1 **Macrolides promote CCL2-mediated macrophage recruitment and clearance of**

2 **nasopharyngeal pneumococcal colonies in mice**

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28

29 **Abstract**

30 ***Background.*** *Streptococcus pneumoniae* (pneumococcus) colonizes mucosal
31 surfaces of the upper respiratory tract (URT), resulting in invasive disease. Macrolides
32 are known for their immunomodulatory effects. We investigated the potency of
33 macrolides to reduce pneumococcal colonization by activating host innate immunity.

34 ***Methods.*** The kinetics of colonization, cellular response, and inflammatory cytokine
35 levels in the URT were assessed after nasal inoculation of pneumococci. EM900 (a
36 novel 12-membered non-antibiotic macrolide having an immunomodulatory effect) was
37 orally administered throughout the experiment. Survival was evaluated for 10 days.
38 Macrolide-mediated CCL2 production from peritoneal macrophages was determined by
39 ELISA. The cell-signaling pathway was analyzed by western blotting and gene
40 silencing assays.

41 ***Results.*** *S. pneumoniae* was significantly reduced from EM900-treated mice 14 days
42 after pneumococcal inoculation. Macrophage recruitment and *Ccl2* mRNA expression
43 were promoted. CCL2 production from peritoneal macrophages was significantly
44 induced by macrolides and was dependent on NF-κB phosphorylation through the
45 MyD88- or TRIF-mediated pathway. Mortality of mice with invasive pneumococcal
46 disease was improved by pre-treatment with EM900.

47 ***Conclusions.*** Macrolides may inhibit invasive pneumococcal infections by

48 accelerating the clearance of pneumococcal nasopharyngeal colonization via promotion

49 of macrophage-mediated innate immunity.

50 **Key words:** *Streptococcus pneumoniae*, macrolides, colonization, CCL2, macrophage

51

52 **Introduction**

53 *Streptococcus pneumoniae* (pneumococcus) is the most common cause of
54 community-acquired pneumonia in all age groups and leads to hospitalization [1].
55 Pneumococcus frequently colonizes the upper respiratory tract (URT), where it persists
56 for four to six weeks [2]. In general, this colonization is simply asymptomatic carriage;
57 however, it occasionally leads to invasive disease [3]. Therefore, control of
58 pneumococcal nasopharyngeal colonization is crucial.

59 Local immune responses play an important regulatory role in resistance to pathogens
60 at the infection site. Recognition of pneumococcus by different sensors of the innate
61 immune system called pattern recognition receptors (PRR) regulates production of
62 inflammatory mediators that orchestrate subsequent adaptive immune responses [4]. In
63 particular, TLR2 senses lipid-modified constituents on *S. pneumoniae*, which
64 contributes to clearance of colonies through the Th17 response and influx of monocytes
65 and macrophages [5]. A recent study found that Nod2 protein, present on phagocytes,
66 senses degradation products of pneumococcus-derived peptidoglycan, leading to the
67 release of the CCL2 chemokine, followed by recruitment of monocytes/macrophages
68 and clearance of pneumococcal colonization [6].

69 The current conjugate vaccine against pneumococcus is highly effective against

nasopharyngeal carriage and invasive disease caused by vaccine-type strains [7]. However, the surface capsular polysaccharide of pneumococcus serves as the basis for serotyping of these organisms; currently, >90 different pneumococcal serotypes have been identified, and serotype replacement by non-vaccine-type strains, which generate disease, has become a serious threat [7]. Additionally, in contrast to conjugate vaccines, the 23-valent pneumococcal polysaccharide vaccine cannot eradicate pneumococcal colonization and sequential bacterial pneumonia [8]. Therefore, new strategies to elicit protection against a broader range of pneumococcal strains are required.

Macrolides are antibiotics that are classically known as protein synthesis inhibitors; they have a broad antimicrobial effect on gram-positive cocci and atypical pathogens. In addition to these antimicrobial effects, several studies demonstrate the immunomodulatory activities of macrolides on host bacteria [9, 10]. The regulation of excessive inflammation by macrolides is well characterized and the role of macrolides in the activation of innate immunity, including their ability to promote neutrophil migration [9], regulate monocyte differentiation into macrophages, and modulate macrophage function, has been established [10]. Recently, the Kitasato Institute for Life Sciences at Kitasato University prepared EM900, a new 12-membered-ring non-antibiotic macrolide derivative of erythromycin that does not have antimicrobial

88 activity but has immunomodulatory effects [11]. EM900 suppresses activation of

89 nuclear factor NF-κB and production of interleukin (IL)-8, IL-1β, and TNF-α from

90 human airway epithelial cells, demonstrating its anti-inflammatory action [12]. We

91 hypothesized that macrolides may be potential candidates for prophylaxis of

92 pneumococcal infection through modulation of host innate immunity. This study

93 investigated the immunomodulatory effects of macrolides in inhibiting pneumococcal

94 nasopharyngeal colonization and subsequent invasive disease in mice.

95

96 **Methods**

97 **Laboratory animals**

98 C57BL/6 (six weeks old, female) specific-pathogen-free mice were purchased from

99 Japan SLC (Hamamatsu, Japan). All animal experiments were performed in accordance

100 with the guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki

101 University School of Medicine.

102

103 **Bacterial strains and growth conditions**

104 *S. pneumoniae* strain P1121 (type 23F clinical isolate) [5, 13], P1547 (*S. pneumoniae*

105 serotype 6A, mouse virulent, clinical isolate from blood) [14], and P173 (*S. pneumoniae*

106 serotype 2, capsulated clinical isolate) were kindly provided by JN Weiser (Professor,

107 University of Pennsylvania). P1121 cells were grown in 10 ml of tryptic soy broth

108 (Difco; BD) until the mid-log phase was reached (OD_{660} = 0.5). After centrifugation, the

109 pellet was resuspended in 100 µl of sterile PBS to achieve a density of 10^9 CFU/ml [5].

110

111 **Experimental murine model of pneumococcal colonization and invasive diseases**

112 In colonization studies, 10 µl (10^7 CFUs) of the pneumococcal suspension was

113 introduced into the nostril of each mouse. After a predetermined number of days, mice

114 were sacrificed by CO_2 asphyxiation. The trachea was exposed and cannulated to

115 introduce 200 µl of sterile PBS. The lavage fluid exiting the nares was collected and

116 P1121 cells were quantified by plating 10 µl serial dilutions of the nasal wash onto

117 tryptic soy agar plates supplemented with catalase (4,741 U/plate) (Worthington

118 Biochemical Corp.) and neomycin (20 µg・ml^{-1}) (Sigma-Aldrich Japan, Tokyo, Japan),

119 and incubating overnight at 37°C in a 5% CO_2 atmosphere [5]. EM900 (10 mg・kg^{-1}), a

120 gift from T Sunazuka (Kitasato University, Tokyo, Japan) [11], was administered orally

121 twice a day from one week before the inoculation and throughout the experiment (We

122 confirmed that MICs of EM900 against pneumococcus used in this study are greater

123 than 100 µg・ml^{-1}). At days 3, 7, and 14 after inoculation, the mice were sacrificed and

124 nasal washing was performed. These lavages were analyzed to evaluate the kinetics of

125 colonization and cellular inflammatory response by flow cytometry, and *Ccl2* and

126 *IL-17A* mRNA expression by quantitative RT-PCR (qRT-PCR). WT mice with or

127 without EM900 treatment for three weeks before infection were challenged intranasally

128 with 5×10^7 CFU of strains P1547 and P173, and survival was observed for 10 days

129 after the challenge.

