed, 2 independent experiments.

Supplementary Experimental Procedures

Sample preparation, and quantification by ICP-MS for total metal analysis

To perform total metal analysis, samples were digested by the wet acid method. In brief, in a 10 % nitric acid washed 2 ml vial, 100 µl of cell lysates from macrophages were acidified with 50 µl of concentrated trace metal grade nitric acid, 20 µl of scandium (500 ppb) were added as internal standard to a final concentration of 10 ppb. The mixture was heated at 80 ºC for 3 h. Then 50 µl of hydrogen peroxide was added, heated at 80 ºC for 1 h and the final volume was made up to 1 ml. Three reagent blanks, and 3 SRMs were analyzed per sample batch.

The digested samples were quantified by external calibration method, using the following calibration points: 0, 0.1, 0.25, 0.5, 1, 2, 5, 10, 15 and 25 ppb; with Sc as internal standard at a concentration of 10 ppb. The collision reaction system was used to remove isobaric interference with 4 ml min^{-1} of He and a discrimination energy of 4 V. The forward power was 1500 watts, the dwell time 100 ms and the isotopes monitored were ^{24}Mg, ^{34}S, ^{44}Ca, ^{45}Sc, ^{55}Mn, ^{56}Fe, ^{57}Fe, ^{63}Cu, ^{66}Zn, ^{68}Zn, and ^{111}Cd.

SEC-ICP-MS normalization of data

To normalize the response of ICP-MS signal from SEC separations on different days, 20 µl of 1 mg ml^{-1} solution of carbonic anhydrase was injected post-column to the LC system, and area of Zn signals from samples was normalized to area of carbonic anhydrase peak. The absorbance at 280 nm of carbonic anhydrase was also followed to ensure integrity of the protein.

Protein in-solution tryptic digestion, proteomics and MASCOT search parameters

Fractions collected from SEC were concentrated by freeze drying. The pellet was re-suspended in 20 µl of ammonium bicarbonate, denatured at 90 °C for 5 minutes and reduced with 3 µl of 50 mM dithiothreitol (DTT) at 40 °C for 1 hr. Then alkylation of Cys residues was performed with 3 µl of 50 mM iodoacetamide in dark at room temperature for 45 min. Three hundred nanograms of trypsin in 20 mM ammonium bicarbonate was then added and incubated overnight at 37 °C. The reaction was stopped by adding 1 µl of formic acid. The non-digested proteins and trypsin were removed by ultrafiltration using a 5 kDa MWCO filter, and filtrate was analyzed by nanoHPLC-ESI-MS-MS as described below.

Fractions from SEC-ICP-MS were prepared as described above for peptide mapping. An Agilent 6300 series MSD Ion Trap XCT Ultra with a capillary binary pump was used for loading into microfluidic HPLC-Chip Zorbax SB 300A-C18 column and nano-flow binary pump to provide analytical flow for RP separation (Agilent Technologies). The ionization system utilized was a microfluidic chip, automatically loaded and positioned into the MS nanospray chamber. Full scan mass spectra were acquired over m/z range 50–500 in positive ion mode. For MS/MS, experimental conditions were: m/z range: 50–250; isolation width: 2 m/z units, fragmentation energy: 30–200%, fragmentation time: 40 ms.

To identify the proteins in each fraction, the results from LC-MS-MS were exported as MASCOT generic file and submitted to the MASCOT MS-MS ion search engine, with the following parameters: database, SwissProt; taxonomy, mammals; enzyme, trypsin; missed cleavages, 1; variable modifications, carbamidomethylation of cysteine; peptide tolerance 1.6 Da; MS/MS tolerance 0.6 Da; peptide charge +1, +2, +3; instrument, ESI-TRAP.

