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2   **Immunomodulators Targeting MARCO Expression Improve Resistance to Post-influenza**

3   **Bacterial Pneumonia**

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5   **Authors:**   Muzo Wu[1], John G. Gibbons[2], Glen M. DeLoid[1], Alice S. Bedugnis[1], Rajesh K.

6   Thimmulappa[3], Shyam Biswal[3], Lester Kobzik[1,*]

7   **Affiliations:**

8   [1]Department of Environmental Health, Harvard School of Public Health, Harvard University,

9   Boston, MA 02115, USA.

10   [2]Biology Department, Clark University, Worcester, MA 01610, USA.

11   [3]Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns

12   Hopkins University, Baltimore, MD 21205, USA.

13

14   Correspondence:
15
16   Lester Kobzik, M.D.
17   Dept. of Environmental Health
18   Harvard T.H. Chan School of Public Health
19   665 Huntington Ave.
20   Boston, MA 02115
21
22   lkobzik@hsph.harvard.edu

23

24

Abstract:

Down-regulation of the alveolar macrophage (AM) macrophage receptor with collagenous structure (MARCO) leads to susceptibility to post-influenza bacterial pneumonia, a major cause of morbidity and mortality. We sought to determine whether immunomodulation of MARCO could improve host defense and resistance to secondary bacterial pneumonia. RNAseq analysis identified a striking increase of MARCO expression between days 9 and 11 after influenza infection and indicated important roles for Akt and Nrf2 in MARCO recovery. In vitro, primary human AM-like monocyte-derived macrophages (AM-MDMs) and THP-1 macrophages were treated with IFNγ to model influenza effects. Activators of Nrf2 (sulforaphane) or Akt (SC79) caused increased MARCO expression and a MARCO-dependent improvement in phagocytosis in IFNγ-treated cells, and improved survival in mice with post-influenza pneumococcal pneumonia. Transcription factor analysis also indicated a role for transcription factor E-box (TFEB) in MARCO recovery. Overexpression of TFEB in THP-1 cells led to marked increases in MARCO. The ability of Akt activation to increase MARCO expression in IFNγ-treated AM-MDMs was abrogated in TFEB-knockdown cells, indicating Akt increases MARCO expression through TFEB. Increasing MARCO expression by targeting Nrf2 signaling or Akt-TFEB-MARCO pathway are promising strategies to improve bacterial clearance and survival in post-influenza bacterial pneumonia.

Keywords: Influenza; bacterial pneumonia; immunomodulators; macrophage receptor with collagenous structure (MARCO); interferon-γ (IFN-γ); Nrf2; Akt; TFEB

Introduction

Secondary bacterial pneumonia contributes to the high mortality from seasonal and pandemic influenza (80) (23). The most common pathogens identified include *Streptococcus pneumoniae, Staphylococcus aureus,* and *Haemophilus influenzae*. Susceptibility to superimposed bacterial infection is highest approximately four to fourteen days after influenza onset (60). Multiple mechanisms underlie increased susceptibility (8, 15, 19, 22, 27, 33, 38, 41, 43-45, 49, 50, 65, 66, 73, 79, 81, 82, 85, 91), including influenza virus-induced interferon-γ (IFNγ), which reduces expression of the phagocytic receptor macrophage receptor with collagenous structure (MARCO) on alveolar macrophages (AMs) (73).

MARCO is a highly-conserved trimeric class A scavenger receptor (7) that mediates phagocytosis of unopsonized particles and bacteria, thereby playing a key role in innate host defense (5). Upregulation of MARCO expression enhances bacterial binding and phagocytosis, and alters cytokine expression (51). Dysregulation of MARCO expression leads to impaired phagocytosis and bacterial clearance, as illustrated by increased lung injury and inflammation in MARCO$^{-/-}$ mice exposed to pathogens or particulates (5, 11, 76). We might anticipate, therefore, that immunomodulators upregulating MARCO expression would improve innate immune function and enhance bacterial clearance in post-influenza bacterial pneumonia. Hence, we analyzed the dynamics of MARCO expression after influenza infection to identify novel immunomodulatory strategies. A striking recovery and increase in MARCO was observed between days 9 and 11 after influenza infection. RNAseq transcriptome profiling of AMs on these days showed that TFEB and Nrf2 were among the most over-represented transcription factors regulating differentially expressed genes during the recovery period of post-influenza MARCO expression.

70   Sulforaphane (SFN), an isothiocyanate present in cruciferous vegetables, increases

71   macrophage MARCO expression and antibacterial function through Nrf2 signaling (25, 77). We

72   investigated the effects of SFN on post-influenza host defenses. Treatment with SFN increased

73   MARCO expression and phagocytosis in IFNγ-treated AM-MDMs, and significantly improved

74   bacterial clearance and survival in a mouse model of secondary pneumococcal pneumonia.

75   TFEB is an evolutionally conserved master gene of lysosomal biogenesis, autophagy, and

76   lysosomal exocytosis (46, 61, 63), and plays an important role in regulating host defenses against

77   pathogens (83) and responses to nutritional stress (64). Transcriptome profiling suggested

78   important roles for Akt and the transcription factor EB (TFEB) in recovery of MARCO

79   expression. Treatment with the Akt activator SC79 improved MARCO expression and bacterial

80   phagocytosis in IFNγ-treated AM-MDMs. However, the effects of Akt-induced MARCO

81   expression in IFNγ-treated AM-MDMs were blocked after TFEB knockdown with siRNAs. Akt

82   activation also improved survival of mice with secondary bacterial pneumonia. These

83   observations identify a novel role for TFEB in the post-influenza host response, as a mediator of

84   Akt activation-induced MARCO upregulation.

85   Our data identify novel regulators of AM MARCO expression after influenza, and

86   broaden the range of potential immunomodulatory strategies to improve host resistance to post-

87   influenza secondary pneumonia.

88

89   **Methods**

90   *Cells*

91   Human alveolar macrophage (AM)-like monocyte-derived macrophages (AM-MDMs) were

92   prepared as previously described (13, 71). Briefly, human monocytes (New York Biologics,

93   Southampton, NY) were isolated from discarded normal blood by negative selection using

94   RosetteSep (Stemcell Technologies, Vancouver, BC, Canada) and matured into AM-like

95   macrophages (1, 88) by 10-11 days of culture in the Roswell Park Memorial Institute – 1640

96   (RPMI-1640) medium containing 2 mM L-glutamine, 20 mM HEPES, 1 mM sodium pyruvate,

97   4500 mg/L glucose, 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, and

98   20 ng/ml human granulocyte/macrophage colony-stimulating factor (GM-CSF, Peprotech, Rocky

99   Hill, NJ, USA). B6 cells are an immortalized cell line derived from alveolar macrophages

100   obtained from normal C57Bl6 mice as detailed in the previous report (89).

101   Human monocyte cell line, THP-1 cells (ATCC TIB-202^{TM}, Rockville, MD), were grown in

102   RPMI-1640 medium containing 2 mM L-glutamine, 20 mM HEPES, 1 mM sodium pyruvate,

103   4500 mg/L glucose, 1500 mg/L sodium bicarbonate, 10% fetal bovine serum, and 1% of

104   penicillin-streptomycin. THP-1 cells were differentiated into macrophages by growing in media

105   containing 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich Co. LLC.) and 20

106   ng/ml GM-CSF for 24 hours.

107   *Bacteria*

108   Green fluorescent protein (GFP) – expressing *Staphylococcus aureus* strain RN6390 was

109   prepared as previously described (13). *Streptococcus pneumoniae* serotype 3 (strain

110   ATCC6303[TM], Rockville, MD) was cultured on 5% sheep blood-supplemented agar plates

111   (VWR International, Radnor, PA). Bacteria were grown overnight and resuspended in sterile

112   phosphate buffered saline (PBS) prior to infection. Concentration of the bacterial suspension was

113   determined by measuring the optical density at A_{600} with a spectrophotometer and by colony

114   forming units (CFUs).

115   ***Immunomodulator Assays***

116   AM-MDMs were prepared from primary human monocytes as previously described (13, 71).

117   AM-MDMs or THP-1 cells were treated with human IFNγ (10, 20, or 50 IU/ml, Peprotech,

118   Rocky Hill, NJ, USA) for 20 to 24 hours to simulate effects of influenza infection, and

119   simultaneously incubated with or without R,S-sulforaphane (5 or 10 µM, LKT Laboratories, Inc.,

120   St. Paul, MN, USA) or with or without SC79 (2, 5 or 8 µg/ml, EMD Millipore, Billerica, MA,

121   USA) before quantitation of MARCO expression or challenge with GFP–expressing *S. aureus*

122   strain RN6390.

123   For the *in vivo* study of immunomodulator effects in post-influenza bacterial pneumonia, wild-

124   type C57BL/6 male mice were treated intraperitoneally with sulforaphane (20 to 25 mg/kg per

125   mouse), or subcutaneously with SC79 (20 mg/kg per day) on days 6 to 9 or 10 after influenza

126   infection.

127   For the *in vitro* study of the EGCG effect in post-influenza bacterial pneumonia, AM-MDMs or

128   THP1 cells were treated with human IFNγ (10, 20, or 50 IU/ml, Peprotech, Rocky Hill, NJ,

129   USA) for 20 to 24 hours to simulate influenza infection, and simultaneously incubated with or

130   without EGCG (10 or 50µM, Sigma Aldrich Co. LLC.) before quantitation of MARCO

131   expression or challenge with GFP–expressing *S. aureus* strain RN6390 by scanning cytometry.

132   *Bronchoalveolar lavage (BAL)*

133   After euthanasia, the mouse trachea was cannulated with a 20-gauge catheter for bronchoalveolar

134   lavage. For colony-forming unit studies, bronchoalveolar lavage was performed on day 2 after *S.*

135   *pneumoniae* infection. Mouse lungs were lavaged with a total of 4 ml of PBS. Serial dilutions of

136   the lavage fluid were spread on blood agar plates and incubated for 17 h at 37 °C before CFU

137   counting. For cytokine/chemokine studies and collection of alveolar macrophages, mouse lungs

138   were first lavaged with 0.5ml PBS via cannulation, followed by 5 lavages with 0.7 ml PBS. The

139   first 1.2 ml of the total 4ml lavage fluid was centrifuged at 200g for 10 minutes at 4°C.

140   Supernatants from BALF collected on days 3, 5, 7, 9, and 11 after influenza infection were used

141   for cytokine/ chemokine analysis. Cell pellets were resuspended with the cells in the remaining

142   2.8 ml lavage for each sample.