130

131 **Flow cytometry and intracellular cytokine staining**

132 Nasal lavages were pooled from 5 mice and the cells were collected by centrifugation at

133 1200 g for 10 min at 4°C. The pellets were washed once using 200 µl washing buffer

134 (PBS supplemented with 1% BSA), collected again by centrifugation, resuspended in

135 200 µl of washing buffer, and transferred to a 96-well polypropylene plate. Nonspecific

136 binding was blocked using a rat anti-mouse antibody directed against the FcγIII/II

137 receptor (CD16/CD32) (BD Biosciences), and the following rat anti-mouse cell surface

138 antibodies were applied: Ly6G (BD Biosciences), CD45 (BD Biosciences), and F4/80

139 (eBioscience). The final dilutions of the antibodies were 1:300 for the FITC- Ly6G

140 antibody and 1:400 for PE-CD45 and APC-F4/80 antibodies. Reactions were performed

141 by incubating the plate on ice in the dark for 45 min. All samples were resuspended in

142 wash buffer and then subjected to flow cytometry analysis by using a BD FACSCalibur

143 flow cytometer (BD Biosciences) [15]. For intracellular staining, cytokine secretion

144 from peritoneal cells pretreated with macrolides was blocked by using GolgiPlug (BD

145 Biosciences). Stimulated peritoneal cells were detached from plates by chilling on ice at

146 4°C and washing with cold PBS. The cells were pelleted, resuspended in 1% BSA, and

147 stained for surface markers as described above. Cells were then fixed and permeabilized

148 using the BD Cytofix/Cytoperm Plus Kit (BD Biosciences). Accumulation of

149 intracellular CCL2 was detected using an Armenian hamster anti–mouse CCL2

150 antibody (eBioscience). An Armenian hamster IgG isotype control was used for the

151 detection of nonspecific binding. All flow cytometry data were analyzed using Flow Jo

152 Mac, version 9.6 (Tree Star) [6].

153

154 **Macrophage depletion**

155 Resident macrophages in the nasopharynx were depleted using liposome-Cl_2MDP

156 (FormuMax) as previously described [5]. For depletion, liposome-Cl_2MDP (20 μl/dose)

157 was administered intranasally at 4 and 8 days before and 1 and 8 days after the P1121

158 challenge. The influence of macrophage depletion was evaluated at 14 days after

159 bacterial inoculation.

160

161 **Isolation and culture of peritoneal macrophages**

162 Three days after intraperitoneal injection of 4% sterile thioglycollate medium (2 ml),

163 peritoneal macrophages were isolated by peritoneal lavage with Hank's buffer (without

164 Ca^{2+} and Mg^{2+}) containing 0.1% gelatin. Contaminating erythrocytes, granulocytes, and

165 dead cells were removed by density gradient centrifugation for 45 min at 800 g in

166 Mono-Poly resolving medium, according to the manufacturer's protocol (MP

167 Biomedicals). Purified peritoneal macrophages were washed 3 times and cultured

168 overnight in Dulbecco's modified Eagle medium [6].

169

170 **ELISA and cytokine array**

171 CCL2 concentrations in culture supernatants of peritoneal macrophages that were

172 pretreated with EM900, clarithromycin (CAM) (Sigma-Aldrich Japan, Tokyo, Japan),

173 azithromycin (AZM) (Sigma-Aldrich Japan, Tokyo, Japan), or solvent only for 12–18 h

174 were assayed using mouse Quantikine ELISA kits (R&D Systems, Minneapolis, MN),

175 according to the manufacturer's protocols. To investigate macrolide-mediated signaling

176 transduction pathways, we utilized specific MAPK inhibitors, i.e., SP600125 (JNK

177 inhibitor), SB203580 (p38 inhibitor), PD98059 (MEK-1 inhibitor), and parthenolide

178 (NF-κB inhibitor) (Sigma-Aldrich Japan, Tokyo, Japan). Additionally, the cytokine

179 expression profile of macrolide-stimulated peritoneal macrophages was analyzed using

180 a mouse cytokine protein array (R&D Systems, Minneapolis, MN), following the

181 manufacturer's instructions. For this experiment, the same amount of cell density and

182 supernatant derived from each culture were used.

183

184 **Lipopolysaccharide (LPS) detection**

185 To exclude the possibility of LPS contamination, the Limulus amebocyte lysate test

186 using the Gel-clot method (Pyrotell) was performed for culture supernatants and

187 reagents. The detection limit of the assay was 0.03 EU/ml (0.003 ng

188 /ml) [16].

189

190 **qRT-PCR assays**

191 RNA was isolated from nasal lavage of the upper respiratory tract with 600 μl of RNA

192 lysis buffer using an RNeasy Mini Kit (QIAGEN) according to manufacturer's protocol

193 [17]. Complementary DNA was generated using a high-capacity reverse transcription

194 kit (Applied Biosystems). Approximately 25 ng of cDNA was then used as a template in

195 reactions with forward and reverse primers and SYBR Green (Applied Biosystems)

196 according to the manufacturer's protocol. Reactions were performed using the

197 StepOnePlus Real-Time PCR system (Applied Biosystems), and quantitative

198 comparisons were obtained using the ΔΔCT method. The primers used are as follows:

199 GAPDH-F: 5′-TGTGTCCGTCGTGGATCTGA-3′; GAPDH-R:

200 5′-CCTGCTTCACCACCTTCTTGAT-3′; CCL2-F:

201 5′-AGCTCTCTCTTCCTCCACCAC-3′; CCL2-R:

202 5′-CGTTAACTGCATCTGGCTGA-3′; IL-17A-F: 5′

203 -TCTCATCCAGCAAGAGATCC-3′; and IL-17A-R:

204 5′-AGTTTGGGACCCCTTTACAC-3′ [6].

205

206 **Phagocytosis assay**

207 FITC-bound *S. pneumonia* was made as reported previously. This suspension of *S.*

208 *pneumoniae* in PBS was added to 2.5 × 10^5 cultured peritoneal macrophages, which

209 were pretreated with macrolides for 24 hours (the ratio bacteria : macrophage = 25:1).

210 Peritoneal macrophages and pneumococcus were co-cultured for 45 minutes at 37 °C

211 under 5% CO_2 in humidified air. Phagocytosis was stopped by putting the plates on ice,

212 following adherent bacteria were removed by washing cells with PBS. To quantify

213 phagocytosed bacteria, fixed cells were used for FACS analysis.

214

215 **Western blotting**

216 Cells were washed and lysed for 5 min in RIPA buffer (Sigma-Aldrich Japan, Tokyo,

217 Japan) containing protease inhibitor cocktail (Sigma-Aldrich Japan, Tokyo, Japan), and

218 the protein concentration was determined using the bicinchoninic acid protein assay kit

219 (Thermo, Rockford, IL, USA). For equivalent loading between samples, the same

220 amount of each sample was fractionated via 4-12% sodium dodecyl sulfate

221 polyacrylamide gel electrophoresis, and transfer, blocking and signal development were

222 performed as described previously [18]. For detection of intact and phosphorylated

223 (activated) forms of NF-κB, rabbit primary antibodies against NF-κB (total NF-κB,

224 #3034; phospho-NF-κB, #3031) were purchased from Cell Signaling Technology

225 (Beverly, MA, USA). Incubation with the primary antibodies was followed by

226 incubation with secondary antibodies conjugated to horseradish peroxidase (sc-2030;

227 Santa Cruz Biotechnology)

228

229 **RNA interference experiments**

230 RNA interference technology was used to knock down the expression of TIR

231 domain-containing adaptors, such as MyD88 and TRIF, in peritoneal macrophages. The

232 efficiency and specificity were validated by quantitative real-time PCR and western

233 blotting. Peritoneal macrophages obtained from mice were grown in 24-well plates ($2 \times$

234 10^5 cells/well) and transfected with 50 nM TIR domain-containing adaptor siRNAs

235 according to the manufacturer's instructions (Invitrogen). Transfected cells were

236 incubated for 72 h before being stimulated by macrolides. Pre-designed RNA

237 oligonucleotides for MyD88 and TRIF were obtained from Invitrogen (Life

238 Technologies Corp.).

239

240 **Statistical analyses**

241 All data were analyzed by using Prism 5 GraphPad Software and are expressed as the

242 mean ± SEM. Differences between the treatment group and the control were tested for

243 significance using the Mann–Whitney U test. The significance of differences among

244 more than three groups was examined using ANOVA followed by Dunnett's or

245 Turkey's post-tests. Survival analysis was performed using the log-rank test, and

246 survival rates were calculated via the Kaplan–Meier method. $P < 0.05$ was considered

247 statistically significant.

