Macrolides Promote CCL2-Mediated Macrophage Recruitment and Clearance of Nasopharyngeal Pneumococcal Colonization in Mice

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Macrolides promote CCL2-mediated macrophage recruitment and clearance of nasopharyngeal pneumococcal colonies in mice

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Abstract

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

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

**Results.** *S. pneumoniae* was significantly reduced from EM900-treated mice 14 days after pneumococcal inoculation. Macrophage recruitment and Ccl2 mRNA expression were promoted. CCL2 production from peritoneal macrophages was significantly induced by macrolides and was dependent on NF-κB phosphorylation through the MyD88- or TRIF-mediated pathway. Mortality of mice with invasive pneumococcal disease was improved by pre-treatment with EM900.
Conclusions. Macrolides may inhibit invasive pneumococcal infections by accelerating the clearance of pneumococcal nasopharyngeal colonization via promotion of macrophage-mediated innate immunity.

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

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

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

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
activity but has immunomodulatory effects [11]. EM900 suppresses activation of nuclear factor NF-κB and production of interleukin (IL)-8, IL-1β, and TNF-α from human airway epithelial cells, demonstrating its anti-inflammatory action [12]. We hypothesized that macrolides may be potential candidates for prophylaxis of pneumococcal infection through modulation of host innate immunity. This study investigated the immunomodulatory effects of macrolides in inhibiting pneumococcal nasopharyngeal colonization and subsequent invasive disease in mice.

Methods

Laboratory animals

C57BL/6 (six weeks old, female) specific-pathogen-free mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were performed in accordance with the guidelines of the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Bacterial strains and growth conditions

*S. pneumoniae* strain P1121 (type 23F clinical isolate) [5, 13], P1547 (*S. pneumoniae* serotype 6A, mouse virulent, clinical isolate from blood) [14], and P173 (*S. pneumoniae* serotype 6A, mouse virulent, clinical isolate from blood) [14].
serotype 2, capsulated clinical isolate) were kindly provided by JN Weiser (Professor, University of Pennsylvania). P1121 cells were grown in 10 ml of tryptic soy broth (Difco; BD) until the mid-log phase was reached (OD_{660} = 0.5). After centrifugation, the pellet was resuspended in 100 µl of sterile PBS to achieve a density of 10^9 CFU/ml [5].

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

In colonization studies, 10 µl (10^7 CFUs) of the pneumococcal suspension was introduced into the nostril of each mouse. After a predetermined number of days, mice were sacrificed by CO₂ asphyxiation. The trachea was exposed and cannulated to introduce 200 µl of sterile PBS. The lavage fluid exiting the nares was collected and P1121 cells were quantified by plating 10 µl serial dilutions of the nasal wash onto tryptic soy agar plates supplemented with catalase (4,741 U/plate) (Worthington Biochemical Corp.) and neomycin (20 µg · ml⁻¹) (Sigma-Aldrich Japan, Tokyo, Japan), and incubating overnight at 37°C in a 5% CO₂ atmosphere [5]. EM900 (10 mg · kg⁻¹), a gift from T Sunazuka (Kitasato University, Tokyo, Japan) [11], was administered orally twice a day from one week before the inoculation and throughout the experiment (We confirmed that MICs of EM900 against pneumococcus used in this study are greater than 100 µg · ml⁻¹). At days 3, 7, and 14 after inoculation, the mice were sacrificed and
nasal washing was performed. These lavages were analyzed to evaluate the kinetics of colonization and cellular inflammatory response by flow cytometry, and Ccl2 and IL-17A mRNA expression by quantitative RT-PCR (qRT-PCR). WT mice with or without EM900 treatment for three weeks before infection were challenged intranasally with 5 × 10^7 CFU of strains P1547 and P173, and survival was observed for 10 days after the challenge.

