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Erythromycin Differentially Inhibits Lipopolysaccharide- or Poly(I:C)-Induced but Not Peptidoglycan-Induced Activation of Human Monocyte-Derived Dendritic Cells

Motoko Yasutomi,* Yusei Ohshima,2* Nemuko Omata,* Akiko Yamada,* Hiromichi Iwasaki,† Yoshimasa Urashaki,† and Mitsufumi Mayumi*†

Erythromycin (EM) has attracted attention because of its anti-inflammatory effect. Because dendritic cells (DCs) are the most potent APCs involved in numerous pathologic processes including innate immunity, we examined effects of EM on the activation of human DCs by pathogen-derived stimuli. Monocyte-derived DCs were pretreated with EM and subsequently stimulated with peptidoglycan, polyriboinosinic-polyribocytidylic acid (poly(I:C)), or LPS. The activation of DCs was assessed by surface molecule expression and cytokine production. To reveal the signaling pathways affected by EM, TLR expression, NF-κB, IFN regulatory factor-3, and AP-1 activation were examined. EM inhibited costimulatory molecule expression and cytokine production that was induced by poly(I:C) and LPS but not by peptidoglycan. EM pretreatment down- and up-regulated mRNA levels of TLR3 and TLR2, respectively, but did not affect that of TLR4. EM suppressed IFN regulatory factor-3 activation and IFN-β production but not AP-1 activation induced by poly(I:C) and LPS. The inhibitory effect of EM on NF-κB activation was observed only in poly(I:C)-stimulated DCs. EM selectively suppressed activation of DCs induced by LPS and poly(I:C) in different ways, suggesting that the immuno-modulating effects of EM depend on the nature of pathogens. These results might explain why EM prevents the virus-induced exacerbation in the chronic inflammatory respiratory diseases and give us the clue to design new drugs to treat these diseases. The Journal of Immunology, 2005, 175: 8069–8076.

The role of macrocid antibiotics in the treatment of upper and lower respiratory tract infections is well established. Independent of their potent antimicrobial activity, 14-membered and 15-membered macrolides possess anti-inflammatory properties that may contribute to the clinical benefits observed in patients with chronic infectious airway inflammation (1, 2). Macrolide antibiotics have been shown to affect a number of processes involved in inflammation, including migration of neutrophils, the oxidative burst in phagocytes, and the production of various cytokines by bronchial epithelial cells, lymphocytes, and monocytes (1, 3–6). Although it is presumed that NF-κB and AP-1, which are crucial regulators of proinflammatory gene expression (4, 5, 7, 8), are potentially the most important targets for at least some anti-inflammatory effects of macrolides, the precise mechanisms remain to be clarified.

The innate immune system has dual roles in host defense, providing a direct and immediate response against microbial invaders as well as playing an instructive role, influencing the nature of adaptive immunity. This instructive role of the innate immune system is served mainly by dendritic cells (DCs) (9). DCs can recognize microbial components using various pattern recognition receptors such as TLRs (10). Different TLRs expressed on DCs can discriminate distinct pathogen-associated molecular patterns and initiate signaling pathways to induce DC maturation and activation (11, 12). After exposure to pathogens, DCs respond by producing immunostimulatory cytokines such as IL-12 and by up-regulating MHC class I, MHC class II, and costimulatory molecules. DCs then mature into efficient APCs. This microbial-induced DC maturation provides crucial elements required for directing the adaptive T cell response toward either a Th1 or Th2 pattern (9) (13, 14).

To date, 11 TLRs have been identified in humans (15). Each TLR recognizes a distinct microbial component; for example, peptidoglycan (PGN), the major Gram-positive bacterial cell wall component, uses TLR2, whereas LPS, a Gram-negative cell wall component, stimulates DCs through TLR4. Double-stranded RNA of viral origin and polyriboinosinic-polyribocytidylic acid (poly(I:C)), a synthetic analog of dsRNA, are recognized by TLR3. Stimulation of TLRs by these ligands activates several signaling cascades, including NF-κB and MAPK, leading to the production of proinflammatory cytokines and the induction of DC maturation (11, 12, 16–18). Erythromycin (EM), a prototype of the 14-membered macrolides, has been shown to inhibit the activation of NF-κB and AP-1 (5–7). In this study, therefore, we examined the immuno-modulating effect of EM on human monocyte-derived DCs and the molecular targets of EM in the TLR signaling pathways. We show here that EM selectively suppresses activation of DCs induced by poly(I:C) and LPS. Furthermore, we discuss the
possibility that EM mainly affects the MyD88-independent pathway.