248

249 **Results**

250 **The effect of EM900 on pneumococcal nasopharyngeal colonization**

251 To examine the importance of immunomodulation in regulating pneumococcal
252 colonization, EM900, a novel 12-membered non-antibiotic macrolide that has an
253 immunomodulatory effect, was administered 7 days prior to *S. pneumoniae* inoculation
254 and continued throughout the observational period. As shown in Figure 1*A*, the density
255 of pneumococcus in the URT was significantly decreased in EM900-treated mice
256 compared to untreated mice 14 days after pneumococcal inoculation. FACS analysis of
257 nasal lavages was performed to measure the number of inflammatory cells recruited to
258 the URT at 7 and 14 days after pneumococcal inoculation (Fig. 1*B*, 1*C*). The number of
259 macrophages in the URT was significantly higher in EM900-treated mice than in
260 untreated mice 14 days after pneumococcal inoculation. These data indicate that EM900
261 promoted macrophage recruitment to the URT and reduced pneumococcal colonization
262 in a macrophage-dependent manner through its immunomodulatory effects.
263

264 **Effect of macrolides on mRNA expression of Ccl2 and IL-17A in the nasal cavity**

265 Previous reports have shown that pneumococcal clearance is dependent on macrophage
266 recruitment mediated by NOD2-CCL2 and TLR2-Th17 lineages［5, 6］. Therefore, we
267 analyzed mRNA expression of *Ccl2* and *IL-17A* in the nasal lavage at day 14 after

268 pneumococcal inoculation with or without EM900 treatment. As shown in Figure 2, the

269 mRNA expression of *Ccl2* but not *IL-17A* was markedly increased by administration of

270 EM900. Next, we performed a macrophage depletion assay by intranasal administration

271 of liposome-Cl_2MDP before pneumococcal inoculation to determine which cells are

272 responsible for CCL2 production in response to EM900. There were no significant

273 differences in the density of pneumococci (Fig. 3*A*) and furthermore, the effect of

274 EM900 for facilitating the recruitment of macrophages and the expression of Ccl2

275 mRNA in URT were disappeared in the mice treated by liposome-Cl_2MDP at day 14

276 after pneumococcus inoculation (Fig. 3*B and 3C*) . Taken together, these results suggest

277 that EM900 exerts its effects on resident macrophages and elicits CCL2 production and

278 macrophage recruitment, resulting in the promotion of pneumococcal clearance in the

279 URT.

280

281 **Effect of macrolides on the function of murine peritoneal macrophages**

282 To investigate the mechanism of macrolide-mediated CCL2 production, we extracted

283 murine peritoneal macrophages and stimulated them with various concentrations of

284 EM900 and two existing macrolides, CAM and AZM. After synthesis of EM900,

285 extraction with chloroform and purification by using a silica gel column are considered

286 to provide a very low possibility of contamination with LPS. Additionally, the LPS
287 content in the culture supernatants and reagents was undetectable in the Limulus
288 amebocyte lysate test using the Gel-clot method, thus assuring that the observed
289 phenomena did not result from LPS contamination. As shown in Figure 4*A*, *Ccl2*
290 mRNA expression in macrolide-stimulated macrophages increased in a
291 concentration-dependent manner. To confirm that CCL2 production from macrophages
292 was in response to macrolides, we stained cells for surface markers and intracellular
293 CCL2 following macrolide stimulation and then analyzed them by flow cytometry.
294 F4/80$^+$ cells stained positive for intracellular CCL2 following stimulation with EM900,
295 CAM, and AZM (Fig. 4*B*). Furthermore, when production of numerous cytokines was
296 screened by cytokine array, it was observed that while CCL2 production was clearly
297 present, pro-inflammatory cytokines associated with tissue injury such as TNF-α were
298 not (Supplementary Fig. 1).
299 To determine the effect of macrolides on the phagocytic function of macrophages, we
300 performed a phagocytosis assay in peritoneal macrophages pretreated with macrolides
301 and FITC-bound *S. pneumoniae*. As shown in Fig. 5A and B, there are significant
302 increase of pneumococcus-phagocytic cells (APC-F4/80 and FITC double positive) in

303 the macrophages pretreated with macrolides, suggesting that macrolides enhanced the

304 phagocytic activity and promoted the clearance of *S.pneumoniae* in URT.

305

306 **Effect of macrolides on intracellular signal transduction pathways that regulate**

307 **CCL2 production**

308 To investigate macrolide-mediated signaling transduction pathways involved in CCL2

309 production, we examined peritoneal macrophages pretreated with specific MAPK

310 inhibitors, i.e., SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD98059 (MEK-1

311 inhibitor), and parthenolide (NF-κB inhibitor), for 30 min and then co-treated with the

312 macrolides for 24 h prior to detection of CCL2 in the supernatant. Only pretreatment

313 with parthenolide inhibited CCL2 production and NF-κB phosphorylation with time

314 after macrolide stimulation (Fig. 6*A* and 6*B*), suggesting that NF-κB was indispensable

315 for macrolide-mediated CCL2 production. To further investigate the ability of

316 macrolides to induce CCL2 production, knockdown of MyD88 and TRIF, one of the

317 TIR domain-containing adaptors existing upstream of NF-κB, was performed using

318 specific siRNA, and NF-κB phosphorylation and CCL2 production examined. Both

319 NF-κB phosphorylation and CCL2 production were significantly reduced by

320 knockdown of MyD88 or TRIF (Fig. 6*C* and 6*D*), indicating that macrolides induce

321 CCL2 production from macrophages through an NF-κB signaling pathway via MyD88
322 or TRIF.
323
324 **Activation of macrolide-mediated innate immunity protects the host from invasive**
325 **pneumococcal disease**
326 To investigate the role of macrophage recruitment in priming innate immunity, we
327 challenged mice with the P1547 (serotype 6A) and P173 (serotype 2) strains, and
328 treated them with EM900. We found that EM900-treated mice were more resistant to
329 sepsis than untreated mice (Fig. 7*A* and 7*B*), which suggests that a reduction in the
330 density of colonization may prevent invasive disease and reduce mortality through
331 activation of innate immune priming by macrolides.
332
333 **Discussion**
334 Invasive pneumococcal infection cannot be completely prevented using the existing
335 pneumococcal vaccine that activates humoral immunity, with the capsular
336 polysaccharide acting as the immune antigen. There have been reports on the
337 development of a live attenuated vaccine with higher protective immune effects [19]
338 and a fusion vaccine that simultaneously activates the innate immune system and the

339 humoral immune system; however, these vaccines have not yet been put into practical

340 use [20, 21]. In this study, we hypothesized that macrolide antibiotics, which have been

341 reported to possess various immunomodulatory capacities, would activate host innate

342 immunity and inhibit pneumococcal nasopharyngeal colonization without serotype

343 dependency.

344 Our results demonstrated that the extent of bacterial colonization 14 days after

345 bacterial inoculation was significantly decreased compared to that in the control group.

346 There was also a significant increase in the number of nasopharyngeal macrophages in

347 the EM900-administered group. Previous reports have shown that macrophages play an

348 important role in the late clearance of pneumococcal nasopharyngeal colonization.