143   *Cytokine analysis*

144   Cytokines/ chemokines in mouse BALF after influenza virus strain PR8 infection were measured

145   by using Mouse Cytokine Array/ Chemokine Array 32-plex Panel (Eve Technologies, Calgary,

146   Alberta, Canada). The 32 cytokines/ chemokines include eotaxin, granulocyte colony-stimulating

147   factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFNγ, IL-1α, IL-

148   1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-

149   17A, interferon gamma-induced protein (IP-10), ketatinocyte chemoattractant (KC), leukemia

150   inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte

151   chemotactic protein 1 (MCP-1), macrophage colony-stimulating factor (M-CSF), monokine

152   induced by gamma interferon (76), macrophage inflammatory protein 1-α (MIP-1α), macrophage

153   inflammatory protein 1- β (MIP-1β), macrophage inflammatory protein 2 (MIP-2), regulated on

154 activation, normal T cell expressed and secreted (RANTES), tumor necrosis factor α (TNFα),

155 and vascular endothelial growth factor (VEGF). The sensitivity of the aforementioned cytokines

156 in the panel ranged from 0.07 to 15.85 pg/ml. Interferon-γ concentrations in BALF were also

157 measured by using Mouse IFNγ ELISA MAX^{TM} Standard kit (BioLegend, San Diego, CA) per

158 manufacturer's protocol.

159 ***RNA sequencing analysis***

160 RNA sequencing was conducted at the Bauer Center Sequencing Core at Faculty of Arts and

161 Sciences Center for Systems Biology, Harvard University. Ribo-depleted RNA was isolated

162 from total RNA of purified murine alveolar macrophages collected on post-influenza day 9 and

163 11. Automated library preparation was performed using the Apollo 324^{TM} System (WaferGen

164 Biosystems, Inc., Fremont, CA, USA) to generate a library of 100-base-pair single-end reads for

165 RNA sequencing. DNA fragments were purified with PCRClean DX Kit from Aline Biosciences

166 (Woburn, MA, USA). Samples were pooled in equal molar amounts, and sequenced with the

167 Illumina HiSeq 2500 System (Illumina, Inc., San Diego, CA, USA).

168 For data filtering and mapping of reads, Trim Galore

169 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to quality trim raw

170 reads and adapters with a minimum quality score of 30, and minimum sequence length of 80.

171 FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) was then used to remove the first 10

172 bases. Remaining reads were mapped to the reference mouse genome from the Ensembl database

173 using the "sensitive" preset parameters in Bowtie 2 (36). A count table of mapped reads was

174 created for each gene, and reads per kilobase of transcript per million mapped reads (RPKM)

175 were calculated from the Bowtie 2 output using the generalized fold change (GFOLD) algorithm

176 (17). The R package of DESeq (3) was used to identify differentially expressed genes and to

177 calculate adjusted p values for comparisons.

178 Two replicates from each group were analyzed using QIAGEN's Ingenuity® Pathway Analysis

179 (IPA®, QIAGEN Redwood City, USA) and MetaCore™ (Thomson Reuters, USA) for

180 identification of over-represented transcription factors and functional analysis of differentially

181 expressed genes. The sequence data are available through the NCBI database using the accession

182 number PRJNA311622.

183 For analysis using a Connectivity Map approach (35), we used the Touchstone database available

184 at clue.io The top DEGs passing a filter of adjusted p value < .01 were used, resulting in

185 submission of 476 and 428 up- and down-regulated DEGs respectively for analysis. The results

186 table was sorted by percentile rank based on their ability to cause a gene expression pattern most

187 similar to the gene signature found by comparing DEGs in day 11 vs day 9 macrophages.

188 ***Quantitative reverse transcription - polymerase chain reaction***

189 Total RNA was extracted according to the manufacturer's manual using Qiagen RNeasy Micro

190 Kit (Valencia, CA, USA). For each sample of FACS-sorted mouse alveolar macrophages, 150 ng

191 of RNA was reverse transcribed into cDNA using *Applied Biosystems*® High Capacity cDNA

192 Reverse Transcription Kits (Grand Island, NY, USA). For each sample of AM-MDMs or THP-1

193 cells, 1 μg of RNA was reverse transcribed. The quantitative reverse transcription – PCR (qRT-

194 PCR) was performed on an *Applied Biosystems*® 7300 *Real Time PCR* System with

195 amplification cycles of 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, 60°C for 30

196 seconds, and 72°C for 10 seconds.

197   *Immunoblot analysis*

198   AM-MDMs were washed with cold phosphate-buffered saline and lysed with protease and

199   phosphatase inhibitors in 1% NP-40 lysis buffer for 30 minutes on ice. Cell lysates were

200   resolved on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels (Life Technologies, Grand Island,

201   NY, USA), and transferred to nitrocellulose membranes. Antibodies used for immunoblot

202   analysis included from Cell Signaling: phosphor-Akt (Ser 473, **Rabbit mAb #4060),** Akt

203   (**Rabbit mAb #4685)**, and β-Actin; poluclonal rabbit anti-MARCO, sc-68913, was obtained

204   from Santa Cruz Biotechnology. Immunoreactive bands were detected by chemiluminescence.

205   Densitometric analysis of the immunoblots was performed using ImageJ (National Institutes of

206   Health).

207   *Stable transduction of THP1 cells*

208   MISSION® Lentiviral Transduction Particles with human TFEB shRNA (Sigma-Aldrich, St.

209   Louis, MO, USA) or human TFEB (BC032448.1) ORF cDNA lentiviral particles (GeneCopoeia,

210   Rockville, MD, USA) were added to 1×10^6 human THP-1 cells at a MOI of 0.3 after cells were

211   incubated in 5µg/ml hexadimethrine bromide for 10 minutes. Cells were then spun at 1,000 x g

212   for 90 minutes at 30°C, and incubated at 37°C for 5 hours before fresh RPMI-1640 media was

213   added. Following overnight incubation at 37°C, cells were washed and grown in culture media

214   containing puromycin 5µg/ml.

215   *Transfection of human monocyte-derived macrophages*

216   AM-MDMs were transfected with ON-TARGETplus Human TFEB siRNA or ON-TARGETplus

217   Non-targeting Pool (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) using PolyJet™

218   DNA In Vitro Transfection Reagent (SignaGen Laboratories, Rockville, MD, USA) according to

219   the manufacturer's protocol.

Mouse model of post-influenza bacterial pneumonia

221   Eight-to-nine-week-old MARCO -/- (4) or wild-type control male mice on a C57BL/6

222   background (Jackson Laboratory, Bar Harbor, ME) were used in the *in vivo* model of post-

223   influenza bacterial pneumonia. Animals were cared for in accordance with the Institutional

224   Animal Care and Use Committee (IACUC) guidelines. Experimental procedures on animals

225   were conducted after approval by the Harvard Medical Area (HMA) Standing Committee on

226   Animals. For the post-influenza pneumonia model, mice were intranasally inoculated with 1 PFU

227   of influenza virus strain A/Puerto Rico/8/34 (PR8) (Virasource, Raleigh-Durham, NC) per

228   mouse in 25 µl PBS after anesthesia with ketamine (200 mg/kg) and xylazine (20 mg/kg). On

229   day 7 after influenza infection, mice were intranasally infected with 300 to 500 CFUs of *S.*

230   *pneumoniae* serotype 3 in 25 µl PBS after anesthesia. Body weight and morbidity were

231   monitored after infections.

Scanning cytometry analysis of MARCO expression and bacterial uptake

233   For analysis of treatment effects on MARCO expression, AM-MDMs were incubated with mAbs

234   PLK-1 (5) or IgG3 isotype control at 4°C after Fc receptor blockade. Cells were then washed and

235   stained with Alexa Fluor® 594 F(ab')2 fragment of goat anti-mouse IgG (Invitrogen™, Grand

236   Island, NY, USA). Dead cells were stained with the Invitrogen™ LIVE/DEAD Fixable Green

237   Dead Cell Stain Kit per manufacturer's instructions. After fixation with 4% formaldehyde, cells

238   were stained with Hoechst nuclear stain and CellMask blue stain (Invitrogen™, Grand Island,

239   NY, USA).

240   For analysis of treatment effects on AM-MDM uptake of GFP-labeled bacteria,

241 extracellular bacteria were stained with Alexa Fluor 594–labeled monoclonal antibody to *S.*

242 *aureus* (ABIN4356218, Novus Biologicals), or lysed with lysostaphin (20 µg/ml, 30 min). For

243 MARCO inhibition experiments, cells were incubated in 200 µg/ml of polyinosinic acid

244 (poly(I)), a class A scavenger receptor inhibitor, at 37°C for 30 minutes prior to bacterial

245 infection. Alternatively, GFP-*S. aureus* suspensions were incubated with or without MARCO

246 blocking peptides (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) for 30 minutes prior to

247 infection. Dead cells were stained with the Invitrogen™ LIVE/DEAD Fixable Red Dead Cell

248 Stain Kit. After fixation with 4% formaldehyde, cells were stained with Hoechst nuclear stain

249 and CellMask blue stain (Invitrogen™, Grand Island, NY, USA).

250   Scanning cytometry BD Pathway 855 High-Content Bioimager (BD Biosciences, San

251 Jose, CA, USA) was used to acquire confocal fluorescence images of MARCO surface labeling

252 and binding and uptake of bacteria, and image data were analyzed using MATLAB® image

253 analysis software (13).

254 ***Fluorescence-activated cell sorting (FACS)***

255 Cells collected from BAL on day 3, 5, 7, 9, and 11 after influenza infection of C57BL/6 mice

256 were stained with fluorescein isothiocyanate (FITC)–conjugated anti-MARCO antibodies (AbD

257 Serotec, Raleigh, NC, USA), Alexa Fluor 647-conjugated F4/80 antibodies (BioLegend, San

258 Diego, CA), PE-Cy7-conjugated CD11c+ antibodies (BioLegend, San Diego, CA), and their

259 corresponding isotypes after Fc Receptor blockade. F4/80+CD11c+ cells were sorted using the

260 BD *FACSAria™ II cell sorter* (BD Biosciences, San Jose, CA, USA) for RNA extraction.

261 ***Chromatin immunoprecipitation (ChIP)***

262 The SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003 from Cell Signaling

263 Technology, Inc. (Danvers, MA) was used for chromatin immunoprecipitation according to the

264 manufacturer's protocol. Briefly, AM-MDMs were formalin-fixed, treated with micrococcal

265 nuclease, and sonicated. Chromatin from the AM-MDMs was immunoprecipitated with anti-

266 TFEB antibody and its corresponding isotype (Cell Signaling Technology, Inc., Danvers, MA).

267 Immunoprecipitated nuclear DNA was extracted, and analyzed by PCR with primers targeting

268 the human MARCO upstream promoter area. The MARCO primers used for the ChIP assay

269 were: forward, 5'-CCCTGGTAGAGATGCCTGAC-3'; reverse, 5'-

270 GAAGCCTTGCTCTGAACCAC-3'.

271 ***Statistical analysis***

272 Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., San

273 Diego, CA). Results are expressed as the mean ± standard deviation (SD). Student's t-test was

274 performed to compare treatment effects between two groups, and one-way analysis of variance

275 (ANOVA) with Bonferroni adjustment for comparison between multiple groups. Mortality of

276 mice was analyzed by the Kaplan-Meier and log-rank methods.