Flow cytometry and intracellular cytokine staining

Nasal lavages were pooled from 5 mice and the cells were collected by centrifugation at 1200 g for 10 min at 4°C. The pellets were washed once using 200 μl washing buffer (PBS supplemented with 1% BSA), collected again by centrifugation, resuspended in 200 μl of washing buffer, and transferred to a 96-well polypropylene plate. Nonspecific binding was blocked using a rat anti-mouse antibody directed against the FcγIII/II receptor (CD16/CD32) (BD Biosciences), and the following rat anti-mouse cell surface antibodies were applied: Ly6G (BD Biosciences), CD45 (BD Biosciences), and F4/80 (eBioscience). The final dilutions of the antibodies were 1:300 for the FITC- Ly6G antibody and 1:400 for PE-CD45 and APC-F4/80 antibodies. Reactions were performed by incubating the plate on ice in the dark for 45 min. All samples were resuspended in
wash buffer and then subjected to flow cytometry analysis by using a BD FACSCalibur flow cytometer (BD Biosciences) [15]. For intracellular staining, cytokine secretion from peritoneal cells pretreated with macrolides was blocked by using GolgiPlug (BD Biosciences). Stimulated peritoneal cells were detached from plates by chilling on ice at 4°C and washing with cold PBS. The cells were pelleted, resuspended in 1% BSA, and stained for surface markers as described above. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm Plus Kit (BD Biosciences). Accumulation of intracellular CCL2 was detected using an Armenian hamster anti–mouse CCL2 antibody (eBioscience). An Armenian hamster IgG isotype control was used for the detection of nonspecific binding. All flow cytometry data were analyzed using Flow Jo Mac, version 9.6 (Tree Star) [6].

Macrophage depletion

Resident macrophages in the nasopharynx were depleted using liposome-Cl2MDP (FormuMax) as previously described [5]. For depletion, liposome-Cl2MDP (20 μl/dose) was administered intranasally at 4 and 8 days before and 1 and 8 days after the P1121 challenge. The influence of macrophage depletion was evaluated at 14 days after bacterial inoculation.
Isolation and culture of peritoneal macrophages

Three days after intraperitoneal injection of 4% sterile thioglycollate medium (2 ml), peritoneal macrophages were isolated by peritoneal lavage with Hank’s buffer (without Ca\(^{2+}\) and Mg\(^{2+}\)) containing 0.1% gelatin. Contaminating erythrocytes, granulocytes, and dead cells were removed by density gradient centrifugation for 45 min at 800 g in Mono-Poly resolving medium, according to the manufacturer’s protocol (MP Biomedicals). Purified peritoneal macrophages were washed 3 times and cultured overnight in Dulbecco’s modified Eagle medium [6].

ELISA and cytokine array

CCL2 concentrations in culture supernatants of peritoneal macrophages that were pretreated with EM900, clarithromycin (CAM) (Sigma-Aldrich Japan, Tokyo, Japan), azithromycin (AZM) (Sigma-Aldrich Japan, Tokyo, Japan), or solvent only for 12–18 h were assayed using mouse Quantikine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer’s protocols. To investigate macrolide-mediated signaling transduction pathways, we utilized specific MAPK inhibitors, i.e., SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD98059 (MEK-1 inhibitor), and parthenolide.
(NF-κB inhibitor) (Sigma-Aldrich Japan, Tokyo, Japan). Additionally, the cytokine expression profile of macrolide-stimulated peritoneal macrophages was analyzed using a mouse cytokine protein array (R&D Systems, Minneapolis, MN), following the manufacturer’s instructions. For this experiment, the same amount of cell density and supernatant derived from each culture were used.

**Lipopolysaccharide (LPS) detection**

To exclude the possibility of LPS contamination, the Limulus amebocyte lysate test using the Gel-clot method (Pyrotell) was performed for culture supernatants and reagents. The detection limit of the assay was 0.03 EU/ml (0.003 ng/ml) [16].