349 Additionally, accumulation of macrophages was promoted in a Nod2-CCL2-dependent

350 manner. This macrophage-dependent defense mechanism is effective against several

351 different serotypes of *pneumococcus* [5, 6, 22]. Notably, *Ccl2* mRNA expression was

352 also significantly increased in the EM900-administered group in our study. Furthermore,

353 when nasopharyngeal macrophage loss was chemically induced before pneumococcal

354 colonization, increased *Ccl2* mRNA expression, macrophage accumulation in the

355 nasopharynx, and inhibition of nasopharyngeal colonization were not observed (data not

356 shown). This finding indicates that EM900 acted on resident macrophages and

357 promoted CCL2 production, resulting in the promotion of macrophage migration into
358 the nasal cavity. Additionally, a similar experiment using an invasive strain with a
359 serotype different from that of the bacterial strain used in the colonization experiment
360 showed that the survival rate was improved significantly in the EM900 pre-administered
361 group compared to the non-administered group. The increase in CCL2 production was
362 observed even with frequently used macrolides, e.g., CAM and AZM, suggesting that
363 macrolides may be able to inhibit pneumococcal colonization by innate immune
364 activation. Therefore, because of their lack of serotype dependency, macrolides may be
365 candidate prophylactic agents for pneumococcal colonization and invasive infection.

366 Macrolides have various immunomodulatory effects, including promotion of
367 monocyte differentiation into macrophages [10] and cytokine production [23-25], in
368 addition to the suppression of excessive production of mucin and inflammatory
369 cytokines [26, 27]. Macrolides are also known to improve the phagocytic ability and
370 migratory ability of inflammatory cells [9, 28]. In this study, we found that macrolide
371 administration increased host innate immunity by promoting local macrophage
372 induction through increased CCL2 production. An *in vitro* study by Vrancic et al.
373 reported increased CCL2 production without any effect on IL-6 and TNF-α expression
374 after pre-administration of azithromycin in monocytes that had been activated with

375 IFN-γ and LPS [23]. In this study, to evaluate the production of other inflammatory
376 cytokines, the culture supernatant of macrophages treated with macrolides was profiled
377 using cytokine arrays. CCL2 production was markedly increased; however, increased
378 production of inflammatory cytokines associated with tissue damage such as TNF-α was
379 not observed (Supplemental Figure 1). Additionally, when intraperitoneal macrophages
380 were stimulated in the same way by ampicillin (a cell wall synthesis inhibitor),
381 increased CCL2 production was not observed (data not shown). From the above results,
382 we conclude that the promotion of CCL2 production was specific to macrolides and had
383 a protective effect on the host.

384 Next, to clarify the mechanism by which CCL2 production was increased by
385 macrolide treatment, analysis of several intracellular signal transduction pathways was
386 performed using RNA interference. We found that EM900, CAM, and AZM induced
387 CCL2 production, dependent on NF-κB, and was shown to act on both MyD88 and
388 TRIF, which are adaptor proteins upstream of the NF-κB pathway. Shinkai et al.
389 stimulated NHBE cells with CAM and found that extra-cellular signal-regulated kinase
390 (ERK) was suppressed after 30–90 min, whereas ERK activation was observed over
391 2–72 h [29]. We believe that the discrepancy between our results and those of Shinkai et
392 al. can be attributed to changes in effects of macrolides based on the influence of

393 various factors such as associated antigens, cells targeted, macrolide concentration and
394 duration of activity, and environmental factors.
395 There are some limitations to this study. First, experiments using CCR2 (CCL2
396 receptor) knockout mice were not performed. Such studies are required to determine the
397 true relevance of CCL2-dependent increases in macrophage-induced migration
398 following macrolide administration, particularly in relation to the inhibition of
399 pneumococcal nasopharyngeal colonization. Second, an accurate *in vivo* evaluation of
400 colonization inhibition mediated by the immunomodulatory effects of CAM and AZM
401 was not possible because of the antimicrobial effects of these macrolides against *S.*
402 *pneumoniae*. However, suppression of the incidence of pneumonia and COPD
403 exacerbation by oral macrolides has been reported clinically [30]; therefore, it is
404 possible that nasopharyngeal colonization is also inhibited. Third, we did not investigate
405 whether the immunomodulatory effect observed in the nasopharynx was also present in
406 the lower respiratory tract. Typically, most immune cells in the lower respiratory tract
407 are alveolar macrophages. Thus, the host immune responses against *S. pneumoniae* in
408 the upper and lower respiratory tracts are different, which should be addressed in future
409 studies.
410 In conclusion, this study has demonstrated that macrolides have the potential to

411 promote the clearance of pneumococcal nasopharyngeal colonies via

412 immunomodulation of macrophages. Macrolide-induced activation of the innate

413 immune system could therefore be a potential candidate for serotype-independent

414 protection against pneumococcal infection. Further investigation is still needed to

415 clarify the effect of macrolides against pneumococcal nasopharyngeal carriage.

416

417 **Notes**

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517

518 **Figure legends**

519 **Figure 1.** EM900 promoted the clearance of pneumococcal nasopharyngeal colonies via

520 macrophage migration to the nasal cavity. (*A*) Colonization density at 3, 7, and 14 days

521 after inoculation with P1121. Horizontal lines indicate mean values. The density of

522 pneumococcus in the URT was significantly decreased in EM900-treated mice 14 days

523 after inoculation. Number of inflammatory cells recruited to the URT at 7 and 14 days

524 after pneumococcal inoculation (two independent experiments): (*B*) $Ly6G^{+}CD45^{+}$

525 neutrophils and (*C*) $F4/80^{+}$ macrophages. Data represent the mean ± SEM of four

526 experiments each with 5 mice. Higher levels of macrophage accumulation were detected

527 in the EM900-treated group 14 days after inoculation. *, $P < 0.05$; **, $P < 0.01$ *versus*

528 control; NS, not significant.

529

530 **Figure 2.** *Ccl2* mRNA expression in the nasal cavity was significantly higher by

531 administration of EM900 irrespective of pneumococcal inoculation. These figures show

532 (*A*) *Ccl2* mRNA expression and (*B*) *IL-17A* mRNA expression 14 days after

533 pneumococcal inoculation. Data represent the mean ± SEM (n=5, two independent

534 experiments). Expression of mRNA was normalized to the constitutive expression of

535 GAPDH mRNA. Significant differences are designated by using ANOVA, followed by

536 Turkey’s post-tests. *, $P < 0.05$ *versus* PBS control; #, $P < 0.05$ *versus* infected mice

537 without EM900 treatment.

538

539 **Figure 3.** The effect of reducing pneumococcal colonization by EM900 was dependent

540 on the accumulation of macrophages in nasal cavity via CCL2 expression. These figures

541 show (*A*) Colonization density, (*B*) F4/80^{+} macrophages and (*C*) *Ccl2* mRNA expression

542 of the nasal cavity at 14 days after inoculation with P1121, with or without intranasal

543 administration of liposome-Cl_2MDP (C.L.) Expression of mRNA was normalized to the

544 constitutive expression of GAPDH mRNA. Data represent the mean ± SEM of three

545 experiments each with at least 5 mice; NS, not significant.

546

547 **Figure 4.** Macrolides induce concentration-dependent CCL2 production from

548 macrophages. These representative results show (*A*) *Ccl2* mRNA expression and (*B*)

549 intracellular CCL2 production of macrophages pretreated with the 100 μM of

550 macrolides. (*A*) White bar represents the macrolide-treated group whereas the black bar

551 represents the control group. Expression of mRNA was normalized to the constitutive

552 expression of GAPDH mRNA. Data represent the mean ± SEM of at least three

553 independent experiments. *, $P < 0.05$ *versus* control; **, $P < 0.01$ *versus* control. (*B*)

554 Lines indicate CCL2 fluorescence intensity on the x axis (FL-2 signal) for duplicate cell

555 samples (at least three independent experiments). Filled peaks denote binding by CCL2

556 isotype control antibodies.