Materials and Methods

Reagents and culture medium

Human recombinant GM-CSF and IL-4 were provided by Kirin Beverage Company and Ono Pharmaceutical Company, respectively. FITC-conjugated mAbs specific for CD83, CD86, CD54, or MHC class II and anti-CDL2, -TLR2, -TLR3, and -TLR4 mAbs were purchased from eBioscience. PE-conjugated mAbs specific for CD14 or IL-4 and FITC-conjugated IFN-γ mAb and anti-CD1a mAb were purchased from BD Biosciences. AIM-V medium and anti-CD54 were from Invitrogen. LPS from Escherichia coli 0111:B4 purified by phenol extraction and gel filtration chromatography and Ultra-pure LPS from Salmonella minnesota R595 (Re) were obtained from Sigma-Aldrich and Calbiochem-Novabiochem, respectively. Staphylococcus aureus PGN was from Fluka. Poly(I:C) (gamma-irradiated) and EM were from Sigma-Aldrich.

Cell purification and culture conditions

Highly purified monocytes (>95% CD14+) were obtained from buffy coats of healthy volunteers as described previously (19). Briefly, PBMCs were separated by Ficol-Paque Plus (Amersham Bioscience). Monocytes were enriched by cold aggregation and deprived of T and NK cells by rosetting with 2-aminoethylisothiouronium bromide-treated (Sigma-Aldrich) sheep RBCs. Enriched monocytes (5 × 10⁶ cells/well) were cultured in plastic 6-well plates (Falcon) for 30 min followed by removal of trace numbers of nonadherent cells. The adherent cells were cultured in 1 ml of AIM-V medium (Invitrogen) supplemented with 40 ng/ml GM-CSF and 40 ng/ml IL-4 at 37°C with 5% CO₂. One milliliter of fresh AIM-V medium containing GM-CSF and IL-4 was added on day 2 and nonadherent cells were harvested on day 5 as immature DCs. Microscopic analysis showed that >98% of the nonadherent cells had cellular projections. Analysis by flow cytometry revealed that the preparations consisted of a homogenous (>96%) population of CD14 +, CD1a +, CD16 +, CD40 +, CD54 +, CD80 +, and HLA-DR + large cells, and <1% of CD3 +, CD19 +, or CD56 + cells could be detected. The immature DCs (1 × 10⁶ cells/ml) were extensively washed, precultivated in the presence or absence of EM (100 μg/ml) for 3 h, and then stimulated with 10 μg/ml PGN, 20 μg/ml poly(I:C), or 2 μg/ml LPS. After 48 h of stimulation, the supernatants were collected and stored at −20°C until they could be assayed for cytokines. For the measurement of IFN-β production, the supernatants were collected after 24 h.

T lymphocytes and allogenic MLR

CD4+ CD45RA naive T cells were negatively selected from PBMCs by using the MACS CD4+ T cell isolation kit and CD45RO MicroBeads according to the protocol recommended by the supplier (Miltenyi Biotec). The purity of naive CD4+ T cells was shown to be >98% by flow cytometry using anti-CD4 and anti-CD45RA mAbs (eBioscience). The DCs containing GM-CSF and IL-4 were added on day 2 and nonadherent cells were harvested on day 5 as immature DCs. Microscopic analysis showed that >98% of the nonadherent cells had cellular projections. Analysis by flow cytometry revealed that the preparations consisted of a homogenous (>96%) population of CD2 +, CD4 +, CD16 +, CD40 +, CD54 +, CD80 +, CD83 +, CD1a +, HLA-DR + large cells, and <1% of CD3 +, CD19 +, or CD56 + cells could be detected. The immature DCs (1 × 10⁶ cells/ml) were extensively washed, precultivated in the presence or absence of EM for 2 days and then stimulated with 10 μg/ml PGN, 20 μg/ml poly(I:C), or 2 μg/ml LPS. After 48 h of stimulation, the supernatants were collected and stored at −20°C until they could be assayed for cytokines. For the measurement of IFN-β production, the supernatants were collected after 24 h.