**qRT-PCR assays**

RNA was isolated from nasal lavage of the upper respiratory tract with 600 μl of RNA lysis buffer using an RNeasy Mini Kit (QIAGEN) according to manufacturer’s protocol [17]. Complementary DNA was generated using a high-capacity reverse transcription kit (Applied Biosystems). Approximately 25 ng of cDNA was then used as a template in reactions with forward and reverse primers and SYBR Green (Applied Biosystems)
according to the manufacturer’s protocol. Reactions were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems), and quantitative comparisons were obtained using the ΔΔCT method. The primers used are as follows: GAPDH-F: 5′-TGTGTCCGTCGATCTGA-3′; GAPDH-R: 5′-CCTGCTACACCCCTTGAT-3′; CCL2-F: 5′-AGCTCTTCCTTCATCCACCAC-3′; CCL2-R: 5′-CGTTAAGCTCATCTGGCTGA-3′; IL-17A-F: 5′-TCTCATCCACCCCTTACAC-3′; and IL-17A-R: 5′-AGTTGGACCCCTTTACAC-3′ [6].

**Phagocytosis assay**

FITC-bound *S. pneumonia* was made as reported previously. This suspension of *S. pneumoniae* in PBS was added to 2.5 × 10^5 cultured peritoneal macrophages, which were pretreated with macrolides for 24 hours (the ratio bacteria : macrophage = 25:1). Peritoneal macrophages and pneumococcus were co-cultured for 45 minutes at 37 °C under 5% CO₂ in humidified air. Phagocytosis was stopped by putting the plates on ice, following adherent bacteria were removed by washing cells with PBS. To quantify phagocytosed bacteria, fixed cells were used for FACS analysis.
Western blotting

Cells were washed and lysed for 5 min in RIPA buffer (Sigma-Aldrich Japan, Tokyo, Japan) containing protease inhibitor cocktail (Sigma-Aldrich Japan, Tokyo, Japan), and the protein concentration was determined using the bicinchoninic acid protein assay kit (Thermo, Rockford, IL, USA). For equivalent loading between samples, the same amount of each sample was fractionated via 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transfer, blocking and signal development were performed as described previously [18]. For detection of intact and phosphorylated (activated) forms of NF-κB, rabbit primary antibodies against NF-κB (total NF-κB, #3034; phospho-NF-κB, #3031) were purchased from Cell Signaling Technology (Beverly, MA, USA). Incubation with the primary antibodies was followed by incubation with secondary antibodies conjugated to horseradish peroxidase (sc-2030; Santa Cruz Biotechnology).

RNA interference experiments

RNA interference technology was used to knock down the expression of TIR domain-containing adaptors, such as MyD88 and TRIF, in peritoneal macrophages. The
efficiency and specificity were validated by quantitative real-time PCR and western blotting. Peritoneal macrophages obtained from mice were grown in 24-well plates (2 × 10^5 cells/well) and transfected with 50 nM TIR domain-containing adaptor siRNAs according to the manufacturer’s instructions (Invitrogen). Transfected cells were incubated for 72 h before being stimulated by macrolides. Pre-designed RNA oligonucleotides for MyD88 and TRIF were obtained from Invitrogen (Life Technologies Corp.).

Statistical analyses

All data were analyzed by using Prism 5 GraphPad Software and are expressed as the mean ± SEM. Differences between the treatment group and the control were tested for significance using the Mann–Whitney U test. The significance of differences among more than three groups was examined using ANOVA followed by Dunnett’s or Turkey’s post-tests. Survival analysis was performed using the log-rank test, and survival rates were calculated via the Kaplan–Meier method. P < 0.05 was considered statistically significant.