557

558 **Figure 5.** Macrolides enhance the phagocytic ability of the macrophages. These

559 representative results show (*A*) FACS analysis of phagocytic reaction and (*B*) %FITC

560 positive cells of F4/80 pretreated with macrolides. FACS plots are gated on F4/80 cells.

561 Values are arithmetic means (±SEM) (n=4, two independent experiments).

562

563 **Figure 6.** Macrolide-mediated CCL2 production is required for phosphorylation of

564 NF-κB (pretreated with the 100 μM of macrolides), whereas MAPKs, i.e., MEK-1, p38,

565 and JNKm are not essential. (*A*) Levels of macrolide-mediated CCL2 production from

566 peritoneal macrophages pretreated with inhibitors of NF-κB, MEK-1, p38, and JNK

567 were measured by ELISA. Data represent the mean ± SEM of three experiments. *, $P <$

568 0.05 *versus* macrolide-pretreated macrophages without a transduction inhibitor. (*B*)

569 Representative results of western blot analysis show phosphorylation of NF-κB p65 in

570 macrophages with time after macrolide stimulation. Both NF-κB phosphorylation (*C*)

571 and CCL2 production (*D*) were significantly reduced by knockdown of MyD88 (i) or

572 TRIF (ii), suggesting that macrolide-mediated CCL2 production from macrophages

573 occurs via a MyD88- or TRIF-mediated pathway that activates NF-κB signaling (two

574 independent experiments). *, $P < 0.05$ *versus* macrolide-pretreated macrophages without

575 RNA interference of MyD88 and TRIF.

576

577 **Figure 7.** Kaplan–Meier survival curves of mice infected with (*A*) P1547 or (*B*) P173

578 and pretreated with (triangle) or without (square) macrolides for 21 days before

579 infection. The log-rank test indicated EM900 treatment improved the survival rate

580 significantly. (n =15-18, two independent experiments). ***, $P<0.001$ *versus* control; *,

581 $P<0.05$ *versus* control.

582

583 **Supplementary Figure 1.** The mouse cytokine array assay detects multiple analytes in

584 cell culture supernatants of peritoneal macrophages pretreated with the 100 μM of

585 macrolides for 12 h ((i) untreated, (ii) EM900, (iii) CAM, (iv) AZM). Optical density

586 was adjusted based on positive controls (the spots of upper and lower left and upper

587 right) among each sample. (a) CCL2 was up-regulated by all macrolides, while

588 cytokines known to induce tissue damage, such as (b) TNF-α and (c) IL-6, were not.

589 The other spots represent the (d) IL-1γ, and (e) MIP-2. Similar results were obtained in

590 two separate experiments.

FIGURE 1.

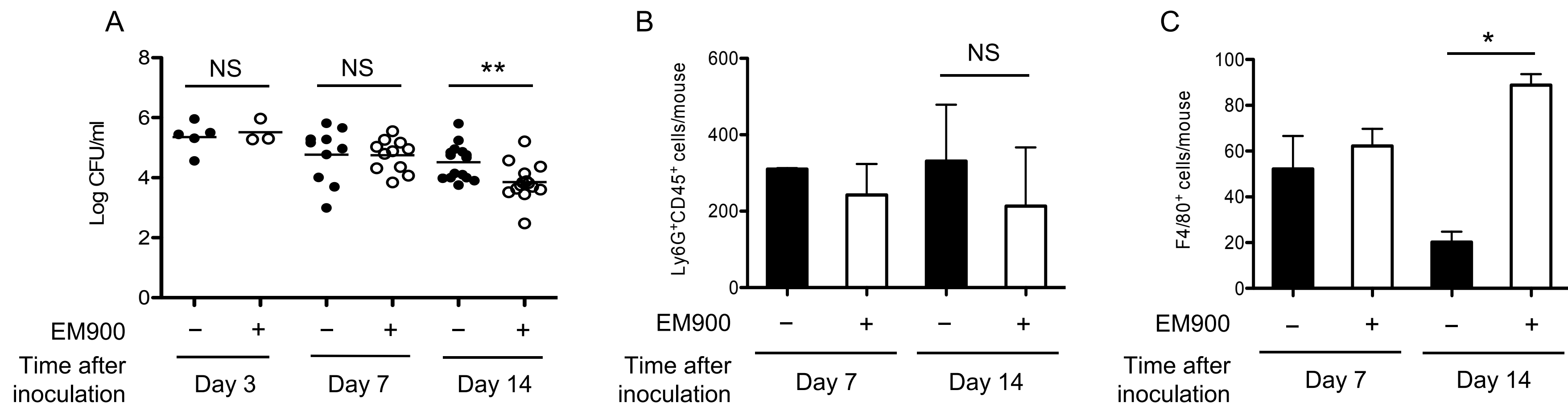


FIGURE 2.

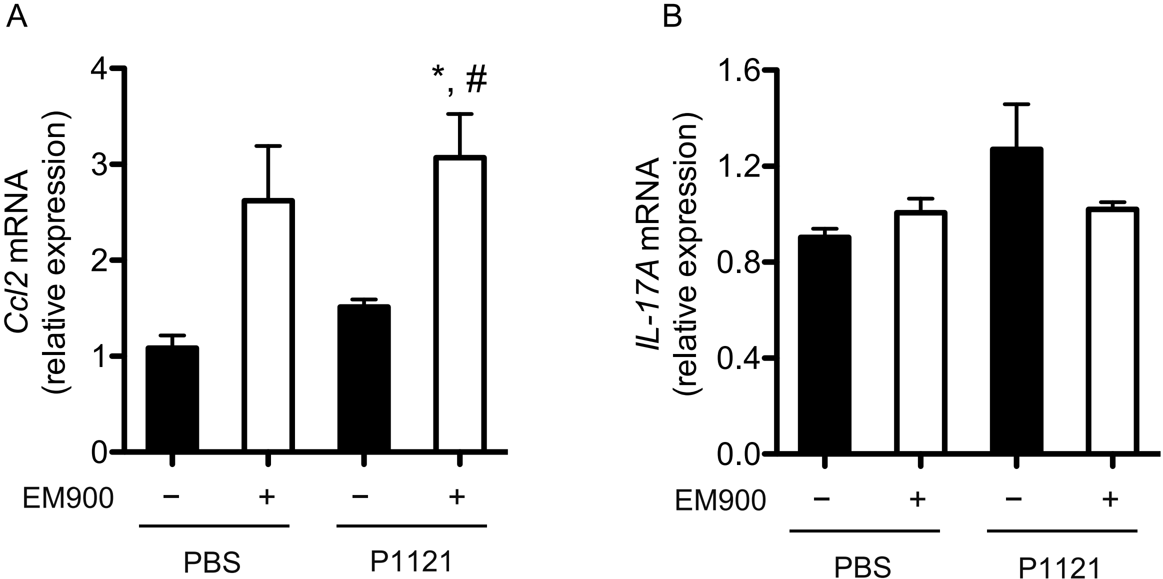
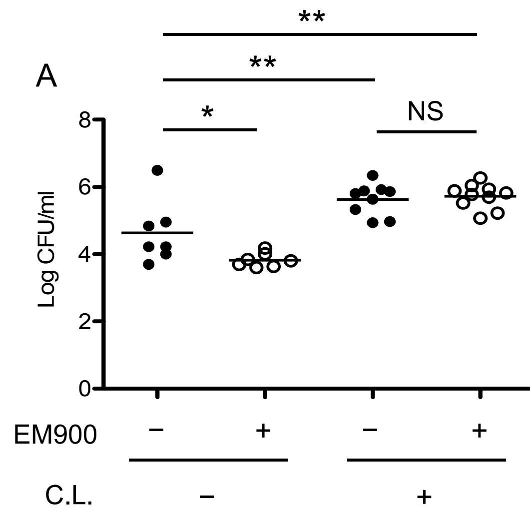
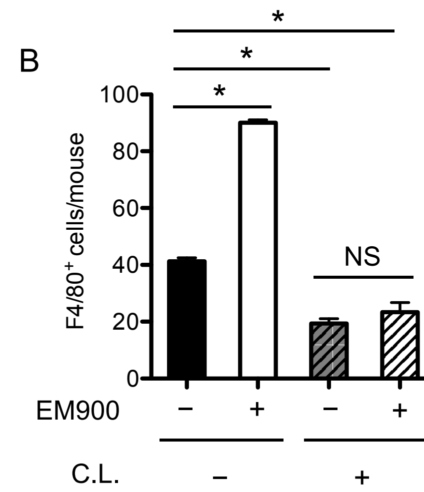


FIGURE 3.