Cytokine measurements

IL-12p70, IL-6, IL-10, IL-13, and IFN-γ were measured by ELISA. Abs pairs and standard recombinant human cytokines for the ELISA were purchased from Pierce Biotechnology and PeproTech, respectively. IFN-β, IL-1β, and TNF-α were measured by an IFN-β ELISA kit (TFB), an IL-1β ELISA kit, and a TNF-α ELISA kit (Biosource), respectively. The detection limits for IL-12p70, IL-6, IL-10, IL-13, IFN-γ, IFN-β, IL-1β, and TNF-α were 15 pg/ml, 31 pg/ml, 15 pg/ml, 250 pg/ml, 15 pg/ml, 2.5 IU/ml, 3.9 pg/ml, and 3.9 pg/ml, respectively.

Analysis of TLR mRNA expression

Immature DCs were lysed after 6 h of culture with 100 μg/ml EM, and total RNAs were isolated using the RNeasy kit (QIAGEN). First-strand cDNA was synthesized from 1 μg of the total amount of RNA using Super Script II reverse transcriptase (Invitrogen Life Technologies). PCR amplification of TLR2, TLR3, TLR4, and TLR7 mRNA was performed using specific primers according to manufacturer’s instructions. For each sample, the difference in threshold cycles between the target gene and β-actin gives the standardized expression level (ΔCt). Subtracting the ΔCt of the control from the ΔCt of an EM-treated sample yields the ΔΔCt value that was used to calculate relative expression levels in EM-treated samples according to the formula 2−ΔΔCt.

Flow cytometry

The cells were incubated with the indicated mAbs, together with human IgG (10 μg/ml) for 30 min on ice in FACS buffer (PBS containing 0.5% BSA and 0.01% sodium azide). After the cells were washed, the FITC-conjugated F(ab′)₂ of goat anti-mouse Ig (DakoCytomation) was added and further incubated for 30 min. β-Actin, and c-Jun were used as internal reference markers. The cells were pretreated with BD Cytofix/Cytoperm (BD Biosciences). The cells were then analyzed on a FACScalibur (BD Biosciences). The specific mean fluorescence intensities were calculated by subtracting the isotype-matched control Ab fluorescence.

Calorimetric analysis for NF-kB activation

Nuclear extracts were prepared from immature DCs that were pretreated with 100 μg/ml EM and subsequently stimulated with LPS or poly(I:C) for the indicated time periods, using a Nuclear Extract kit (Active Motif). Binding of NF-κB subunits to the NF-κB binding consensus sequences was measured with the ELISA-based Trans-AM NF-κB family transcription factor assay kits (Active Motif), respectively, according to the protocol recommended by the supplier. The active forms of NF-κB subunits in nuclear extracts can be detected using Abs specific for an epitope that is accessible only when the subunit is activated and bound to its target DNA.

Western blotting

To detect IFN regulatory factor-3 (IRF-3) dimerization, the cells were lysed with 50 μM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 0.1 mg/ml leupeptin, and 1 mM PMSF. The whole cell lysates were prepared by centrifugation and subjected to native PAGE (10 μg of protein/lane). Native PAGE was performed using a 7.5% acrylamide gel (0.375 M Tris (pH 8.8), 1% SDS, and 0.01% sodium azide). After the gels were soaked in water, the proteins were transferred to polyvinylidene difluoride membrane (Millipore) as described previously (20). The immunoreactive proteins were detected using rabbit anti-human IRF-3 rabbit polyclonal Ab (IBL) and the ECL-Plus detection system (Amersham Biosciences). The whole cell lysates (10–30 μg of protein/lane) were also electrophoresed by SDS-PAGE, transferred, and immunoblotted with specific primary Abs: rabbit polyclonal Abs to c-Fos, c-Jun, or IκBα; a mAb to phosphorylated IκBα; a rabbit polyclonal Ab to IL-1R-associated kinase (IRAK) from Santa Cruz Biotechnology; a rabbit polyclonal Ab to IL-1R-associated kinase (IRAK) from Santa Cruz Biotechnology; and a rabbit polyclonal Ab to B (Santa Cruz). Blots were then incubated with the appropriate HRP-conjugated secondary Abs and bands were revealed using the ECL-Plus detection system.