277

278

279 **Results**

280 ***MARCO mRNA expression of sorted alveolar macrophages after influenza virus infection***

281 Lung cells from influenza-infected mice were collected by lavage on days 3, 5, 7, 9, and 11 after

282 infection. Lavaged F4/80+CD11c+ cells were sorted as alveolar macrophages (6, 34) (Figure

283 1A), and *Marco* expression was measured by qRT-PCR. Corresponding to the increased IFNγ

284 levels on post-influenza days 7 and 9 (Figure 1B), *Marco* expression was down-regulated on

285 days 7 (0.46 fold, p<0.0001, Figure 1C) and 9 (0.51 fold, p<0.0001). However, there was a

286 surprising 4.86-fold increase (p<0.0001) in *Marco* expression between day 9 and 11. Moreover,

287 *Marco* expression was up-regulated 2.48 fold (p<0.0001) on day 11 compared to un-infected

288 controls.

289 To explore the basis for these changes in *Marco* expression, levels of large panel of

290 cytokines were measured in lavage fluids using a multiplexed array assay. The results are

291 summarized in Figure 2 and identified several cytokines whose expression declines from day 7

292 onward. Consistent with prior studies (73), IFNγ exhibited the greatest increase on day 7

293 compared to day 5 (261-fold), and also when day 7 was compared to days 9 or 11 (524-fold and

294 66-fold, respectively). A subset of the cytokines assayed showed a similar pattern (e.g., IL-4, 10,

295 12p70, fold-change data not shown), but no single cytokine showed a unique change in

296 magnitude or pattern that might easily explain (by itself) the marked increase in *Marco*

297 expression between day 9 and 11.

298

299 ***RNA sequencing analysis of sorted alveolar macrophages on day 9 and 11 after influenza***

300 ***infection***

301   To explore potential mechanisms for up-regulation of *Marco* expression more broadly, RNAseq

302   analysis was performed on F4/80+CD11c+ alveolar macrophages purified by flow sorting from

303   mice on post-influenza days 9 and 11. 1537 differentially expressed genes (DEGs) were

304   identified between day 9 and day 11 samples, using criteria of adjusted p < 0.01; (Table S1).

305   Notably, these included Marco, v-akt murine thymoma viral oncogene homolog 1 (Akt1 or Akt),

306   mitogen-activated protein kinase 3 (Mapk3), and another Nrf2-regulated gene, heme oxygenase

307   1 (Hmox1). Enrichment analysis using MetaCore™ showed that the candidate genes were

308   associated with process networks relevant to the resolution phase of influenza (Table S2), and

309   also showed that NRF2 and TFEB are among the most over-represented transcription factors

310   (based on binding sites in genes differentially expressed in AMs between day 9 and day 11,

311   Table S3). Finally, analysis of the DEGs using the CLUE portal to query the Connectivity Map

312   Touchstone dataset (clue.io/touchstone) identified agents that cause gene expression changes that

313   correlate strongly with the gene signature linked to up-regulated MARCO on day11. Six of the

314   top 10 compounds (out of the 2,837 compounds evaluated) activate NRF2 (by rank: 1) celastrol

315   (16), 5 & 6) topoisomerase inhibitors SN38 and topotecan (54), 7) mycophenolate (75) 8)

316   triptolide (90), 9) trichostatin (9)) We further investigated immunomodulation of both of these

317   targets, focusing on sulforaphane activation of NRF2 (47) and Akt-mediated activation of TFEB.

318

319   ***Sulforaphane increases MARCO expression of IFNγ-treated human macrophages***

320   To model the effects of influenza *in vitro*, we treated primary AM-MDMs with IFNγ for 24

321   hours, which resulted in down-regulation of MARCO expression, (0.54±0.20 fold change vs.

322   control, p<0.001, Figures 3A and 3B). SFN treatment up-regulated MARCO expression and

323   diminished the IFNγ-mediated reduction in MARCO expression (0.92±0.25 vs. 0.54±0.20 fold

324   change, p<0.01). Cell death following IFNγ treatment was also significantly reduced in SFN-

325   treated groups (SFN = 10 μM) compared to untreated controls (1.3±1.5% vs. 4.5±2.0%, p<0.01,

326   Figures 3A and 3C). Similarly, treatment of differentiated human THP-1 macrophages with IFNγ

327   (5 – 50 IU/ml) resulted in a dose-dependent down-regulation of MARCO expression and

328   increased cell death, which were reversed by treatment with SFN (Figs. 4A & B).

329

330   ***Sulforaphane improves bacterial phagocytosis in IFNγ-treated AM-MDMs through MARCO***

331   ***upregulation***

332   AM-MDMs were challenged with *S. aureus* following ~24 hours of IFNγ pre-treatment.

333   Phagocytosis of fluorescent GFP-labeled *S. aureus* by AM-MDMs was impaired by IFNγ

334   treatment (p<0.05, Figures 3D, 3E, and 3F), but significantly improved when treated

335   simultaneously with SFN (p<0.01). Cell death following IFNγ and *S. aureus* infection was also

336   reduced by concurrent SFN treatment (data not shown).

337   To further delineate the causal link between sulforaphane treatment, MARCO expression,

338   and bacterial phagocytosis, we treated AM-MDMs with either the broad class A scavenger

339   receptor blocker, polyinosinic acid [poly(I)], or a specific MARCO blocking peptide prior to *S.*

340   *aureus* infection. The rescue effect of SFN on bacterial phagocytosis after IFNγ treatment was

341   significantly inhibited by both poly(I) (5.19 bacteria per cell, 95% CI 2.62 to 7.76, vs. 3.25, 95%

342   CI 0.47 to 6.04, p<0.01, Figure 3E) and the specific MARCO blocking peptide (5.21, 95% CI

343   4.39 to 6.02, vs. 3.04, 95% CI 1.75 to 4.33, p<0.05, Figure 3F). Taken together with the previous

344   findings these results indicate that SFN improves bacterial phagocytosis in IFNγ-treated

345   macrophages through up-regulation of the class A scavenger receptor MARCO. In contrast to

346   uniform results seen with the THP-1 cell line, considerable donor-to-donor variability was seen

347   with primary AM-MDM cells. However, AM-MDMs from all donors showed a similar pattern,

348   with IFNγ causing a reduction in bacterial phagocytosis that was reversed by SFN, and MARCO

349   blockade resulting in inhibition of phagocytosis that was not abrogated by SFN (Figure 3E and

350   3F).

351   We also tested the effects of another Nrf2 activator, epigallocatechin gallate (EGCG) (24,

352   86), on MARCO expression and bacterial phagocytosis in AM-MDMs from 2 donors. EGCG

353   also reversed declines in MARCO expression and *S. aureus* phagocytosis caused by IFNγ

354   (Figure 5).

355

356   ***Sulforaphane improves bacterial clearance and host survival in post-influenza bacterial***

357   ***pneumonia***

358   We investigated the effects of SFN using the *in vivo* model of post-influenza bacterial

359   pneumonia summarized schematically in Fig. 6A. To study the effects of SFN on bacterial

360   clearance, mice were subcutaneously injected with SFN or vehicle on post-influenza days 6 to 9

361   (i.e. day -1 to 2 post *S. pneumoniae* infection). SFN treatment significantly improved clearance

362   of *S. pneumoniae,* measured as bacterial colony forming units (CFU) in bronchoalveolar lavage

363   fluid, compared to untreated controls ($\log_{10}$ CFU = 6.09±0.60 vs. 7.57±0.20, p=0.036, Figure

364   6B). To study the effects of SFN on host survival in post-influenza bacterial pneumonia, mice

365   were injected with SFN or vehicle on post-influenza days 6 to 9 or 10 (i.e. day -1 to 2 or 3 post *S.*

366   *pneumoniae* infection) (Figure 6A). SFN-treated mice exhibited lower body weight loss and

367   faster weight recovery after post-influenza bacterial infection compared to controls (data not

368   shown). Notably, SFN improved survival in wild-type (53.3% vs. 5.9%, p<0.0001, Figure 6C)

369   but not *Marco* $^{-/-}$ mice (Figure 6D), indicating that *Marco* expression was required for the

370   beneficial effect of SFN. Median survival times for SFN-treated and vehicle-treated wild-type

371   mice were >12 days and 4.7 ± 0.2 days, respectively.

372

373

374   ***Role of Akt activation on MARCO expression and bacterial phagocytosis in post-influenza***

375   ***bacterial pneumonia.***

376   Akt and MAPK3 were among the top five genes identified as enriched network objects analysis

377   from the Metacore analysis of the RNAseq results (in order, with # of networks involved:

378   ERK1/2 (33), MAPK3 (30), PI3KIA (29), AKT (27), NF-kB (24)). Both were also previously

379   found to be altered by influenza (18). qRT-PCR showed that *Akt* and *Mapk3* mRNA expression

380   levels in murine alveolar macrophages gradually declined after influenza infection (Figures 7A

381   and 7B), but were increased on post-influenza day 11 compared to the reduced levels from day 5

382   to day 9. The nadirs of *Akt* and *Mapk3* expression occurred on day 7 and day 9, respectively.

383   These patterns of *Akt* and *Mapk3* expression were concordant with that of MARCO expression,

384   and inversely corresponded to IFNγ levels in BALF.

385   Both Akt inhibitor perifosine (10 to 50 μM) (26, 74) (Figure 8A) and MAPK/ERK

386   inhibitors U0216 and PD98059 (2, 53) (data not shown), reduced MARCO expression on AM-

387   MDMs supporting a role for Akt and MAPK signaling pathways in MARCO expression in

388   human macrophages.

389   Western blot analysis of AM-MDMs showed that IFNγ treatment decreased both pAkt

390   and MARCO. Moreover, treatment with the Akt activator SC79 (30) increased MARCO levels

391   in the presence of IFNγ (Figure 8B). Scanning cytometry also showed that the SC79 increased

392   MARCO expression in IFNγ-treated AM-MDMs (p<0.001, n=6, Figures 8C and 8D). Treatment

393   with MAPK/ERK activators failed to change MARCO expression (data not shown). Since the

394   available MAPK activators did not increase MARCO expression *in vitro*, we chose to focus on

395   Akt. SC79 treatment increased phagocytosis of GFP-*S. aureus* by IFNγ-treated AM-MDMs

396   (Figure 9A and 9B). Finally, the rescue effect of SC79 in increasing bacterial phagocytosis was

397   significantly inhibited by the class A scavenger receptor, poly(I) (Figure 9C).

398   To study the effects of SC79 on *in vivo* survival from secondary pneumonia, mice were

399   treated with SC79 (20 mg/kg) or vehicle from days 6 to 9 or 10 (i.e. day -1 to day 2 or 3 post *S.*

400   *pneumoniae* infection) (Figure 9D). SC79-treated mice had a significantly higher survival rate

401   compared to vehicle-treated mice (25% vs. 4%, p=0.001, Figure 9E). The median survival times

402   for SC79-treated and vehicle-treated mice were 6.8 ± 1.1 and 4.9 ± 0.3 days, respectively.