Results
The effect of EM900 on pneumococcal nasopharyngeal colonization

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

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

Previous reports have shown that pneumococcal clearance is dependent on macrophage recruitment mediated by NOD2-CCL2 and TLR2-Th17 lineages [5, 6]. Therefore, we analyzed mRNA expression of \textit{Ccl2} and \textit{IL-17A} in the nasal lavage at day 14 after
pneumococcal inoculation with or without EM900 treatment. As shown in Figure 2, the mRNA expression of Ccl2 but not IL-17A was markedly increased by administration of EM900. Next, we performed a macrophage depletion assay by intranasal administration of liposome-Cl\textsubscript{2}MDP before pneumococcal inoculation to determine which cells are responsible for CCL2 production in response to EM900. There were no significant differences in the density of pneumococci (Fig. 3A) and furthermore, the effect of EM900 for facilitating the recruitment of macrophages and the expression of Ccl2 mRNA in URT were disappeared in the mice treated by liposome-Cl\textsubscript{2}MDP at day 14 after pneumococcus inoculation (Fig. 3B and 3C). Taken together, these results suggest that EM900 exerts its effects on resident macrophages and elicits CCL2 production and macrophage recruitment, resulting in the promotion of pneumococcal clearance in the URT.

Effect of macrolides on the function of murine peritoneal macrophages

To investigate the mechanism of macrolide-mediated CCL2 production, we extracted murine peritoneal macrophages and stimulated them with various concentrations of EM900 and two existing macrolides, CAM and AZM. After synthesis of EM900, extraction with chloroform and purification by using a silica gel column are considered
to provide a very low possibility of contamination with LPS. Additionally, the LPS content in the culture supernatants and reagents was undetectable in the Limulus amebocyte lysate test using the Gel-clot method, thus assuring that the observed phenomena did not result from LPS contamination. As shown in Figure 4A, Ccl2 mRNA expression in macrolide-stimulated macrophages increased in a concentration-dependent manner. To confirm that CCL2 production from macrophages was in response to macrolides, we stained cells for surface markers and intracellular CCL2 following macrolide stimulation and then analyzed them by flow cytometry. F4/80+ cells stained positive for intracellular CCL2 following stimulation with EM900, CAM, and AZM (Fig. 4B). Furthermore, when production of numerous cytokines was screened by cytokine array, it was observed that while CCL2 production was clearly present, pro-inflammatory cytokines associated with tissue injury such as TNF-α were not (Supplementary Fig. 1).

To determine the effect of macrolides on the phagocytic function of macrophages, we performed a phagocytosis assay in peritoneal macrophages pretreated with macrolides and FITC-bound S. pneumoniae. As shown in Fig. 5A and B, there are significant increase of pneumococcus-phagocytic cells (APC-F4/80 and FITC double positive) in
the macrophages pretreated with macrolides, suggesting that macrolides enhanced the phagocytic activity and promoted the clearance of *S. pneumoniae* in URT.

**Effect of macrolides on intracellular signal transduction pathways that regulate CCL2 production**

To investigate macrolide-mediated signaling transduction pathways involved in CCL2 production, we examined peritoneal macrophages pretreated with specific MAPK inhibitors, i.e., SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD98059 (MEK-1 inhibitor), and parthenolide (NF-κB inhibitor), for 30 min and then co-treated with the macrolides for 24 h prior to detection of CCL2 in the supernatant. Only pretreatment with parthenolide inhibited CCL2 production and NF-κB phosphorylation with time after macrolide stimulation (Fig. 6A and 6B), suggesting that NF-κB was indispensable for macrolide-mediated CCL2 production. To further investigate the ability of macrolides to induce CCL2 production, knockdown of MyD88 and TRIF, one of the TIR domain-containing adaptors existing upstream of NF-κB, was performed using specific siRNA, and NF-κB phosphorylation and CCL2 production examined. Both NF-κB phosphorylation and CCL2 production were significantly reduced by knockdown of MyD88 or TRIF (Fig. 6C and 6D), indicating that macrolides induce
CCL2 production from macrophages through an NF-κB signaling pathway via MyD88 or TRIF.