EMSA

Nuclear extraction was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer’s instructions (Pierce). The AP-1 probe was prepared by biotin-labeling the double-stranded AP-1 consensus sequence (Santa Cruz Biotechnology) using the Biotin 3′-End DNA Labeling Kit (Pierce). Nuclear protein-DNA binding reactions were conducted for 20 min at room temperature in a 20-μl volume containing 10 mM Tris, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 1 μg/μl poly(dI·dC) 1% Nonidet P-40, (all the reagents are included in the LightShift Chemiluminescent EMSA Kit; Pierce), 100 fmol biotin-labeled AP-1 probe, and 5 μg of nuclear protein. Binding reactions were analyzed using 5% native PAGE. After blotting to a nylon membrane, labeled oligonucleotides were used as a probe.
were detected with the LightShift Chemiluminescent EMSA Kit following the instructions of the manufacturer (Pierce).

Statistical analysis
All data are expressed as mean ± SEM. Differences between groups were examined for statistical significance using the Wilcoxon matched-pairs signed-ranks test. A p value <0.05 denoted a statistically significant difference.

Results
Effects of EM on the differentiation of monocytes into DCs
Immature DCs were derived from monocyte precursors isolated from peripheral blood by culturing with GM-CSF and IL-4. By day 5, a homogenous population of immature DCs was recovered, based upon expression of defining surface markers including high amounts of MHC class II, CD54, and CD1a, low levels of CD86, and practically no CD14 or CD83. The expression levels of the surface molecules, including lineage markers such as CD1a and CD14, were not changed by exposure to EM, irrespective of the duration (Fig. 1 and data not shown), suggesting that EM did not alter the differentiation of monocytes into immature DCs, at least in terms of surface marker phenotypes.

EM selectively inhibited the cytokine production of DCs stimulated with various TLR ligands
We next evaluated the capacity of EM-treated DCs to produce cytokines in response to various TLR ligands. Immature DCs were pretreated with increasing concentrations of EM for 3 h and were subsequently stimulated with PGN, poly(I:C), or LPS for 48 h in the presence of EM. As shown in Fig. 2, there were striking differences in the cytokine-producing patterns of DCs that were exposed to the different stimuli; poly(I:C) and PGN preferentially induced IL-12p70 and proinflammatory cytokine production, respectively, whereas LPS induced production of all cytokines tested, including IL-10, an anti-inflammatory cytokine (Fig. 2 and Table I). It is of note that treatment with EM did not affect the cell viability, but all cytokine production in response to poly(I:C) was suppressed by EM in a concentration-dependent manner (Fig. 2 and Table I). EM pretreatment also marginally but consistently inhibited LPS-mediated IL-12p70, IL-1ß, and IL-10 production. However, EM did not show any inhibitory effects on PGN-mediated cytokine production.

![CD14, CD1a, CD83](image1)

**FIGURE 1.** Effects of EM on expression of surface markers of monocyte-derived DCs. Monocyte-derived DCs were prepared by culturing with GM-CSF and IL-4 for 5 days. The cells were treated with EM (100 μg/ml) for 24 h. Surface phenotypes of EM-treated DCs (thick lines) and non-treated ones (thin lines) were assessed by flow cytometry using specific mAbs (solid lines) or isotype control mAbs (dotted lines).

![EM Effects](image2)

**FIGURE 2.** EM selectively suppressed cytokine production of DCs stimulated with poly(I:C) and LPS but not with PGN. Monocyte-derived DCs were pretreated with the indicated concentrations of EM for 3 h and were then stimulated with PGN (○), poly(I:C) (■), or LPS (●) or without any (□). After 48 h of stimulation, supernatants were tested by ELISA to determine the production of IL-12p70, IL-10, and IL-6. Shown are the mean ± SEM of five experiments. *, p < 0.05; **, p < 0.03.