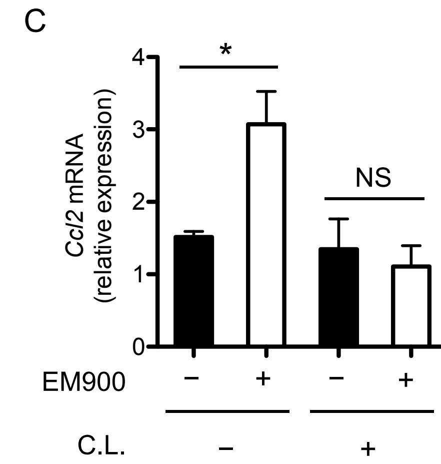


FIGURE 4.

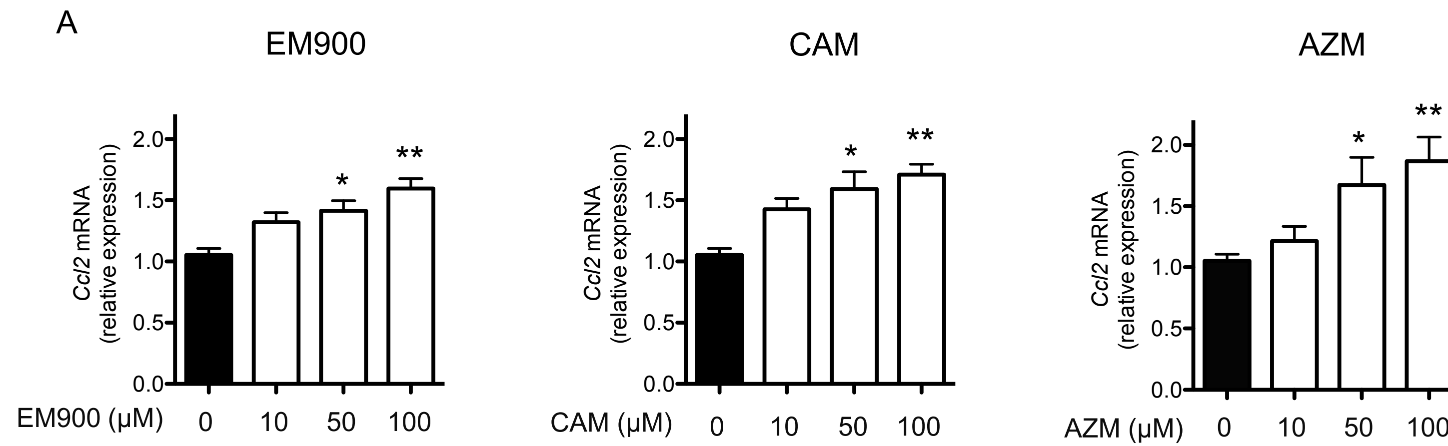
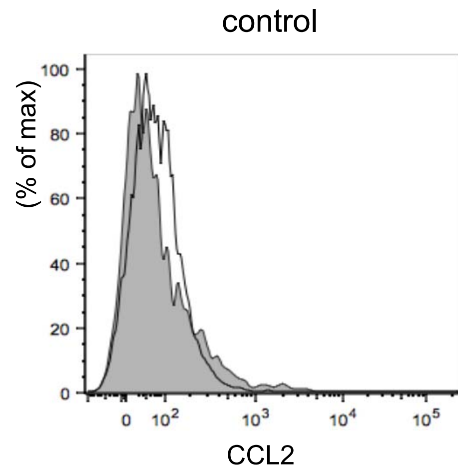
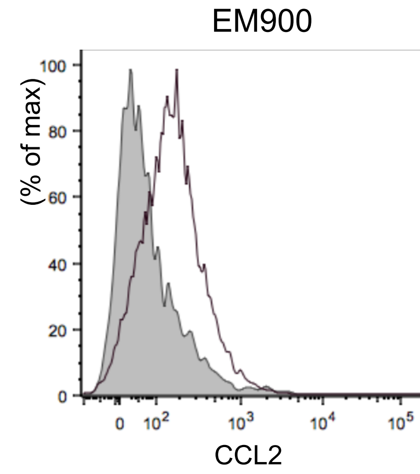
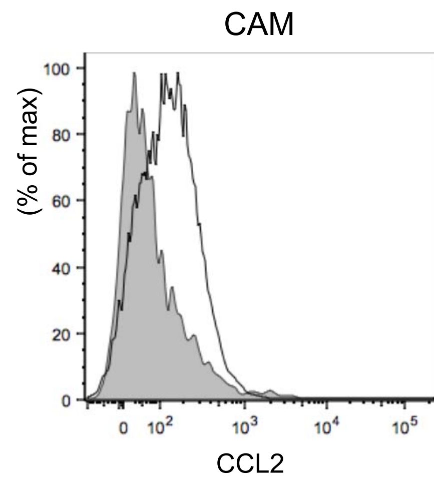


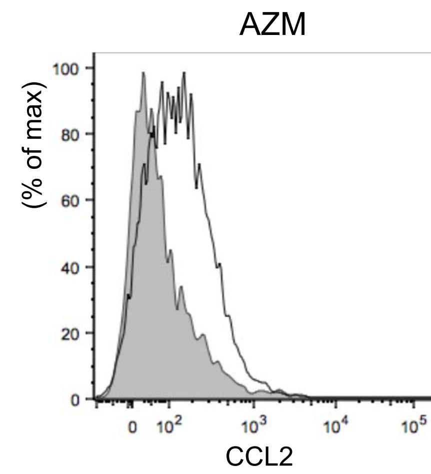
FIGURE 4.