Denaturation of MT fraction and treatment with Cd for SEC-ICP-MS analysis

A large volume of cell lysate (4 ml instead of 200 µl of usual experiments) from activated infected bone marrow macrophages was prepared by scaling up the described procedure. An aliquot was analyzed by SEC-ICP-MS to see the profile **(Figure S7)**, and the rest was concentrated to a volume of 1ml by using a MWCO filter (3kDa). This solution was injected multiple times into the SEC column, and the fraction between 19 - 23 minutes was collected, freeze dried, re-suspended in ammonium acetate (50 mM), denatured by heat at 95°C for 5 minutes, and reduced by adding DTT to a final concentration of 10 mM. This solution was exposed to 5 mM $CdCl_2$ for 30 minutes with stirring, followed by ultra-filtration using a 3kDa MWCO filter and re-injected into the SEC-ICP-MS. Zn and Cd signals were monitored under standard instrumental conditions.

ICP-MS and SEC-ICP-MS quality control to avoid external Zn contamination

All total metal analysis experiments were carried out using trace metal grade reagents, on acid washed plastic vials and reagent blanks were used to correct the results. The analysis was done through a metal free auto sampler, rather than a HPLC flow injection method. The concentration of Zn in the blanks was always below 100 ppt, the blank estimate concentration on the calibration curves was always below 50 ppt, while the detection limits were below 30 ppt.

For chromatographic analysis, the mobile phase was cleaned using a Chelex 100 resin, using the batch method. In brief, an acid washed portion of 3 g of Chelex-100 was added to a liter of mobile phase, stirred for 30 min and passed through a 0.45 µm membrane. This decreased the Zn concentration below 200 ppt (measured as total). As an additional cleaning step a trapping column was packed with Chelex 100 resin, and placed just before the injector to avoid

contamination from the HPLC pump. By doing this the base line for the ICP-MS Zn signal was below one thousand counts per second, which represents sub ppb levels.

Mice

Mice were housed in isolator cages in Department of Laboratory Animal Medicine, University of Cincinnati, accredited by American Association for Accreditation of Laboratory Animal Care (Frederick, MD).

Macrophage isolation, activation and *Hc* infection

Bone marrow from femurs and tibias of ~12 week old mice was cultured in RPMI (BioWhittaker, MD) with 10% fetal bovine serum (HyClone Laboratories, Utah) and differentiated into macrophages with 10 ng/ml GM-CSF (Peprotech) for 7 days at 37°C and 5% CO_2. Cells were plated for 24 h with 10 ng/ml GM-CSF or TNF-α (Peprotech) before infection. Peritoneal macrophages were isolated by peritoneal lavage using Hank's balanced salt solution (HBSS), RBCs lysed, washed and rested for 24 h in media, after which they were treated for 24 h with 10 ng/ml GM-CSF or TNF-α before infection. Non-adherent cells were removed by washing, fresh media was added and macrophages were activated with cytokines at the time of infection.

Silencing

Silencing was performed by preparing an siRNA complex with TransIT TKO (Mirus Bio, LLC) in Opti-MEM Reduced Serum Medium (Life Technologies) followed by incubation for 30 min. Macrophages were then transfected with the siRNA complex in antibiotic free RPMI media with

10% FBS and incubated for 12h followed by activation with GM-CSF for 24h. After this time, the media was replaced, GM-CSF added and macrophages were infected for 24h prior to analysis.

Gene expression analysis

RNA was extracted using RNeasy mini kit (Qiagen) or Trizol (Invitrogen, CA) and genomic DNA was digested using RNase-free DNAse set (Qiagen) or DNase I recombinant, RNase free (Roche). cDNA was prepared using Reverse Transcription Systems kit (Promega, WI). qRT-PCR was performed in ABI Prism 7500, using Taqman assay with primer and probe sets from Applied Biosystems, CA; hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as internal standard and target gene expression was normalized to resting peritoneal macrophages in peritoneal macrophage experiments and to activated bone marrow macrophages in bone marrow macrophage experiments.

SDS PAGE and western blotting

Cell lysates were prepared using Denaturing Cell Extraction Buffer (Invitrogen). To assess STAT3 activation, macrophages were treated with GM-CSF and infected for 10 min, lysates were prepared and immunoblotted using antibodies for STAT3 (Millipore) and p-STAT3 Tyr705 (Cell Signaling). In silencing experiments, *Stat3* and *Stat5* were silenced, followed by activation and infection. Cell lysates were prepared 24 h post infection to assess p-STAT3 and p-STAT5 Tyr 694 (Cell Signaling). SDS-PAGE was performed on 10% Precise Protein Gel (Pierce). β-ACTIN (Santa Cruz) was the loading control.