EM selectively suppressed up-regulation of CD54 and CD86 on DCs induced by poly(I:C) and LPS but not by PGN
To examine the effect of EM on phenotypic maturation of DCs stimulated via different TLRs, the expression of CD83, CD86, and CD54 on DCs was analyzed by means of flow cytometry. All of the stimuli examined up-regulated the expression of CD83, CD86, and CD54 on DCs (Fig. 3). Interestingly, EM selectively inhibited up-regulation of CD54 and CD86 induced by poly(I:C) or LPS but not by PGN. These results support the notion that EM preferentially inhibits TLR3- and TLR4-mediated signaling pathways in DCs.

EM selectively impaired APC function of poly(I:C)-primed DCs
Different TLR ligands have been shown to differentially bias helper T cell responses by inducing distinct cytokine and costimulatory molecules in DCs (13, 14, 21). We wondered whether EM affects helper T cell differentiation indirectly through modulating the functional maturation of DCs. To this end, DCs were primed with TLR ligands in the presence or absence of EM and then were cocultured with allogenic naïve CD4+ T cells without adding EM. As shown in Fig. 4A, the DCs primed with TLR ligands induced proliferation of naïve T cells, and pretreatment with EM did not affect the T cell proliferation. However, EM pretreatment significantly decreased IFN-γ production by naïve T cells upon stimulation with poly(I:C)-primed DCs (p < 0.05) and also tended to suppress IFN-γ upon stimulation with LPS-primed DCs (p = 0.06) (Fig. 4B). In contrast, EM treatment had no effect on IL-13 production from naïve T cells under any culture conditions (Fig. 4C). In parallel with the results from the primary coculture, intracellular cytokine staining demonstrated that the pretreatment of DCs with EM hampered the development of IFN-γ-producing...
EM-modulated TLR expression on DCs

To reveal the inhibitory mechanisms of EM, we first examined the mRNA expression of the TLRs by RT-PCR. As shown in Fig. 5, A and B, 6 h of EM treatment up- and down-regulated the mRNA expression of TLR2 (97% increase) and of TLR3 (60% decrease), respectively, whereas EM did not affect the level of TLR4 mRNA. Next, the effects of EM on the protein expression levels of TLRs were analyzed by flow cytometry. Concordant with other reports (22–24), we detected little, if any, TLR2 and TLR4 proteins on DCs, confirming that they were not up-regulated by EM treatment (data not shown). In contrast, TLR3 was detected only by intracellular staining and, consistent with the message levels, EM down-regulated intracellular TLR3 expression at the protein level (Fig. 5C).

EM suppressed poly(I:C) induced NF-κB activation

Because the activity of NF-κB is controlled by IκB, we examined whether EM influenced the degradation of IκB-α by means of Western blot analysis (25). As shown in Fig. 6A, PGN, poly(I:C), and LPS reduced the amount of total IκB-α, indicating its degradation. EM pretreatment before stimulation with poly(I:C) prevented IκB-α degradation. However, IκB-α degradation induced by PGN and LPS was not affected by EM. A multi-well colorimetric assay to quantify DNA binding activity of the transcription factors showed that stimulation with PGN, poly(I:C), and LPS preferentially up-regulated the activities of NF-κB p65 and p50 (Fig. 6B). Inhibitory effects of EM were detected only in the poly(I:C)-induced up-regulation of NF-κB p65 and in the p50 activities.

Effects of EM on AP-1 activation

Induction of AP-1 activity generally requires phosphorylation of c-Jun by JNK and transcriptional induction of the c-fos gene (26). We examined whether EM influenced the phosphorylation of c-Jun and the accumulation of c-Fos proteins. As shown in Fig. 7, A and B, phosphorylation of c-Jun induced by poly(I:C) or LPS was not affected by EM. LPS induced the accumulation of c-Fos protein, whereas poly(I:C) did little, if any (Fig. 7, C and D). EM pretreatment did not affect the c-Fos expression after stimulation with poly(I:C), but it slightly enhanced the LPS-induced up-regulation of c-Fos.

Next, the modulation of DNA-binding activities of AP-1 complex by EM was tested by EMSA. Substantial induction of AP-1 DNA-binding activity was found in the nuclear extracts from DCs stimulated with poly(I:C) or LPS. Consistent with the results of Western blot analysis, EM pretreatment did not inhibit or rather enhanced the AP-1 DNA binding activities (data not show).