Activation of macrolide-mediated innate immunity protects the host from invasive pneumococcal disease

To investigate the role of macrophage recruitment in priming innate immunity, we challenged mice with the P1547 (serotype 6A) and P173 (serotype 2) strains, and treated them with EM900. We found that EM900-treated mice were more resistant to sepsis than untreated mice (Fig. 7A and 7B), which suggests that a reduction in the density of colonization may prevent invasive disease and reduce mortality through activation of innate immune priming by macrolides.

Discussion

Invasive pneumococcal infection cannot be completely prevented using the existing pneumococcal vaccine that activates humoral immunity, with the capsular polysaccharide acting as the immune antigen. There have been reports on the development of a live attenuated vaccine with higher protective immune effects [19] and a fusion vaccine that simultaneously activates the innate immune system and the
humoral immune system; however, these vaccines have not yet been put into practical use [20, 21]. In this study, we hypothesized that macrolide antibiotics, which have been reported to possess various immunomodulatory capacities, would activate host innate immunity and inhibit pneumococcal nasopharyngeal colonization without serotype dependency.

Our results demonstrated that the extent of bacterial colonization 14 days after bacterial inoculation was significantly decreased compared to that in the control group. There was also a significant increase in the number of nasopharyngeal macrophages in the EM900-administered group. Previous reports have shown that macrophages play an important role in the late clearance of pneumococcal nasopharyngeal colonization. Additionally, accumulation of macrophages was promoted in a Nod2-CCL2-dependent manner. This macrophage-dependent defense mechanism is effective against several different serotypes of *pneumococcus* [5, 6, 22]. Notably, *Ccl2* mRNA expression was also significantly increased in the EM900-administered group in our study. Furthermore, when nasopharyngeal macrophage loss was chemically induced before pneumococcal colonization, increased *Ccl2* mRNA expression, macrophage accumulation in the nasopharynx, and inhibition of nasopharyngeal colonization were not observed (data not shown). This finding indicates that EM900 acted on resident macrophages and
promoted CCL2 production, resulting in the promotion of macrophage migration into
the nasal cavity. Additionally, a similar experiment using an invasive strain with a
serotype different from that of the bacterial strain used in the colonization experiment
showed that the survival rate was improved significantly in the EM900 pre-administered
group compared to the non-administered group. The increase in CCL2 production was
observed even with frequently used macrolides, e.g., CAM and AZM, suggesting that
macrolides may be able to inhibit pneumococcal colonization by innate immune
activation. Therefore, because of their lack of serotype dependency, macrolides may be
candidate prophylactic agents for pneumococcal colonization and invasive infection.

Macrolides have various immunomodulatory effects, including promotion of
monocyte differentiation into macrophages [10] and cytokine production [23-25], in
addition to the suppression of excessive production of mucin and inflammatory
cytokines [26, 27]. Macrolides are also known to improve the phagocytic ability and
migratory ability of inflammatory cells [9, 28]. In this study, we found that macrolide
administration increased host innate immunity by promoting local macrophage
induction through increased CCL2 production. An in vitro study by Vrancic et al.
reported increased CCL2 production without any effect on IL-6 and TNF-α expression
after pre-administration of azithromycin in monocytes that had been activated with
IFN-γ and LPS [23]. In this study, to evaluate the production of other inflammatory 
cytokines, the culture supernatant of macrophages treated with macrolides was profiled 
using cytokine arrays. CCL2 production was markedly increased; however, increased 
production of inflammatory cytokines associated with tissue damage such as TNF-α was 
not observed (Supplemental Figure 1). Additionally, when intraperitoneal macrophages 
were stimulated in the same way by ampicillin (a cell wall synthesis inhibitor), 
increased CCL2 production was not observed (data not shown). From the above results, 
we conclude that the promotion of CCL2 production was specific to macrolides and had 
a protective effect on the host.