403

404   ***TFEB is an over-represented transcription factor regulating susceptibility to post-influenza***

405   ***bacterial pneumonia***

406   Since TFEB, the most over-represented transcription factor in our transcriptome data, was also

407   recently identified as an important regulator of innate immunity in *C. elegans* and in murine

408   macrophages (83), we investigated the role of TFEB in our post-influenza model. TFEB is

409   known to translocate to the nucleus and bind to E-box sequences (5'-CANNTG-3') (69).

410   Examination of the MARCO promoter region (-2,000 to +200) showed multiple E-box

411   sequences, and thus potential binding sites for TFEB (Figure 10). To investigate whether TFEB

412   binds to the MARCO promoter, AM-MDMs were subjected to chromatin immunoprecipitation

413   using anti-TFEB antibody. PCR with primers targeting the upstream sequence (-824 to -622) of

414   human MARCO gene showed enhanced detection, consistent with binding of TFEB to the

415   MARCO promoter area (Figure 11A).

416   A possible association between MARCO, Akt, and TFEB is suggested by the findings

417   that increased mTORC1 activity down-regulates Akt activity and MARCO expression (78, 87),

418   and sequesters TFEB in the cytoplasm through phosphorylation (59). We used RNA interference

419   to knock down TFEB in THP-1 macrophages and AM-MDMs prior to treatment with the Akt

420   activator SC79 (30). THP-1 cells stably transduced with lentivirus-delivered shRNA against

421   TFEB showed an almost five-fold reduction in MARCO mRNA expression (p=0.002, Figure

422   11B) and a seventy-percent reduction in *Hmox1* (data not shown) compared to cells transduced

423   with non-targeting shRNA. Mouse B6 cells (derived from alveolar macrophages) transduced

424   with shRNA against TFEB also showed reduced *Marco* mRNA expression compared to non-

425   targeting shRNA-transduced controls (data not shown). In contrast, after lentiviral transduction,

426   TFEB-overexpressing THP-1 cells showed a >300-fold increase in *Marco* expression (p<0.0001,

427   Figure 11C) and a five-fold increase in *Hmox1* expression (data not shown). To test the effect of

428   TFEB levels on the interaction of Akt and MARCO expression, we treated TFEB knockdown

429   cells with the Akt activator SC79. SC79-induced MARCO expression in IFNγ-treated

430   macrophages was abrogated in primary AM-MDMs transfected with TFEB siRNA (p<0.01, n=5,

431   Figures 11D and 11E), and in THP-1 knockdowns (data not shown), suggesting a link between

432   Akt signaling, TFEB nuclear translocation, and MARCO expression (Figure 12).

433

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Discussion

Alveolar macrophages are critical for maintaining lung homeostasis and promoting survival following influenza infection (12, 20, 62), including defense against secondary pneumonia. We investigated the role of the AM scavenger receptor MARCO in these defenses to identify potential immunomodulators that could be used to reduce the risk of post-influenza bacterial pneumonia. Initially, elevated IFNγ levels following influenza infection corresponded to synchronous decreases in AM MARCO expression. However, as IFNγ levels returned to normal on post-influenza day 11, there was a striking increase in MARCO expression. Transcriptome profiling of purified lung macrophages indicated important roles for NRF2 and Akt/TFEB-related signaling in the unexpectedly high rebound level of MARCO. High levels of IFNγ after influenza virus infection correlated with decreased MARCO expression *in vivo* and caused impaired phagocytosis in AM-MDMs *in vitro*. Activating the Nrf2 and Akt signaling pathways with sulforaphane and SC79, respectively, increased MARCO expression and improved bacterial phagocytosis in the presence of IFNγ. In an *in vivo* model of post-influenza bacterial pneumonia, both SC79 and sulforaphane improved host survival. The sulforaphane-induced improvement of survival was observed in wild-type but not in MARCO knockout mice, suggesting that MARCO is an indispensable mediator for Nrf2 effects in post-influenza bacterial pneumonia.

Some limitations of our study merit discussion. Although previous studies have shown that GM-CSF-matured human macrophages (AM-MDMs) used on our *in vitro* studies resemble primary AMs by numerous criteria, (13, 71), and IFNγ treatment of AM-MDMs reproduces the post-influenza down-regulation of lung macrophage MARCO expression and impaired bacterial phagocytosis seen *in vivo*, it is possible that responses of true primary lung macrophages may

458   differ. In addition, although our mouse model of post-influenza is similar to that used by many

459   other investigators (20, 28, 41, 67, 68, 72), results may be dependent upon influenza dose as well

460   as the dose and type of bacteria used for secondary infection (29, 38, 45, 70). In our secondary

461   model we did not measure viral load on day 7, the day of secondary bacterial infection. In studies

462   using much higher inocula of virus (designed to cause primary influenza pneumonia), we

463   observed a marked decrease in viral load by day 6 (21). Here, with a much lower inoculum that

464   causes minimal inflammation and weight loss, it is likely that virus is cleared by day 7. However,

465   whether there remains some residual virus has not been directly addressed in our data. We

466   focused on IFNγ-mediated effects but our multiplex cytokine results could be interpreted as

467   showing that changes in the levels of several other cytokines (individually or in combinations)

468   may contribute to the striking increase in MARCO levels from day 9 to 11. Another issue that

469   merits mention is our use of S. aureus for in vitro studies, while the in vivo studies use S.

470   pneumoniae. Both pathogens are common agents in secondary post-influenza pneumonia, and

471   both are engaged by MARCO and other scavenger receptors. However, in vivo secondary

472   pneumonia models using pneumococcus may be more relevant since they require much lower

473   inocula (e.g. 500 CFU), whereas inocula of 10^8 are commonly required for post-influenza studies

474   with S. aureus. Moreover, using the GFP-S. aureus in vitro allowed the use of fluorescent assays

475   reported, a strategy not possible for pneumococcus given the lack of available fluorescence

476   tagged S. pneumoniae. Future studies with both in vitro and in vivo analyses using the same

477   organism could add to understanding of any pathogen-specific aspects.   Finally, there is a rich

478   literature that identifies multiple mediators as causal for post-influenza secondary pneumonia (8,

479   15, 22, 31-33, 38, 40, 42, 44, 52, 55-58, 72, 79, 82, 85), including type I interferons (37). The

480   proposed multiple mechanisms are not necessarily mutually exclusive, and may offer alternative

481   targets for immunomodulator interventions.

482   Notably, we found that the MARCO rescue effect of Akt activation is dependent upon the

483   transcription factor TFEB, a lysosome and autophagy master regulator. These results are

484   congruent with previous studies in which PI3K inhibitors reduced scavenger receptor-mediated

485   phagocytosis (71). The class A scavenger receptor inhibitor poly(I) blocked the SC79-mediated

486   increase in bacterial phagocytosis, indicating a link between Akt signaling and scavenger

487   receptors. It has previously been shown that IFNγ produced by influenza infection induces

488   hyper-activated mTORC1 (87), which in turn inhibits Akt activity (10), and reduces both

489   MARCO expression and unopsonized phagocytosis (87). It is also possible that IFNγ inhibits

490   Akt activity through undiscovered mTORC1-independent pathways. Taken together, these data

491   suggested that IFNγ production after influenza is associated with attenuated Akt signaling and

492   MARCO expression in alveolar macrophages. The rebound phenomenon of MARCO expression

493   on post-influenza day 11 may be due to a feedback-based increase of Akt activity from the

494   previously inhibited state. It is worth noting that the magnitude of changes in Akt expression

495   (Fig. 7A) is relatively small. Moreover, phosphorylation state rather than expression level often

496   regulates Akt activity. Future studies are warranted to further delineate these potentially

497   important details.

498   Notably, both TFEB and Nrf2 are among the over-represented transcription factors

499   regulating differentially expressed genes during the recovery period of post-influenza MARCO

500   expression (Table S3), indicating that both are essential in restoration of homeostasis and/or host

501   defense against post-influenza complications. There may be some interplay between these two

502   transcription factors since: 1) as we have shown here, TFEB mediates Akt signaling in MARCO

503   upregulation; 2) activation of either insulin receptor (84) or epidermal growth factor receptor

504   tyrosine kinase (48) induces Nrf2 activation through PI3K/Akt signaling; 3) PI3K/Akt signaling

505   pathway may be activated by sulforaphane, leading to increased expression of Nrf2-regulated

506   genes (14, 39); 4) THP-1 cells stably transduced with shRNA against TFEB showed reduced

507   expression of *Hmox1*, an Nrf2-regulated gene, compared to the non-targeting shRNA control,

508   whereas TFEB-overexpressing THP-1 cells showed increased *Hmox1* expression when

509   compared to the control. We speculate that the ability of sulforaphane to also reverse IFNγ-

510   mediated cell death (Figs. 3C, 4A) may also reflect activation of pro-survival Akt signaling, but

511   our data do not directly test this postulate. Taken together, these data suggest that TFEB, Nrf2,

512   and MARCO expression are correlated with each other and all associated with Akt signaling.

513   The mechanism(s) by which MARCO expression is inhibited by IFNγ is unclear.

514   Previous data showed that tyrosine kinases, protein kinase C, PI3K signaling, and MAPK

515   pathway are involved in macrophage scavenger receptor-mediated phagocytosis (71).

516   Interactions between Nrf2 and PI3K Akt signaling are known (48, 84). These data indicate that

517   Nrf2 signaling and regulation of MARCO expression are both coupled to the MAPK and

518   PI3K/Akt signaling pathways, but the specific pathways for IFNγ effects need further

519   characterization.

520   Figure 12 summarizes our working hypotheses regarding the role of TFEB in mediating

521   Akt-dependent signaling to upregulate MARCO, the major opsonin-independent phagocytic

522   receptor on alveolar macrophages, in the post-influenza alveolar milieu. Our data also suggest

523   that expression of the scavenger receptor MARCO may be linked to additional TFEB-regulated

524   autophagy and/or lysosomal genes. Our results show that, in addition to Nrf2, Akt signaling and

525   TFEB may be effective therapeutic targets in post-influenza bacterial pneumonia.

527   **Figure Legends**
528

529   **Figure 1. MARCO expression is spontaneously restored and increased in the later phase of**

530   **influenza virus infection.**

531   **(A)** Bronchoalveolar lavage samples were collected after influenza infection, and

532   F4/80+CD11c+ cells (alveolar macrophages) were sorted and subjected to RNA

533   extraction and qRT-PCR for evaluation of *MARCO* expression.

534   **(B)** ELISA of bronchoalveolar lavage fluids collected from influenza-infected mice showed

535   that IFNγ levels significantly increased on day 7 and day 9 compared to uninfected

536   controls. N = 3-5 per group.

537   **(C)** Quantitative RT-PCR of sorted alveolar macrophages showed that *MARCO* expression

538   was down-regulated on day 7 ($p<0.0001$) and day 9 ($p<0.0001$) post-influenza virus

539   infection, but up-regulated on day 11 ($p<0.0001$) compared to uninfected controls. N= 3-

540   9 per group. Data are shown as fold change after normalization to the housekeeping gene

541   *TBP* expression; for both B, C: p values: *< 0.05, **<0.01, ***<0.001, or ****<0.0001.

