In Vitro Therapeutic Effect of Epigallocatechin Gallate on Nicotine-Induced Impairment of Resistance to Legionella pneumophila Infection of Established MH-S Alveolar Macrophages

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Epigallocatechin gallate (EGCg), a major form of tea catechins, has a variety of biological activities. Tobacco smoking, nicotine in particular, is one of the risk factors for respiratory infections. In the present study, a possible immunotherapeutic effect of EGCg on the nicotine-induced impairment of alveolar macrophages regarding antimicrobial activity, as well as immune function, was examined. The treatment of MH-S macrophages with nicotine significantly enhanced Legionella pneumophila replication in the cells and selectively down-regulated the production of interleukin (IL)–6, IL-12, and tumor necrosis factor (TNF)–α induced by infection but did not alter IL-10 production. The EGCg treatment of nicotine-suppressed macrophages reconstituted the resistance to the infection. Furthermore, EGCg diminished the nicotine-induced inhibition of cytokine production. Experiments with TNF-α treatment, neutralization of cytokines with antibodies, and analysis of interferon (IFN)–γ messenger RNA showed that the mechanism of the EGCg-induced recovery of anti–L. pneumophila activity impaired by nicotine may be due to the recovery of TNF-α and IFN-γ production by the macrophages.

It is widely accepted that tobacco smoking is associated with and most probably is a causative agent of many diseases, including infections [1–3], allergies [4, 5], cancer [6, 7], and cardiovascular disease [8, 9]. In particular, it is reported that smoking accelerates pneumonia caused by bacteria, such as Legionella, Chlamydia, Mycobacteria, and Streptococcus species [3, 10–14]. Although tobacco smoking has been linked to these serious illnesses and to the disruption of normal physiological function, the mechanism of the pathogenesis of tobacco smoking is still unclear. Much of the health risk of tobacco has been associated with the tars and with the generation of nitrosamines in the smoke [15, 16], and these compounds are often considered to be mutagens. Recent work has supported the concept that the deleterious effects of smoking may also be due, in part, to its immunosuppressive activity [17, 18], such that a generalized down-regulation of immunity may lead to tobacco-related illness. Various studies have analyzed which components of tobacco may be immunosuppressive. Recent evidence has supported the role of nicotine as a possible immunomodulator [19, 20]. For example, it has been shown that nicotine has a modulatory activity on the production of various cytokines [21–23]. However, the specific biological mechanisms by which exposure to nicotine increases the risk of bacterial pneumonia are poorly understood.

Legionella pneumophila, a facultative intracellular pathogen, is the etiologic agent of the bacterial pneumonia legionnaires disease. The mechanism by which L. pneumophila infection of the lung is controlled is not yet clear, but it is widely accepted that the activation of macrophages to suppress intracellular bacterial growth is an essential effector mechanism of the resolution of legionellosis [24]. Th1 cells are essential for the development of cell-mediated immunity (CMI) and may play a pivotal role in the defense against L. pneumophila infection. It is known that the Th1 cytokine interferon (IFN)–γ can activate macrophages and monocytes to inhibit L. pneumophila growth [25, 26] and that Th1 cells play an essential role in the development of CMI to pathogens [27]. Both IFN-γ and interleukin (IL)–12, which has a major role in the differentiation of the T helper cell phenotypes, are produced by macrophages. In addition, it has been reported that the inflammatory cytokine tumour necrosis factor (TNF)–α is required for the prompt resolution of pneumatic legionellosis, and a direct role for TNF-α in the activation of phagocytes has been indicated [28]. Other inflammatory cytokines, such as IL-6, are also known to control infections [29, 30]. In contrast, Th2 cytokines, IL-10 in particular, may facilitate the growth of L pneumophila in permissive mononuclear phagocytes, due, in part, to IL-10–mediated inhibition of TNF-α secretion and IFN-γ–mediated mononuclear phagocyte activation [31]. Nevertheless, all these cytokines—IL-6, IL-10, IL-12, TNF-α, and even IFN-γ—are known to be produced by macrophages in response to a bacterial infections and may play a critical role in the resolution of the infection.
role in the host defense against infection. Therefore, the modulation of production of such key cytokines by macrophages may eventually affect the outcome of the infection.