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

There are some limitations to this study. First, experiments using CCR2 (CCL2 receptor) knockout mice were not performed. Such studies are required to determine the true relevance of CCL2-dependent increases in macrophage-induced migration following macrolide administration, particularly in relation to the inhibition of pneumococcal nasopharyngeal colonization. Second, an accurate \textit{in vivo} evaluation of colonization inhibition mediated by the immunomodulatory effects of CAM and AZM was not possible because of the antimicrobial effects of these macrolides against \textit{S. pneumoniae}. However, suppression of the incidence of pneumonia and COPD exacerbation by oral macrolides has been reported clinically \cite{30}; therefore, it is possible that nasopharyngeal colonization is also inhibited. Third, we did not investigate whether the immunomodulatory effect observed in the nasopharynx was also present in the lower respiratory tract. Typically, most immune cells in the lower respiratory tract are alveolar macrophages. Thus, the host immune responses against \textit{S. pneumoniae} in the upper and lower respiratory tracts are different, which should be addressed in future studies.

In conclusion, this study has demonstrated that macrolides have the potential to
promote the clearance of pneumococcal nasopharyngeal colonies via immunomodulation of macrophages. Macrolide-induced activation of the innate immune system could therefore be a potential candidate for serotype-independent protection against pneumococcal infection. Further investigation is still needed to clarify the effect of macrolides against pneumococcal nasopharyngeal carriage.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.
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15. Nakamura S, Davis KM, Weiser JN. Synergistic stimulation of type I interferons


Figure legends

**Figure 1.** EM900 promoted the clearance of pneumococcal nasopharyngeal colonies via macrophage migration to the nasal cavity. (A) Colonization density at 3, 7, and 14 days after inoculation with P1121. Horizontal lines indicate mean values. The density of pneumococcus in the URT was significantly decreased in EM900-treated mice 14 days after inoculation. Number of inflammatory cells recruited to the URT at 7 and 14 days after pneumococcal inoculation (two independent experiments): (B) Ly6G⁺CD45⁺ neutrophils and (C) F4/80⁺ macrophages. Data represent the mean ± SEM of four experiments each with 5 mice. Higher levels of macrophage accumulation were detected in the EM900-treated group 14 days after inoculation. *, P < 0.05; **, P < 0.01 versus control; NS, not significant.

**Figure 2.** *Ccl2* mRNA expression in the nasal cavity was significantly higher by administration of EM900 irrespective of pneumococcal inoculation. These figures show (A) *Ccl2* mRNA expression and (B) *IL-17A* mRNA expression 14 days after pneumococcal inoculation. Data represent the mean ± SEM (n=5, two independent experiments). Expression of mRNA was normalized to the constitutive expression of GAPDH mRNA. Significant differences are designated by using ANOVA, followed by
Turkey’s post-tests. *, P < 0.05 versus PBS control; #, P < 0.05 versus infected mice without EM900 treatment.

**Figure 3.** The effect of reducing pneumococcal colonization by EM900 was dependent on the accumulation of macrophages in nasal cavity via CCL2 expression. These figures show (A) Colonization density, (B) F4/80+ macrophages and (C) Ccl2 mRNA expression of the nasal cavity at 14 days after inoculation with P1121, with or without intranasal administration of liposome-ClzMDP (C.L.) Expression of mRNA was normalized to the constitutive expression of GAPDH mRNA. Data represent the mean ± SEM of three experiments each with at least 5 mice; NS, not significant.

**Figure 4.** Macrolides induce concentration-dependent CCL2 production from macrophages. These representative results show (A) Ccl2 mRNA expression and (B) intracellular CCL2 production of macrophages pretreated with the 100 μM of macrolides. (A) White bar represents the macrolide-treated group whereas the black bar represents the control group. Expression of mRNA was normalized to the constitutive expression of GAPDH mRNA. Data represent the mean ± SEM of at least three independent experiments. *, P < 0.05 versus control; **, P < 0.01 versus control. (B)
Lines indicate CCL2 fluorescence intensity on the x axis (FL-2 signal) for duplicate cell samples (at least three independent experiments). Filled peaks denote binding by CCL2 isotype control antibodies.