542
543   **Figure 2. Cytokine multiplex assay of bronchoalveolar lavage fluids from influenza-**
544   **infected C57BL/6 mice.**
545
546   Day 0: uninfected control; D3, D5, D7, D9, D11: post-influenza day 3, 5, 7, 9, 11. There were

547   three mice in each group, represented by individual symbols; columns show mean values.

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553 **Figure 3. Sulforaphane significantly improves MARCO expression, bacterial phagocytosis,**
554 **and reduces cell death in IFNγ-treated human monocyte-derived macrophages.**
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556   **(A)** Representative images of scanning cytometry analysis of AM-MDMs after treatment with

557   IFNγ and/or SFN. Red, MARCO on the cell surface; green, dead cells.

558   **(B)** Quantitation of results of scanning cytometry analysis of AM-MDMs treated with or

559   without IFNγ and SFN. Fluorescence index (FI) = % positive x mean fluorescence

560   intensity of all cells (MFI). Number of donors = 6. Data are represented as mean ± SD.

561   **(C)** SFN treatment reduced cell death in IFNγ-treated AM-MDMs. Number of donors = 6.

562   Data are represented as mean ± SD.

563   **(D)** Representative images of scanning cytometry analysis of AM-MDMs after treatment with

564   IFNγ and/or SFN, followed by GFP-*S. aureus* infection. Extracellular bacteria were

565   labeled with red-fluorescent dye.

566   **(E)** Individual donor results showing decreased phagocytosis in response to IFNγ,

567   improvement by SFN, and blockade of SFN-effect with the scavenger receptor inhibitor,

568   poly(I). Number of donors = 6. Colors indicate different donors.

569   **(F)** Individual donor results showing decreased phagocytosis in response to IFNγ,

570   improvement by SFN, and blockade of SFN-effect with the MARCO peptide for

571   competition studies. Number of donors = 6. Colors indicate different donors. for B, C, E,

572   F: p values: *< 0.05, **<0.01, ***<0.001.

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577 **Figure 4. Sulforaphane significantly improves MARCO expression and reduces cell death**

578 **in IFNγ-treated THP1 macrophages.**

579 **(A)** Scanning cytometry analysis showed that IFNγ reduced MARCO expression on THP1 cells

580 in a dose-dependent manner, but simultaneous treatment with sulforaphane upregulated and/or

581 restored MARCO expression in IFNγ-treated THP1 cells. Fluorescence index (FI) of MARCO

582 expression = % of THP1 cells expressing MARCO x MFI (mean fluorescence intensity of all

583 cells). Data represent means of four replicates from two independent experiments.

584 **(B)** SFN treatment also reduced cell death in the IFNγ-treated THP1 cells.

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587 **Figure 5. Epigallocatechin gallate (EGCG) increases MARCO expression and bacterial**
588 **phagocytosis in IFNγ-treated cells.**
589 **(A)** The Nrf2 activator, EGCG, improved MARCO expression in IFNγ--treated AM-MDMs.

590 AM-MDMs were incubated with or without of IFNγ (20, 50 IU/ml) and EGCG (50µM). Data

591 shown are mean fold-changes in MFI (mean fluorescence intensity) in cells from 2 donors.

592 **(B)** EGCG improved phagocytosis of GFP-*S. aureus* in IFNγ-treated macrophages. Fluorescence

593 index (FI) of phagocytosis = % of cells with phagocytosed bacteria x number of bacteria per cell.

594
595 **Figure 6. Sulforaphane improves clearance of S. pneumoniae and host survival in wild-type**
596 **C57BL/6 mice with post-influenza bacterial pneumonia.**
597   **(A)** Summary of secondary pneumonia model starting infection with influenza virus strain

598   A/Puerto Rico/8/34 (PR8) on day 0, followed by *S. pneumoniae* infection on day 7. Mice

599   received daily treatments with either vehicle or sulforaphane from post-influenza day 6 to

600   day 9 or 10.

601   **(B)** Treatment with SFN significantly improved bacterial clearance measured as CFU in

602   bronchoalveolar lavage fluids on day 9 in post-influenza pneumococcal pneumonia (p =

603   0.036).

604   **(C)** Survival studies of wild-type C57BL/6 mice and (D) *MARCO* knockout mice with post-

605   influenza bacterial pneumonia. Sulforaphane significantly improved host survival in

606   wild-type mice (p<0.0001), but not that of *MARCO* knockouts. Number of vehicle-treated

607   wild-type mice = 34, number of SFN-treated wild-type mice = 30, number of vehicle-

608   treated *MARCO* knockouts = 20, number of SFN-treated *MARCO* knockouts = 20.

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613 **Figure 7. Influenza infection and decreased expression of Akt and MAPK3.**
614 Numbers in the X axis indicate days after PR8 influenza virus infection in wild-type adult male

615 C57BL/6 mice.

616 **(A)** Quantitative RT-PCR of sorted alveolar macrophages showed that *Akt* expression was

617 significantly down-regulated on day 5 to day 9 post-influenza virus infection, but there was no

618 significant difference on day 11 compared to uninfected controls. There were three to five mice

619 in each group. Data are represented as mean ± SD.

620 **(B)** Quantitative RT-PCR of sorted alveolar macrophages showed that *MAPK3* expression was

621 significantly down-regulated on day 5 to day 9 post-influenza virus infection. The level of

622 *MAPK3* expression significantly increased on day 11 from day 9, though it was still lower than

623 controls. There were three to five mice in each group. Data are represented as mean ± SD; for

624 both A, B: p values: *< 0.05, **<0.01, ***<0.001, or ****<0.0001.

Figure 8. Akt signaling is involved in post-influenza MARCO expression.

    (A) MARCO expression of AM-MDMs *in vitro* (measured by scanning cytometry) is reduced by the Akt inhibitor, perifosine. Number of donors = 3. Data are represented as mean ± SD.

    (B) AM-MDMs were treated overnight with IFNγ (20 IU/ml) and/or SC79 (0, 2, 5, 8 µg/ml). Western blot analysis showed that IFNγ treatment decreased levels of MARCO. The Akt activator, SC79, increased MARCO expression in a dose-dependent manner in the presence of IFNγ.

    (C) Representative scanning cytometry images showed that SC79 improved MARCO expression in IFNγ-treated AM-MDMs. AM-MDMs were treated simultaneously with 20 IU/ml of IFNγ and 8 µg/ml of SC79 for 20 to 24 hours. Red, MARCO proteins on the cell surface; green, dead cells.

    (D) Quantitation results of scanning cytometry analysis of AM-MDMs with or without IFNγ or SC79. Results from scanning cytometry analysis showed that Interferon-γ treatment of human monocyte-derived macrophages reduced MARCO expression in a dose-dependent manner (data with IFNγ 10 IU/ml not shown). Fluorescence index (FI) of MARCO = % positive x mean fluorescence intensity of each cell. Number of donors = 6. Data are represented as mean ± SD; for both A, D: p values: **<0.01, ****<0.0001.

Figure 9. The Akt activator, SC79, improves AM-MDM phagocytosis of *S. aureus*.

    (A) Average number of phagocytosed GFP-*S. aureus* per cell after treatment with IFNγ ± SC79. SC79 treatment increased the average number of phagocytosed GFP-*S. aureus* per cell in IFNγ-treated AM-MDMs. Number of donors = 6. Colors indicate different donors.

648 **(B)** Percentage of cells infected with GFP-*S. aureus* after treatment with IFNγ and/or SC79.

649 SC79 treatment increased the proportion of cells with phagocytosed bacteria in IFNγ-

650 treated AM-MDMs. Number of donors = 6. Colors indicate different donors.

651 **(C)** The ability of SC79 to increase bacterial phagocytosis after IFNγ treatment was

652 significantly inhibited by the class A scavenger receptor inhibitor, poly(I). Number of

653 donors = 4. Colors indicate different donors. for A - C: p values: *< 0.05, **<0.01.

654 **(D)** Summary of secondary pneumonia model starting infection with influenza virus (PR8) on

655 day 0, followed by *S. pneumoniae* infection on day 7. Mice received daily treatments

656 with either vehicle or SC79 from post-influenza day 6 to day 9 or 10. Data are a

657 combination of four independent experiments. Thirteen mice were treated with

658 subcutaneous injection of SC79 in the dose of 20 mg/kg from post-influenza day 6 to day

659 9. Fifteen mice received subcutaneous injection of SC79 in the dose of 20 mg/kg from

660 post-influenza day 6 to day 10. There was no significant difference in host survival

661 between four-day and five-day SC79 treatments.

662 **(E)** Survival studies of wild-type C57BL/6 mice with post-influenza bacterial pneumonia.

663 Number of vehicle-treated mice = 25, number of SC79-treated mice = 28.

664 **Figure 10. Multiple E-box sequences are identified in the MARCO promoter area.**

665 (A) Logo representation (WebLogo 3) of the E-box sequences (CANNTG) within 2000 b.p.

666 upstream of the transcription start site of human MARCO gene. (B) The CANNTG sites at -

667 2000b.p. to +200b.p. of the transcription start site of the human MARCO gene. Sequences in

668 blue indicate primers used in the ChIP assay.

669

670 **Figure 11. TFEB modulation changes *MARCO* expression levels.**

671   **(A)** ChIP assay of AM-MDMs (± treatment of SC79) showed direct binding of TFEB to the

672   upstream promoter area of human MARCO.

673   **(B)** Relative gene expression of *TFEB* and *MARCO* in TFEB knockdown THP1 cells

674   compared to the control transduced cells. Data are shown as fold change after

675   normalization to the housekeeping gene *TBP* expression. Bars represent the mean and

676   error bars represent the standard deviation of triplicates from one representative

677   experiment of two.

678   **(C)** Relative gene expression of *TFEB* and *MARCO* in TFEB-overexpressing THP1 cells

679   compared to control. Data are shown as fold change after normalization to the

680   housekeeping gene *TBP* expression. Bars represent the mean and error bars represent the

681   standard deviation of triplicates from one representative experiment of three.

682   **(D)** Representative scanning cytometry images of AM-MDMs with or without IFNγ and/or

683   SC79. AM-MDMs were transfected with human TFEB siRNA or non-targeting pool. NT:

684   non-target, TFEB kd: TFEB knockdown.

685   **(E)** Quantitation results of scanning cytometry analysis of TFEB AM-MDMs knockdowns.

686   Akt activator-induced MARCO expression in IFNγ-treated macrophages is blocked in

687   TFEB AM-MDM knockdowns. Fluorescence index (FI) = % positive x mean

688   fluorescence intensity of all cells (MFI). NT: non-target, TFEB kd: TFEB knockdown.

689   Number of donors = 5. Colors indicate different donors; for B, C, E: p values: *< 0.05,

690   **<0.01, ***<0.001.