B









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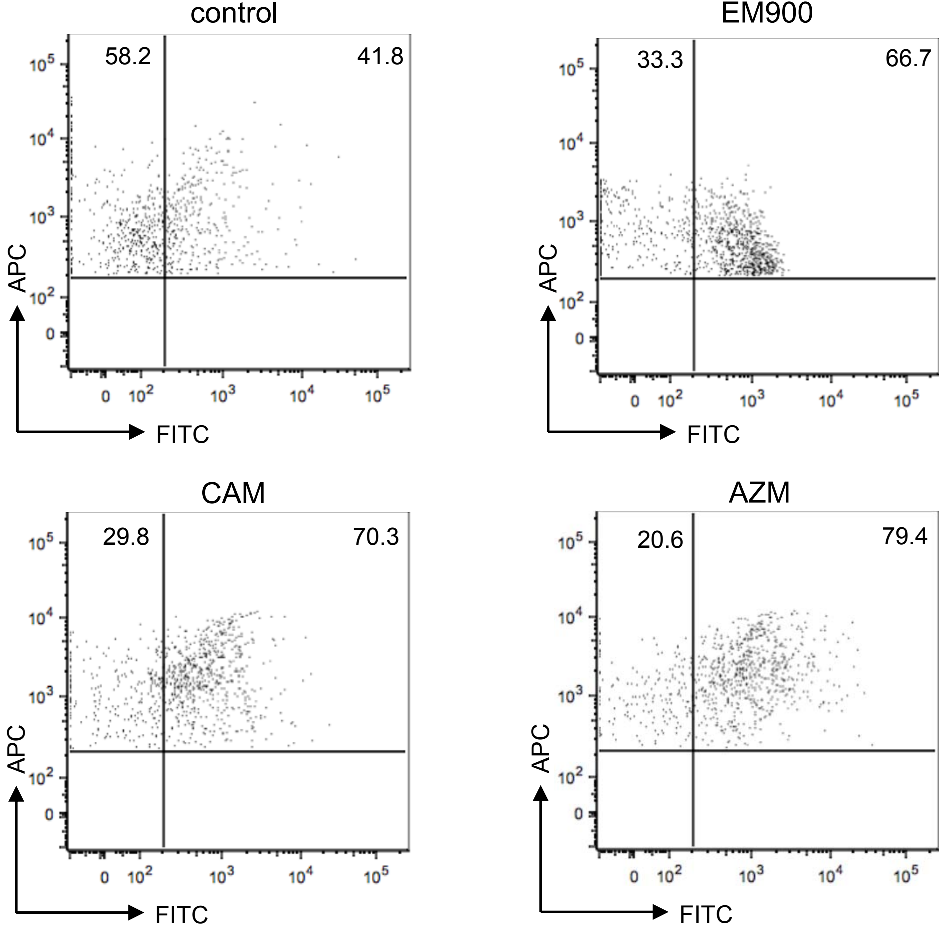


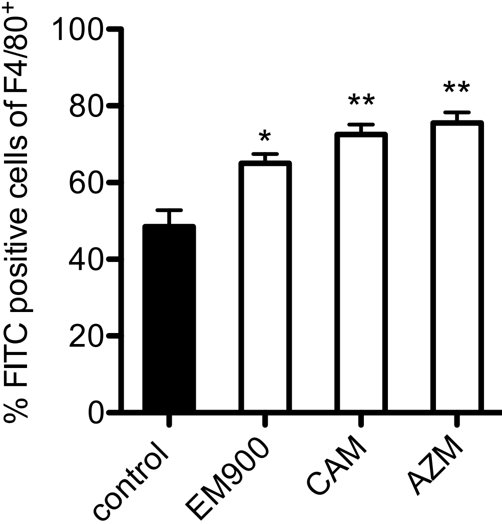
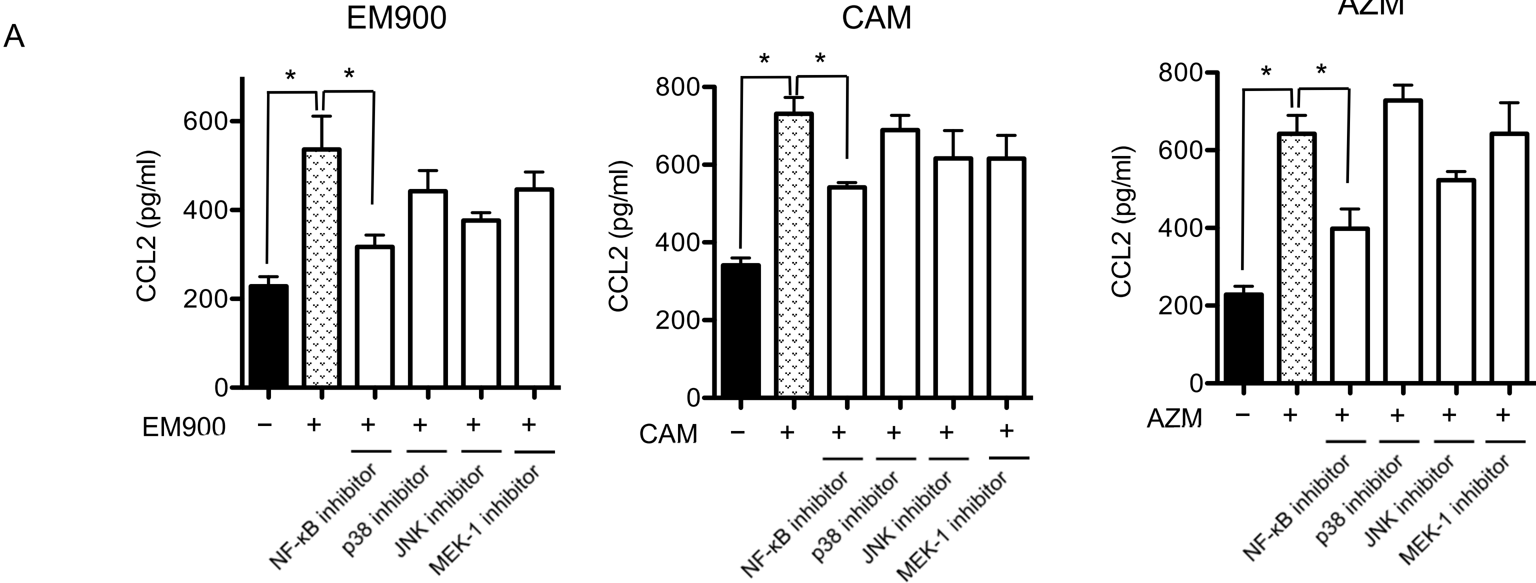


FIGURE 6.