Confocal microscopy procedures, equipment and settings

Peritoneal and bone marrow macrophages were cultured on coverslips with cytokines. *H. capsulatum* was stained with PKH-26 (Sigma-Aldrich) as per manufacturer's protocol and were infected with PKH-26 stained yeasts. For microscopy, cells were washed twice with HBSS containing 0.5 mM ethylene diamine tetraacetate (EDTA) to chelate extracellular metals and stained for 30 min with nuclear stain, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (300 nM) and Golgi stain, BODIPY TR ceramide (Molecular Probes) (5 µM) and/or labile Zn stain, Zinpyr-1 (Santa Cruz Biotechnology) (10 µM) in RPMI only. Cells were washed and mounted on slides; images were acquired immediately on a Zeiss LSM710 confocal connected to Zeiss Axio-observer.Z1 inverted microscope and visualized using ZEN 2011 software.

Macrophage controls: Bone marrow macrophages were plated on glass coverslips and treated with 2 µM N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) overnight or 100 µM $ZnSO_4$ and 20 µM pyrithione (Sigma) for 30 min. Cells were washed twice with HBSS containing 0.5 mM EDTA and stained for 30 min with 10 µM Zinpyr-1. Cells were washed again and imaged as described above.

***H. capsulatum* control:** Yeasts cultured in Ham's F12 were washed twice in HBSS and stained with PKH-26 dye as described in Methods. The yeasts were again washed twice in HBSS containing 0.5 mM EDTA and with stained with Zinpyr-1 and DAPI for 30 min. After two washes in HBSS, yeasts were imaged as described above.

All images were acquired as 8 bit images at room temperature using a Zeiss LSM 710, Zeiss Axio observer.Z1 inverted microscope with 63X oil immersion/1.4 NA objective. Images were acquired with an optical slice thickness of 1 to 1.5 µm and lateral pixel dimensions of 220 nm for regular images and 66 nm for zoom images. Acquisition settings were held constant for Zn

fluorescence (green channel) in sample groups - Figures 3A-3E for all images**;** similar in Figure 3F and 3G for regular size images; and constant in Supplementary Figure 3 for all images. Zinpyr-1 was excited using a 488 nm argon laser and emission was measured from 490 nm to 552 nm, DAPI was excited using a 405 nm violet laser diode and emission was measured from 410 to 487 nm and PKH-26 as well as BODIPY TR ceramide were excited using a 561 nm HeNe laser and emission was measured from 566 to 705 nm.

Specific depletion of Zn in macrophage culture media for ROS studies

Serum-free or serum-containing RPMI medium was mixed with 3g/L of Chelex-100 under sterile conditions. This solution was stirred overnight and filtered through a 0.45 µm filter. This treatment was repeated once more, and the resultant solution was analyzed to assess the percent of removal of the following elements: Zn, Cu, Cr, Mn, Co, and Fe. Then the levels of Cu, Cr, Mn, Co and Fe were restored in RPMI (serum free and serum containing) using inorganic salts of high purity of the corresponding element. The percent of Zn removal from serum-free and serum containing RPMI was 94% and 87 % respectively.

Growth inhibition assay

10^5 bone marrow macrophages were plated on a 96-well plate in culture media with or without GM-CSF (Act-BM macrophage and BM macrophage respectively) for 24 h. The Act-BM macrophages were activated again with GM-CSF and both groups were infected with *H. capsulatum* at a multiplicity of 5 yeasts per macrophage. Growth of yeasts was assayed 24 h post infection. Macrophages were lysed hypotonically and yeasts plated on Mycosel agar 5 (Beckton Dickinson) with 5% sheep blood and 5% glucose. Colonies were counted 7 days after incubation

at 37°C; the detection limit was 100 colony forming units (CFU) and data expressed CFU X 10^6 per ml. Where *H. capsulatum* was cultured in ROS generating media, 200µM of xanthine substrate and 40mU or 60mU of XO was added to normal or low Zn media to generate ROS, with or without 10µM Zn. Enzyme and substrate solutions were prepared in Zn free solution or ddiH_2O.