Effects of EM on IRF-3 activation and IFN-β production induced by poly(I:C) and LPS

TLR signaling pathways are separated into two groups: a MyD88-dependent pathway that leads to the activation of IRAK and a MyD88-independent pathway associated with the activation of IRF-3 and subsequent induction of IFN-β. The latter pathway is involved in TLR3 and TLR4 signaling but not in TLR2 signaling (27–29). Consistent with these findings, DCs accumulated the IRF-3 phosphodiomer, an active form of IRF-3, and produced IFN-β in response to poly(I:C) or LPS but not PGN (Fig. 8, A and

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**Table 1. Effects of EM on proinflammatory cytokine production**

<table>
<thead>
<tr>
<th>EM pretreatment</th>
<th>TNF-α (ng/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Nil</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PGN</td>
<td>22.5 ± 11.0</td>
<td>26.5 ± 9.4</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>0.28 ± 0.08</td>
<td>0.20 ± 0.05*</td>
</tr>
<tr>
<td>LPS</td>
<td>16.9 ± 3.2</td>
<td>14.3 ± 1.9</td>
</tr>
</tbody>
</table>

*a* Monocyte-derived DCs were pretreated with 100 μg/ml EM for 3 h and were then stimulated. After 48 h of stimulation, supernatants were tested by ELISA. Shown are the mean ± SEM of five experiments. *, *p* < 0.05.

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**FIGURE 3.** EM selectively inhibited the up-regulation of costimulatory molecules on DCs stimulated with poly(I:C) and LPS but not with PGN. Monocyte-derived DCs were pretreated with EM (100 μg/ml) for 3 h and were then stimulated with PGN, poly(I:C), or LPS. After 48 h of stimulation, the surface phenotypes of EM-treated DCs (thick lines) and nontreated DCs (thin lines) were assessed by flow cytometry using specific mAbs (solid lines) or by isotype control mAbs (dotted lines). Values represent the specific mean fluorescence intensity of the EM-treated DCs (top) and that of nontreated DCs (bottom) (A). The specific mean fluorescence intensity (ΔMFI) of CD86 (B) and CD54 (C) of EM-treated DCs (■) and nontreated DCs (□) was calculated by subtracting the isotype-matched control Ab fluorescence. Shown are the mean ± SEM of five experiments. *, *p* < 0.05.
EM pretreatment consistently inhibited the IRF-3 phosphodimer formation induced by poly(I:C) and LPS stimuli and suppressed IFN-β production.

Activation of the MyD88-dependent pathway is known to cause activation of IRAK, as indicated by autophosphorylation and subsequent degradation of IRAK (30). EM-pretreated DCs showed LPS-induced IRAK-1 degradation similar to that of nontreated cells (Fig. 8C), suggesting that TLR-mediated MyD88-dependent signaling was not inhibited by EM. Considering these results...

**FIGURE 4.** Effects of EM on the acquisition of T cell stimulatory activity of TLR ligand-primed DCs. Monocyte-derived DCs were pretreated with (■) or without (□) EM (100 μg/ml) for 3 h and were then primed with PGN, poly(I:C), or LPS for 48 h. Cells were then extensively washed and were cocultured with allogenic naive CD4+ T cells (DCs, 2 × 10^6 cells/well; naive T cells, 2 × 10^5 cells/well). Cell proliferation was determined by thymidine uptake (A). IFN-γ (B) and IL-13 (C) production was measured in the supernatants of the coculture. Shown are the mean ± SEM of five experiments. *, p < 0.05. After 5 days of the coculture, intracellular cytokine profiles of the T cells were analyzed by flow cytometry (D). The percentages of the respective cytokine-producing T cells are indicated. Results represent four experiments.