A



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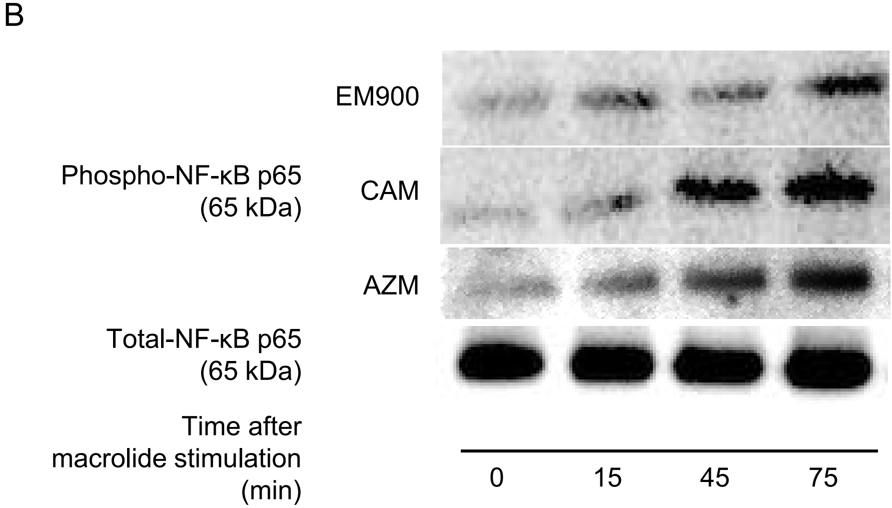
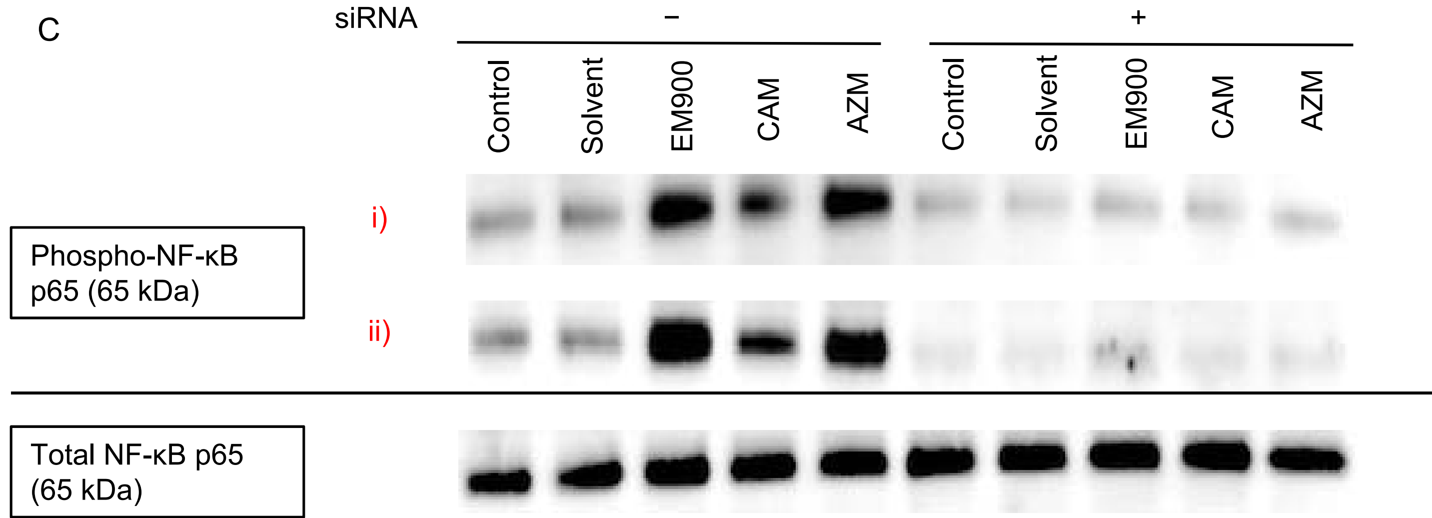
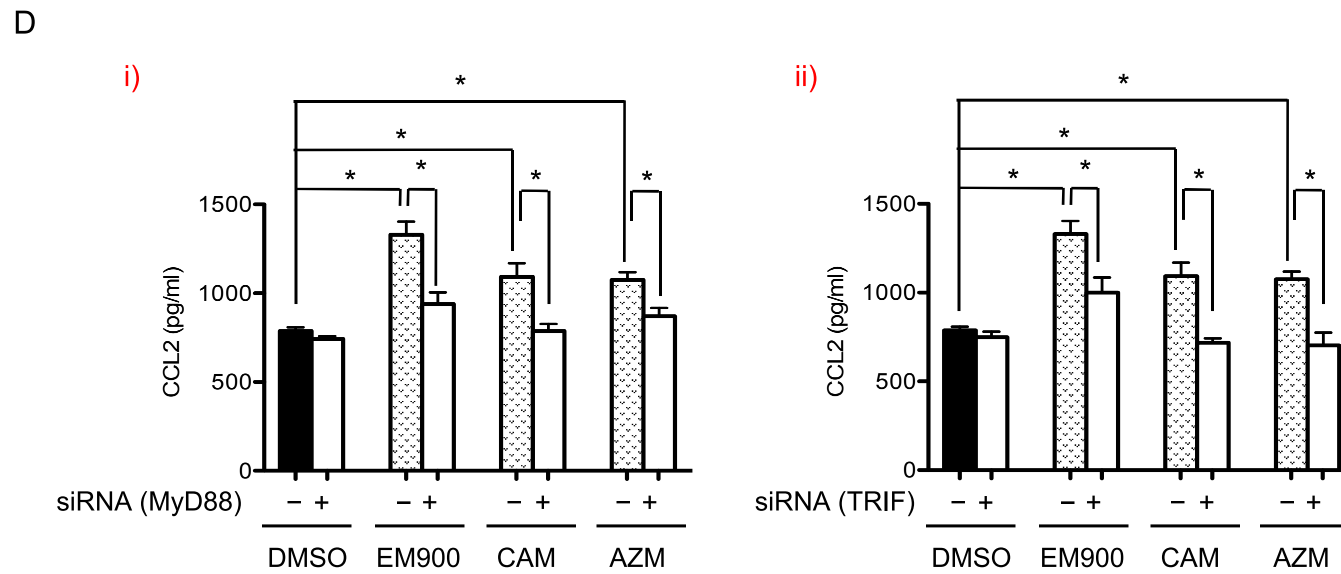


FIGURE 6.





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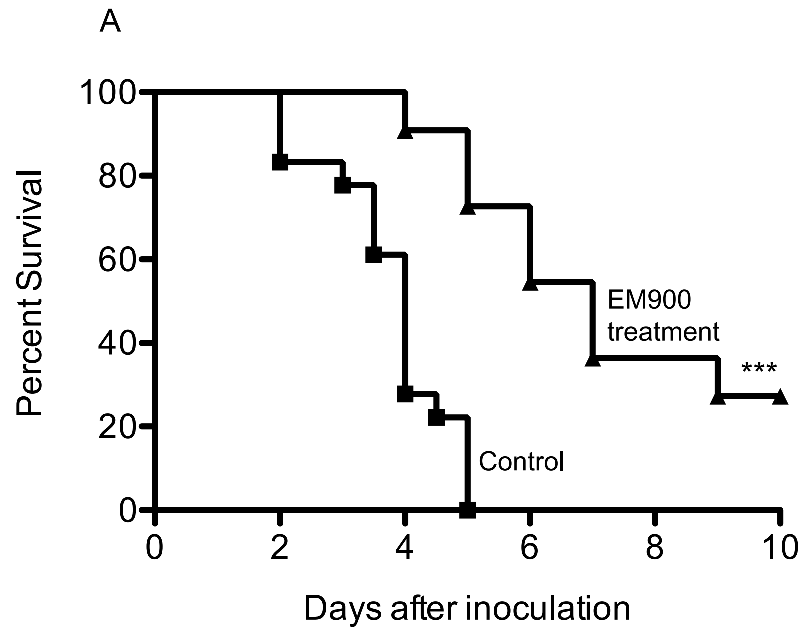

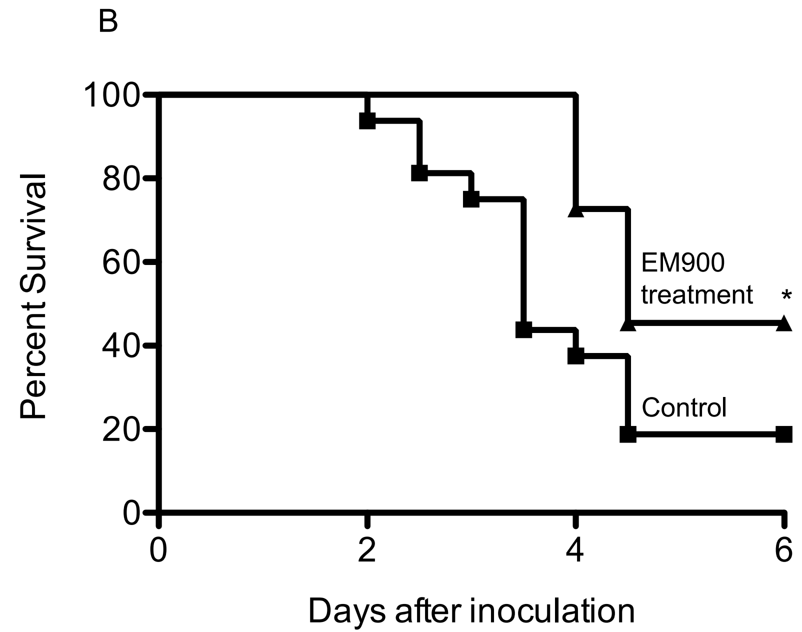

Supplementary Figure 1.