**Figure 5.** Macrolides enhance the phagocytic ability of the macrophages. These representative results show (A) FACS analysis of phagocytic reaction and (B) %FITC positive cells of F4/80 pretreated with macrolides. FACS plots are gated on F4/80 cells. Values are arithmetic means (±SEM) (n=4, two independent experiments).

**Figure 6.** Macrolide-mediated CCL2 production is required for phosphorylation of NF-κB (pretreated with the 100 μM of macrolides), whereas MAPKs, i.e., MEK-1, p38, and JNKm are not essential. (A) Levels of macrolide-mediated CCL2 production from peritoneal macrophages pretreated with inhibitors of NF-κB, MEK-1, p38, and JNK were measured by ELISA. Data represent the mean ± SEM of three experiments. *, P < 0.05 versus macrolide-pretreated macrophages without a transduction inhibitor. (B) Representative results of western blot analysis show phosphorylation of NF-κB p65 in macrophages with time after macrolide stimulation. Both NF-κB phosphorylation (C) and CCL2 production (D) were significantly reduced by knockdown of MyD88 (i) or
TRIF (ii), suggesting that macrolide-mediated CCL2 production from macrophages occurs via a MyD88- or TRIF-mediated pathway that activates NF-κB signaling (two independent experiments). *, P < 0.05 versus macrolide-pretreated macrophages without RNA interference of MyD88 and TRIF.

**Figure 7.** Kaplan–Meier survival curves of mice infected with (A) P1547 or (B) P173 and pretreated with (triangle) or without (square) macrolides for 21 days before infection. The log-rank test indicated EM900 treatment improved the survival rate significantly. (n =15-18, two independent experiments). ***, P<0.001 versus control; *, P<0.05 versus control.

**Supplementary Figure 1.** The mouse cytokine array assay detects multiple analytes in cell culture supernatants of peritoneal macrophages pretreated with the 100 μM of macrolides for 12 h ((i) untreated, (ii) EM900, (iii) CAM, (iv) AZM). Optical density was adjusted based on positive controls (the spots of upper and lower left and upper right) among each sample. (a) CCL2 was up-regulated by all macrolides, while cytokines known to induce tissue damage, such as (b) TNF-α and (c) IL-6, were not. The other spots represent the (d) IL-1γ, and (e) MIP-2. Similar results were obtained in
two separate experiments.
FIGURE 1.

A. Log CFU/mL

B. Ly6G+CD45+ cells/mouse

C. F4/80+ cells/mouse

EM900

Time after inoculation

Day 3  Day 7  Day 14

NS

EM900

Time after inoculation

Day 7  Day 14

NS

EM900

Time after inoculation

Day 7  Day 14

**
FIGURE 2.
**FIGURE 3.**

A. Log CFU/ml

B. F4/80+ cells/mouse

C. Ccl2 mRNA (relative expression)
FIGURE 4.
FIGURE 4.
FIGURE 5.

A

control

EM900

CAM

AZM

B

% FITC positive cells of F4/80^+
FIGURE 6.

A

B

EM900

CAM

AZM

Phospho-NF-κB p65
(65 kDa)

Total-NF-κB p65
(65 kDa)

Time after macrolide stimulation (min)

0 15 45 75
FIGURE 6.

C

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<tr>
<td>AZM</td>
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Phospho-NF-κB p65 (65 kDa)

i)

ii)

Total NF-κB p65 (65 kDa)

D

i)

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<th>siRNA (MyD88)</th>
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CCL2 (pg/ml)

DMSO: 

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</table>

CCL2 (pg/ml)
FIGURE 7.

A

Percent Survival

Control

Days after inoculation

0 2 4 6 8 10

EM900 treatment

***

B

Percent Survival

Control

Days after inoculation

0 2 4 6

EM900 treatment

*
Supplementary Figure 1.