**FIGURE 5.** Effects of priming with EM on the expression of TLRs of DCs. A, Total RNA was purified from monocyte-derived DCs treated with EM (100 μg/ml) for 6 h, and the expression of TLR2, TLR3, and TLR4 mRNAs was determined by RT-PCR. The PCR products were electrophoresed in 1.5% agarose gels. Results represent six experiments. B, Expression levels of TLR2, TLR3, and TLR4 mRNAs were determined by real-time quantitative PCR and were standardized by measuring expression of β-actin mRNA. The indicated relative expression levels were calculated with the formula 2^(-ΔΔCt). C, Intracellular levels of TLR3 in EM-treated DCs (thick lines) and in nontreated DCs (thin lines) were assessed by flow cytometry using specific mAbs (solid lines) or isotype control mAbs (dotted lines). Values represent the specific mean fluorescence intensity of the EM-treated DCs (bottom) and that of nontreated DCs (top). Results represent three experiments.

**FIGURE 6.** EM selectively suppressed NF-κB activation induced by poly(I:C) but not by PGN. Monocyte-derived DCs were pretreated with EM and were then stimulated with PGN, poly(I:C), or LPS for the indicated periods of time. A, Equal amounts of whole cell lysates (10 μg) were separated on SDS-PAGE, and Western blots were performed as indicated to evaluate degradation of IκB-α. Blots were stripped and reblotted to evaluate actin. Results represent three experiments. B, Nuclear extracts (7 μg) were tested for binding of the activated NF-κB subunits to an NF-κB consensus sequence using the Trans-AM NF-κB family transcription factor assay kit. Shown are the mean ± SEM of three experiments.
and
C
(reblotted to evaluate total c-Jun (A and B) and total actin (C and D). Results represent four experiments.

Collectively, it appears that EM may affect the MyD88-independent/IRF-3-mediated pathway.

Discussion
There is now emerging evidence that NF-κB and AP-1 are involved in the immunomodulating effects of macrolide antibiotics (4, 5, 8). However, it remains unclear where the molecular basis for the effects lies in the signaling pathway for transcription factor activation. TLR signaling pathways have been shown to consist, at least, of a MyD88-dependent pathway that seems to be common to all TLRs and a MyD88-independent pathway that is peculiar to the TLR3 and TLR4 signaling pathways (15, 29–32). Because to date no interaction between MyD88 and TLR3 upon poly(I:C) stimulation has been observed, TLR3 signaling appears to depend mainly on the MyD88-independent pathway (30). TLR2 signaling has been shown to completely depend on MyD88, whereas LPS-induced TLR4 signaling diverges into two signaling cascades: a MyD88-dependent pathway that leads to the production of proinflammatory cytokines with quick activation of NF-κB and MAPK and a MyD88-independent pathway associated with the activation of IRF-3, subsequent induction of IFN-β, and maturation of DCs, with delayed activation of NF-κB and MAPK (16, 29, 33–35). In this study, EM selectively inhibited TLR3- and TLR4-mediated cytokine production and up-regulation of costimulatory molecules on monocyte-derived DCs but not on TLR2-mediated ones. This indicates that interaction of EM either with NF-κB proteins per se or with transcription factor binding sites could not account for the inhibitory activity of EM. It is conceivable that a pathway common to TLR3 and TLR4 signaling, but not shared with TLR2 signaling, might be a target for the action of EM. The recent analysis of Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)/Toll/IL-1R domain–containing adaptor molecule-1 (TICAM-1)-deficient mice showed the essential role of TRIF/TICAM-1 in the MyD88-independent pathways of TLR3 and TLR4 signaling (29, 33, 34). TRIF/TICAM-1-deficient mice were defective in TLR3-mediated NF-κB and IRF-3 activation, as well as TLR4-mediated IRF-3 activation. Interestingly, TRIF-deficient mice had a diminished response to LPS in terms of producing cytokines whose production was considered to be mediated by the MyD88-dependent pathway. In contrast, all TLR4-mediated responses were abrogated only in TRIF/TICAM-1-MyD88 double-deficient cells. These findings indicate that the inhibitory activity of EM on cytokine production may be the result of a suppressed TRIF/TICAM-1 cascade. To support this notion, IRF-3 activation and IFN-β production, which occur downstream of TRIF/TICAM-1 signaling, were decreased by EM, but IL-1β-α degradation, which occurs 10 min after stimulation with LPS and is related to early NF-κB activation.
through the MyD88-dependent pathway, was not affected by EM treatment (data not shown). The faint inhibitory effect of EM on LPS-induced IL-12 production might be explained by compensatory NF-κB activation from MyD88-dependent signaling.