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692

693 **Figure 12. TFEB mediates Akt activation-induced MARCO expression.**

694   **(A)** A schematic illustration depicting postulated activities between Akt, TFEB, and

695   MARCO. Influenza infection stimulates T cell production of IFNγ, which in turn may

696   induce activation of mTORC1 and ensuing Akt inhibition. It is also possible that IFNγ

697   inhibits Akt activity through mTORC1-independent pathways. Activated mTORC1

698   sequesters TFEB in the cytoplasm, and also down-regulates MARCO expression through

699   Akt inhibition or other downstream effectors, such as c/EBPβ inhibition (87). Akt

700   inhibition results in MARCO down-regulation, leading to impaired bacterial phagocytosis

701   and increased susceptibility to bacterial infection.

702   **(B)** Treatment with SC79 activates Akt signaling and upregulates MARCO in the presence of

703   IFNγ through the effects of TFEB.

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Figure 1
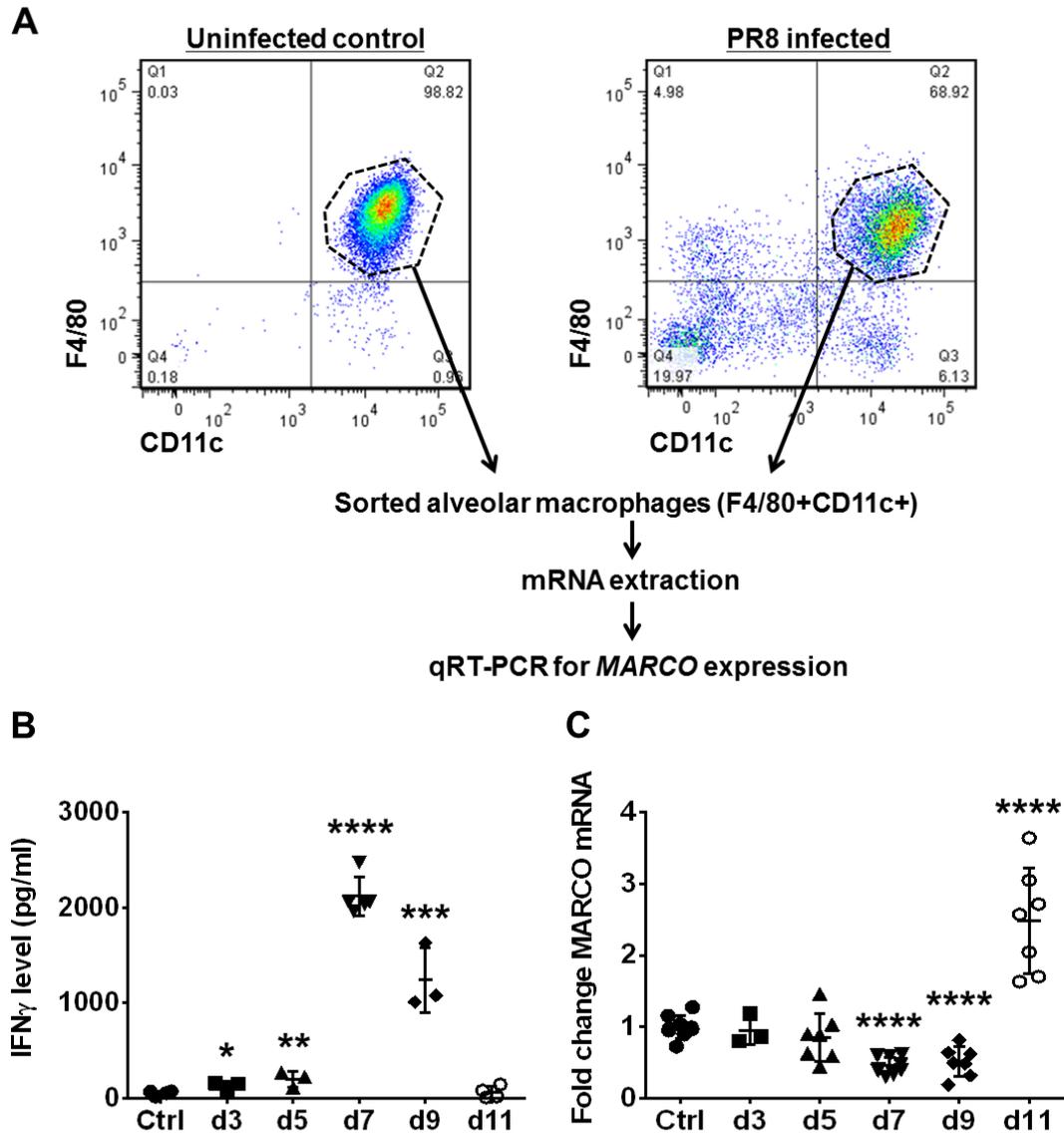
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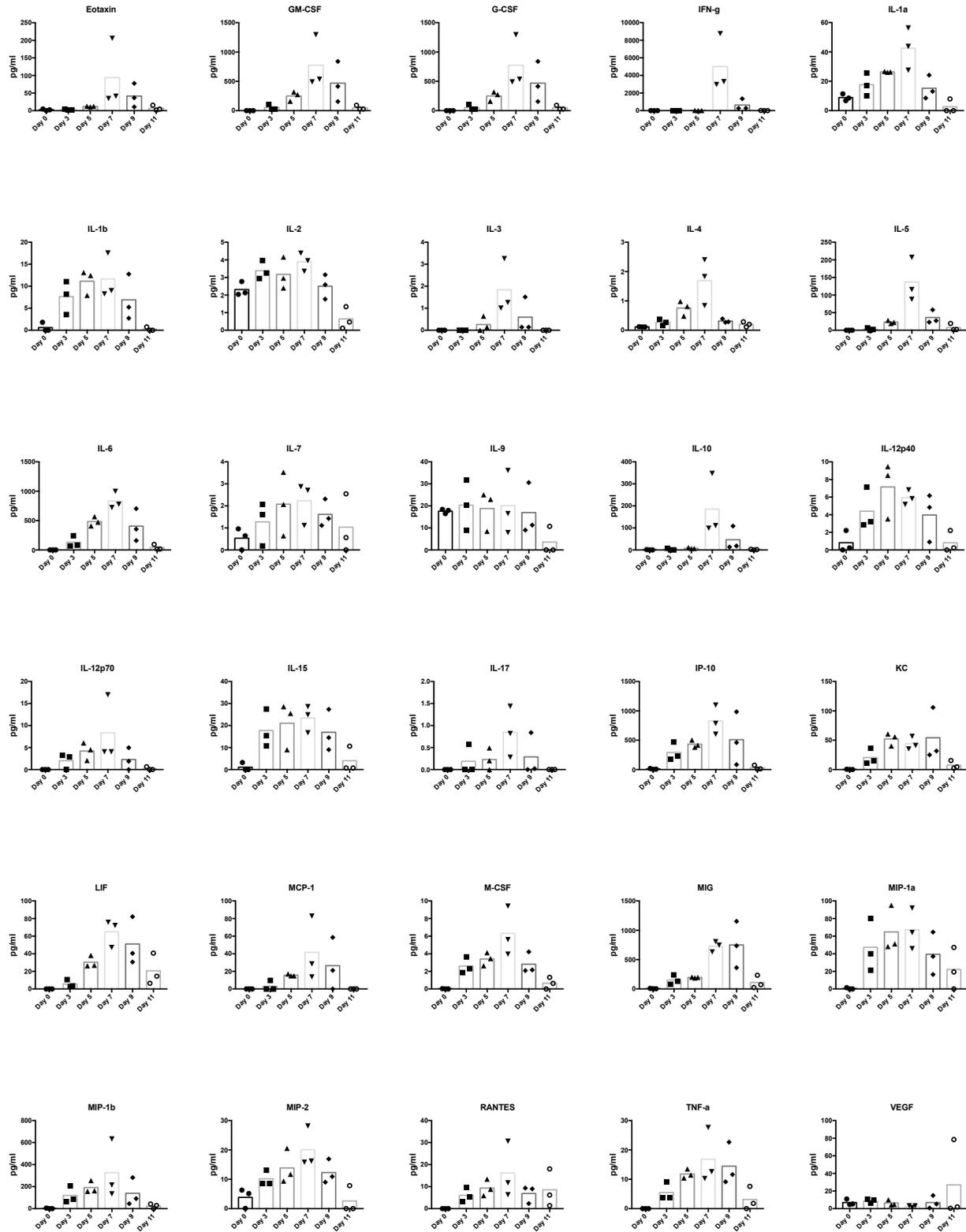
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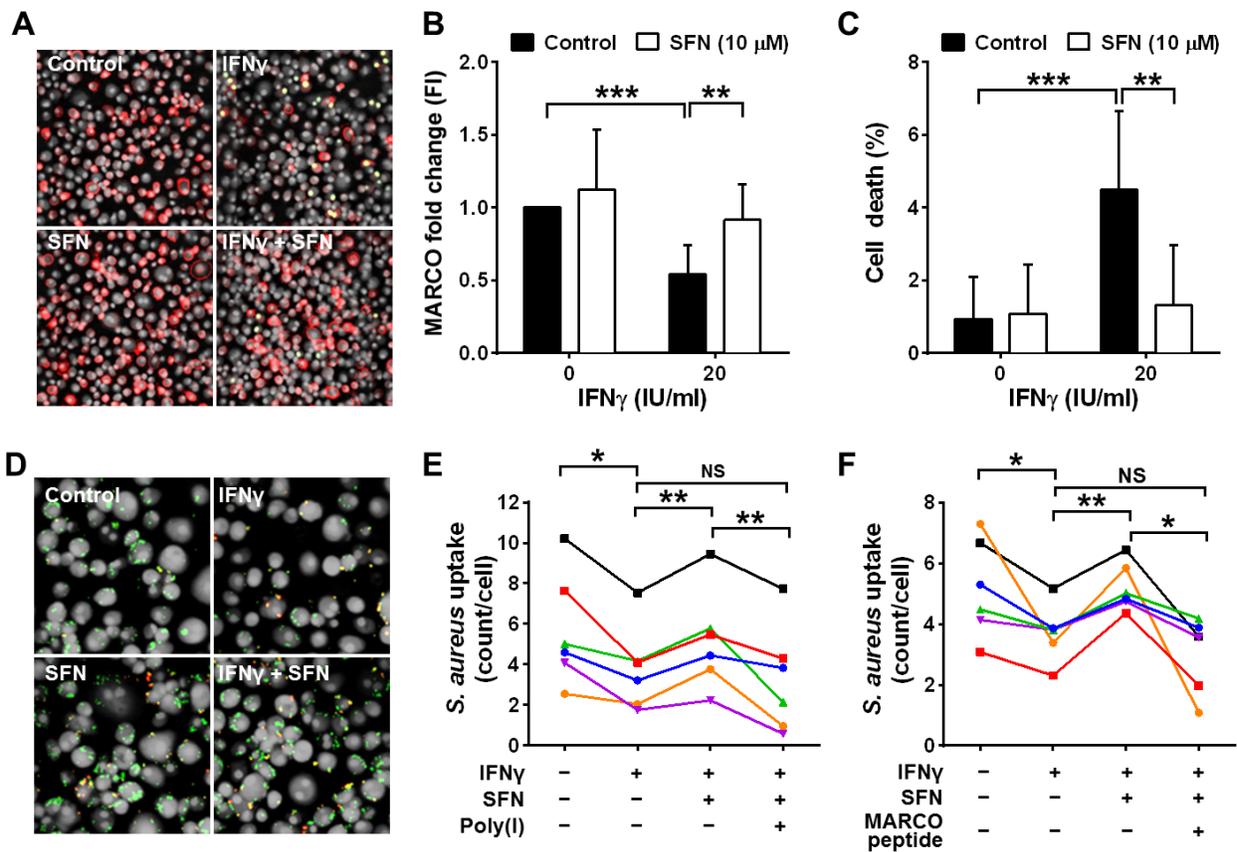
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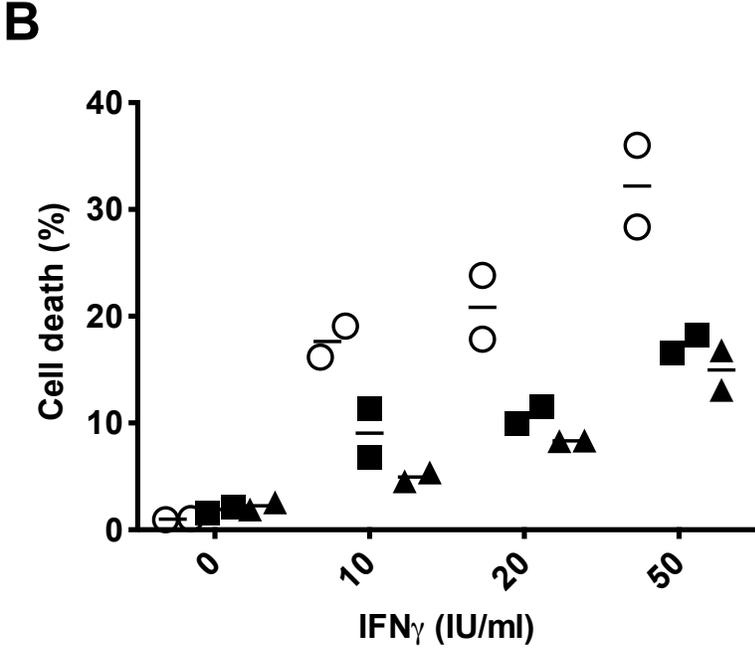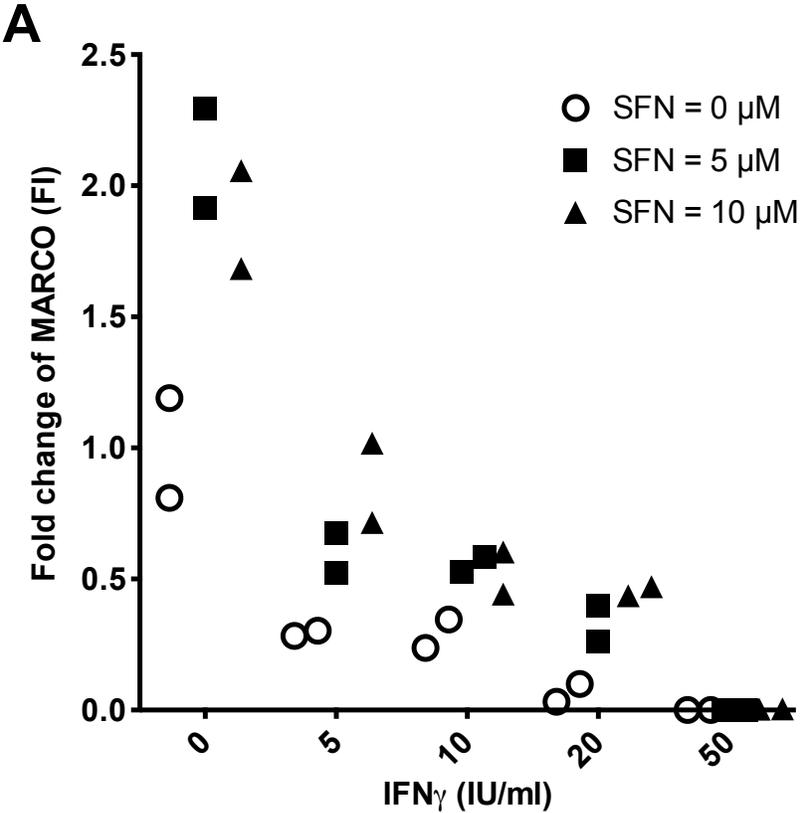
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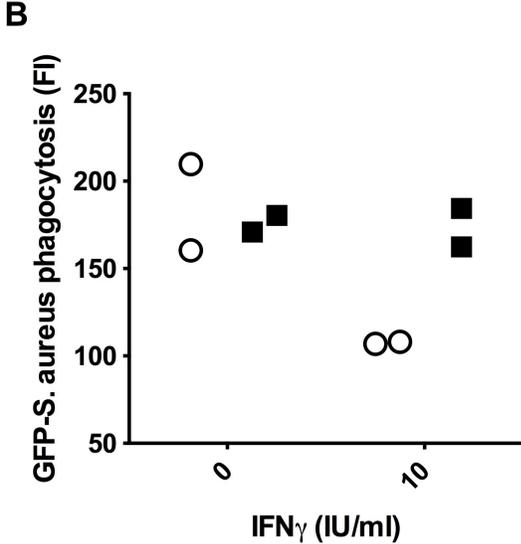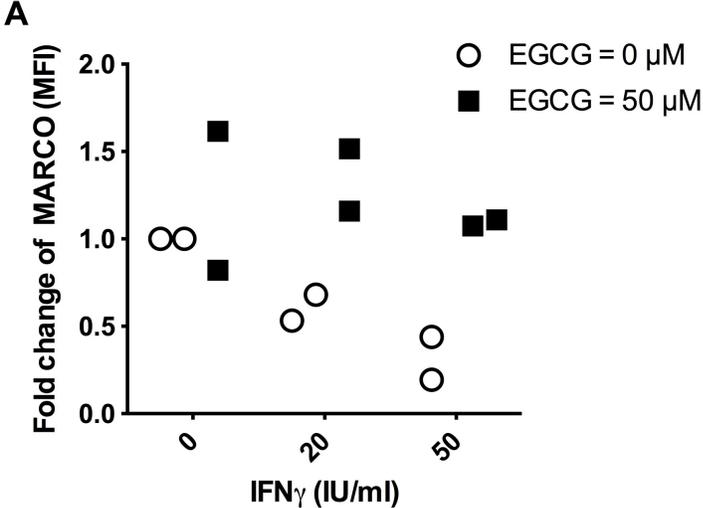
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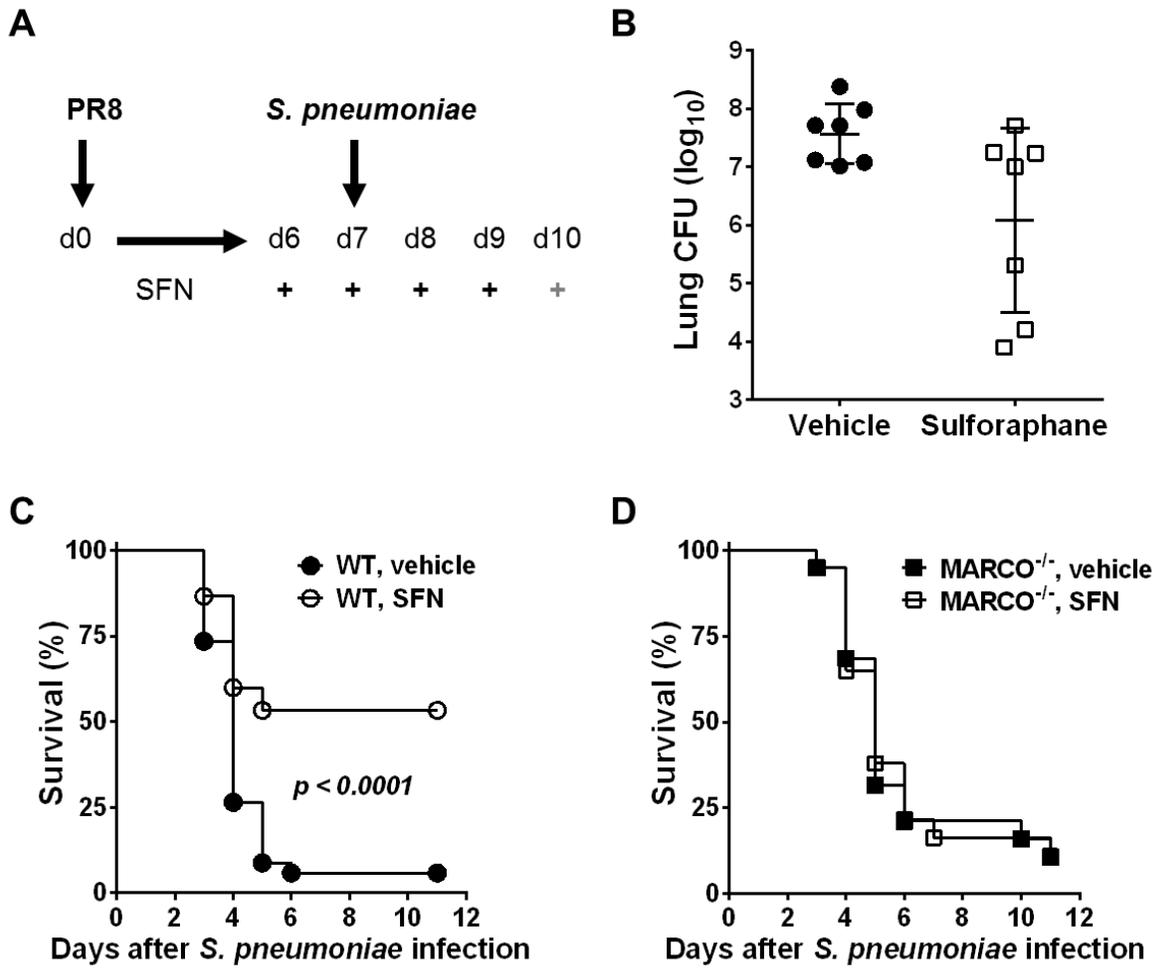
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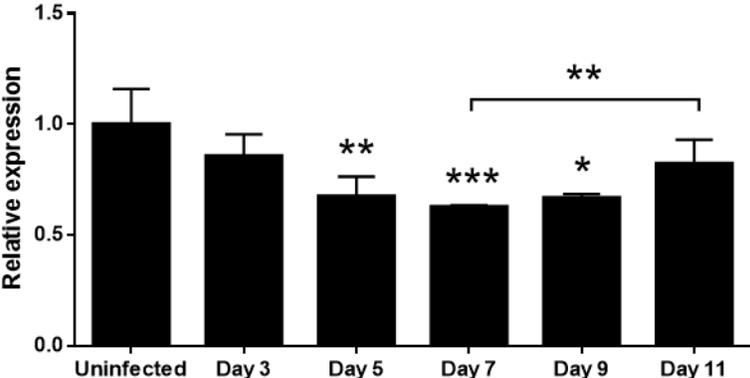
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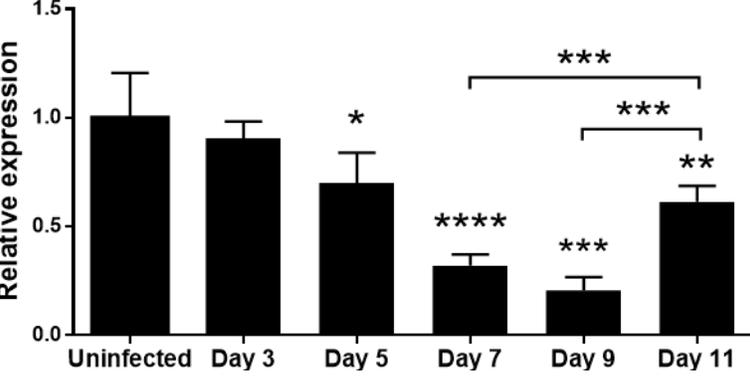
Figure 8