It is known that the interaction of LPS with TLR4 on DCs leads to phosphorylation and activation of three major MAPKs, i.e., ERK, JNK, and p38 MAPK (36). The ERK and p38 MAPK pathways are shown to have opposite effects on DC maturation in response to LPS (37). However, the relationships and interaction among these MAPKs and TRIF/IRF-3 signaling pathways are not fully understood. A p38 MAPK inhibitor restores the activity of ERK suppressed by LPS during the differentiation of monocytes into DCs (38). Anthrax lethal toxin inhibits LPS-mediated IRF-3 activation and subsequent cytokine production by interfering the activation of p38 MAPK (39). Of the various MAPK pathways, the p38 MAPK signaling cascade seems to have the most important role in the generation of type I IFN-mediated signals (40). Because EM inhibited LPS-mediated IRF-3 activation and IFN-β production, EM might also inhibit p38 MAPK activation, resulting in a reciprocal up-regulation of ERK activity. The expression of c-Fos is induced by ternary complex factors, which are activated through phosphorylation by ERK (41). It is conceivable that the enhanced AP-1 activity in EM-pretreated cells was the result of the enhanced ERK/c-Fos cascade (Fig. 7).

It has been reported that TLR2 and TLR4 mRNA are coordinately expressed in DCs stimulated by inflammatory signals, whereas TLR3 mRNA expression is regulated independently (23, 42). Type I IFN is known to up-regulate TLR3 mRNA in DCs (43). EM inhibited IFN-β production of DCs in response to poly(I:C) and selectively suppress the constitutive TLR3 expression at the mRNA and protein levels. Thus, the suppression of IFN-β-driven TLR3 expression as well as the constitutively one might partially account for the inhibitory effects of EM on poly(I:C)-induced activation of DCs.

It has been shown that different TLR ligands instruct DCs to induce distinct Th responses via modulation of cytokine-producing ability and costimulatory molecule expression (13, 14, 21). Consistent with this notion, naive T cells stimulated with allogeneic DCs that were primed with different TLR ligands exhibit a distinct cytokine-producing profile: poly(I:C)-primed DCs induced naive T cells to produce higher IFN-γ and lower IL-13 than did PGN-primed DCs. EM pretreatment significantly decreased IFN-γ production and Th1 differentiation of naive T cells, when they were stimulated with poly(I:C)- or LPS-primed DCs. Contrarily, EM pretreatment did not affect the cytokine production profile of naive T cells stimulated with PGN-primed DCs. Because IL-12 is a Th1-promoting cytokine and CD54-mediated costimulation enhances the acquisition of IFN-γ-producing ability, the selective effects of EM on IL-12 and CD54 expression could partially account for the impaired ability of TLR-primed DCs to induce IFN-γ-producing T cells (44, 45). Thus, EM might attenuate Th1-type immune responses in RNA virus infection and endotoxin exposure by modulating DC activation. Several studies have shown that respiratory virus infections and colonization of *Pseudomonas aeruginosa*, a Gram-negative bacteria, are associated with the exacerbation of chronic obstructive pulmonary diseases and diffuse panbronchiolitis, respectively (46, 47). The modulation of Th1 responses by EM may serve as one explanation for the beneficial effects of macrolides in patients with these Th1-mediated chronic pulmonary diseases (48, 49). In fact, the IFN-γ level in the bronchoalveolar lavage fluid from patients with diffuse panbronchiolitis is significantly decreased after long-term EM treatment (49).

In conclusion, EM selectively suppressed TLR-mediated cytokine production, including an anti-inflammatory cytokine, IL-10, and altered T cell responses through modulating DC function. Thus, EM acts as an immunomodulator rather than a simple antibiotic. Because accumulating evidence supports the idea that DCs are deeply involved in chronic airway inflammation, the immunomodulating effect of EM on DCs may be beneficial to control these diseases (50). Our findings may provide clues for designing new drugs to treat chronic airway inflammation, such as that seen in patients with diffuse panbronchiolitis or chronic obstructive pulmonary diseases.

**Disclosures**

The authors have no financial conflict of interest.

**References**