Figure 9

Figure 10

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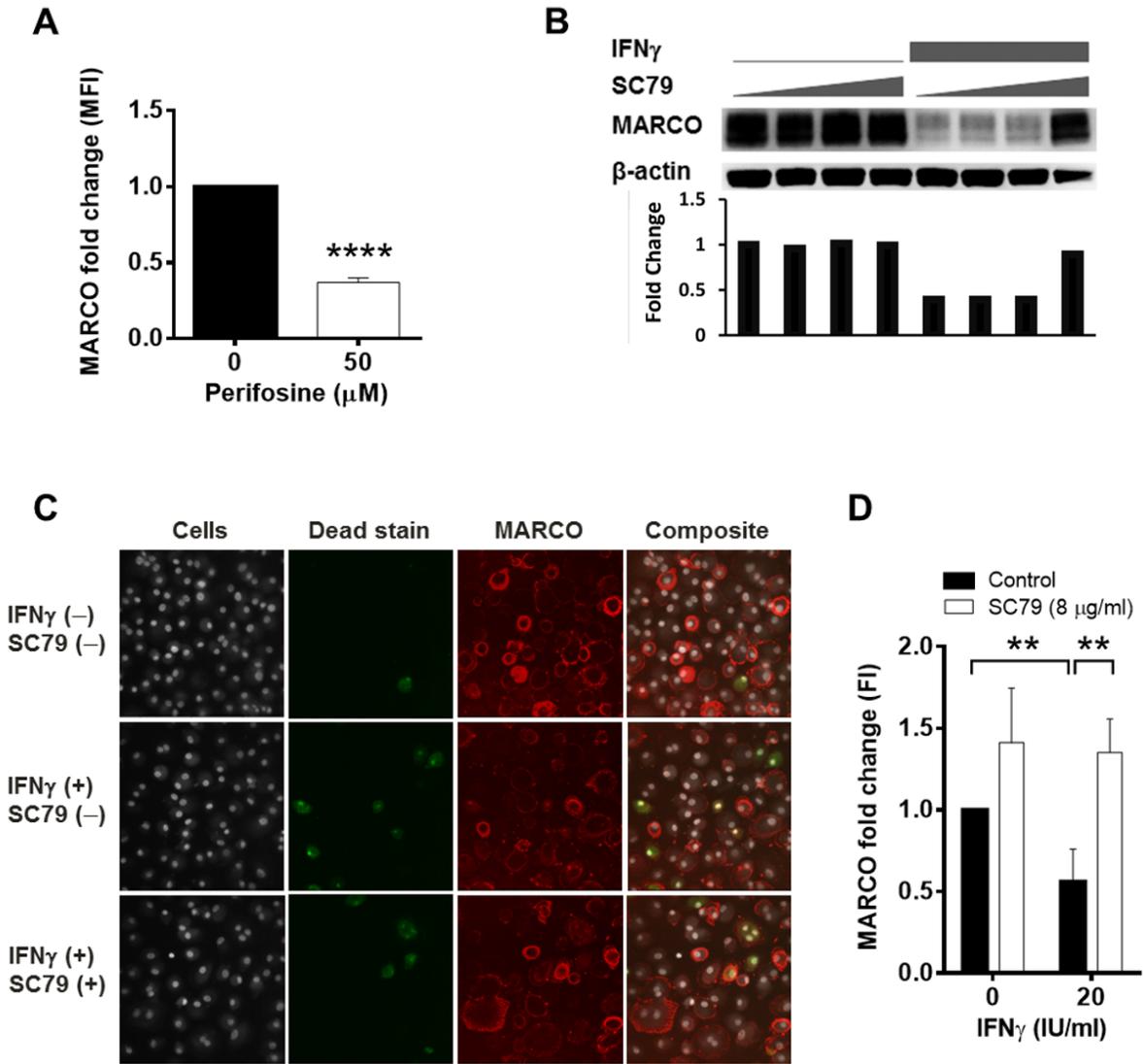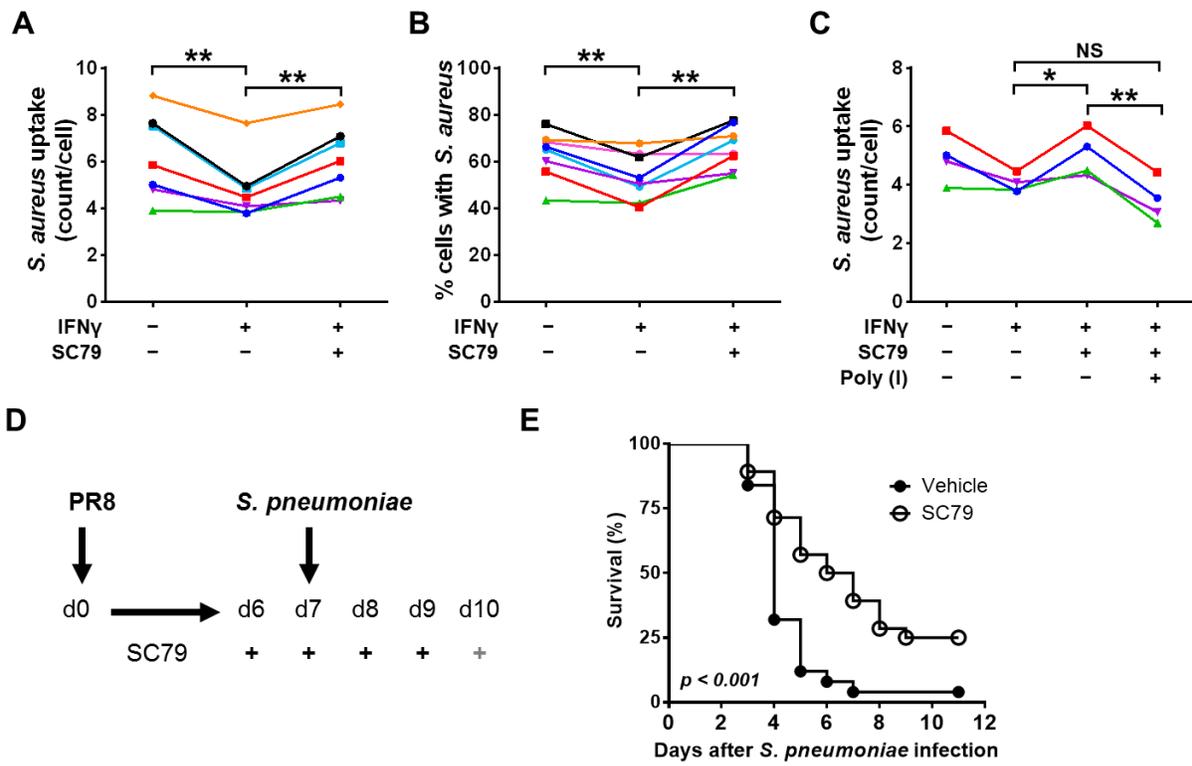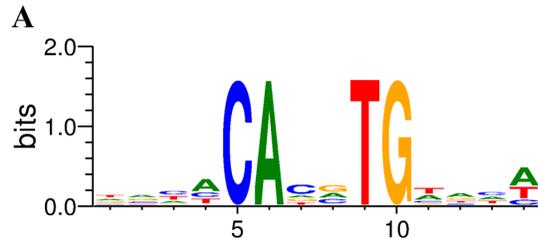
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CTTCTCAAACTGTAATGTGTATATAAATCACCTGGGGATCTAGTTAAACTGTAGATTTGGATTTGATCT
GATGGGGTGGGACCAGAGACTCATTGTTAGAACAAGTGAACTTGCATTTCTAACAAGTTCTCAGGTGA
TACTAATACTGCTGGTCCATGGACCACACTTTGAGCAGCAAGGATGAACTCTAAAGGTTGAGGCAGGT
CAGAGAAGGGAGATTTCACTGTGGGCTGGAGAGGTCCTGGGACAGAGAGCTGAGTCTGGGCCCATGG
CAGGGCTTGGTTGGCCCTCTCTGGAGCCATCCAGCTTTTGGGTCTCAGGGACCTGGGAGTGACGGGTG
CATTCAGAGGCCCGTAACTTGTGTCCCAAAGCCTCCTCGATCCCCCTTAACAAGCAGCAGCACTGTGT
GGGAGATCCACATGTGAATAGCCCGTGTTTGAGAAATGTCCAATCCTGATCATGTCAGGAAACATCCT
GCAAATTCTGAAATCAGAGCCAAAGGGAAGTGCTGCGAGGTTTACAACCAGCTGCAGTGGTTCGATG
GGAAGGATCTTTCTCCAAGTGGTTCCTCTTGAGGGGAGCATTTCTGCTGGCTCCAGGACTTTGGCCATC
TATAAAGCTTGGCAATGAGAAATAAGAAAATTCTCAAGGAGGACGAGCTCTTGAGTGAGACCCAACA
AGCTGCTTTTCACCAAATTGCAATGGAGCCTTTCGAAATCAATGGATGAGGAGTGTAAATTTCACACT
GTACCCTCTAGAAGCACAGTGCAACTTGCATCCCTCTTTCCTGCCATCACCTCCAGCCTCCCTCATTAG
AACAAGCCTC

Figure 11

A

200 b.p. | Input | TFEB | IgG | Input | TFEB | IgG

SC79   −   −   −   +   +   +

B

C

D

IFNγ	−	+	+	+
SFN	−	−	+	+
	NT	NT	NT	TFEB kd

Cells

Dead cells

MARCO

Composite

E

Figure 12