Epigallocatechin gallate (EGCg) is a major form of tea catechins and has a variety of biological activities, including antioxidant and antimicrobial activities, against some pathogens [32–35]. The immunomodulatory effect of EGCg has been increasingly recognized [36, 37], because the bioactivity of EGCg in plasma after drinking tea is known to be high [38–40]. Furthermore, our recent studies have shown that EGCg selectively alters the immune responses of macrophages to L. pneumophila and leads to an enhanced anti-L. pneumophila activity of macrophages [40]. In the present study, therefore, an alveolar macrophage infection model with L. pneumophila [41] was used to determine the possible immunotherapeutic activity of EGCg on the nicotine-induced impairment of alveolar macrophages.

Materials and Methods

Nicotine and EGCg. Nicotine hydrogen tartrate salt and EGCg were purchased from Sigma. The nicotine solution (10 mg/mL) in pyrogen-free water was freshly prepared for each experiment, was sterilized through a membrane filter, was kept on ice, and was shielded from light. EGCg was dissolved in pyrogen-free water at a concentration of 5 mg/mL, was sterilized through a membrane filter, and was stored at −20°C in a tube covered with foil to protect it from light. Both nicotine and EGCg were diluted with RPMI 1640 medium that contained 10% heat-inactivated fetal calf serum (FCS; Hyclone Laboratories) to prepare a solution for experiments.

Bacteria. L. pneumophila M124, serogroup 1, was obtained originally from a patient with fatal legionellosis [42]. The bacteria were cultured on charcoal blood agar plates (BCYE medium; Becton Dickinson) for 3 days at 37°C. The bacterial suspensions were prepared in pyrogen-free saline, and the concentration of bacteria was determined by spectrophotometry.

Macrophages. An MH-S murine alveolar macrophage cell line purchased from the American Type Culture Collection was used in the present study. The cells were maintained in RPMI 1640 medium that contained 10% FCS. The MH-S cells were adhered to 24-well tissue culture plates at a concentration of 5 × 10^5 cells/mL for 2 h in 5% CO2 at 37°C. The resulting cell monolayers were washed with Hanks’s balanced salt solution, supplied with 10% FCS-RPMI 1640 medium without antibiotics, and then used for experiments.

Macrophage infection. The macrophage monolayers were infected with L. pneumophila (infectivity ratio, 10 bacteria/cell) for 30 min, were washed to remove nonphagocytized bacteria, and were incubated in RPMI 1640 medium that contained 10% FCS without antibiotics. The cultures then were incubated for 48 h at 37°C in 5% CO2.

Macrophage treatment. The macrophage cultures infected with bacteria were treated with various concentrations (0.1–100 μg/mL) of nicotine for up to 48 h at 37°C in 5% CO2. The infected macrophage cultures were also treated with various concentrations (0.5–50 μg/mL) of EGCg for up to 48 h at 37°C in 5% CO2 in the presence or absence of nicotine. In some experiments, macrophage cultures infected with bacteria and treated with nicotine were incubated with recombinant mouse TNF-α (0.5–5 ng/mL; R&D Systems) for up to 48 h. The cultures infected with bacteria and treated with nicotine and/or EGCg also were incubated with either anti-mouse TNF-α IgG (20 μg/mL), anti-mouse IFN-γ IgG (10 μg/mL), anti-mouse TNF-α and IFN-γ IgG, or control hamster IgG (BD Pharmingen). The concentrations of recombinant TNF-α and antibodies have been confirmed elsewhere to show the activation of macrophages and complete neutralization of cytokines produced in culture supernatants, respectively [43].

Viable bacteria in cell cultures (colony-forming units assay). The number of viable bacteria (in colony-forming units) in cell lysates was determined by standard plate counts on BCYE medium, as described elsewhere [44]. After incubation, the cell monolayers were lysed with 0.1% saponin, and the number of viable bacteria in the lysates was determined.

ELISA. The amount of IL-6, IL-10, IL-12 p40/p70, TNF-α, and IFN-γ in the culture supernatants of macrophage cultures was determined by sandwich ELISA with the use of matched antibody pairs and protein standard for ELISA (IL-6, IL-10, IL-12 p40/p70, and IFN-γ; BD Pharmingen) and Duoset ELISA development system (TNF-α; R & D Systems). Concentrations were calculated from the standard curve performed for each cytokine protein.

Reverse-transcription (RT) polymerase chain reaction (PCR). Total RNA was extracted from cells by the microspin technique with the RNeasy Mini Kit (Qiagen), in accordance with the manufacturer’s manual. RT of total RNA (1 μg) was performed with avian myeloblastosis virus transcriptase in a commercial reaction mixture (Reverse Transcription System; Promega). The resulting cDNA was subjected to PCR with primers for β2-microglobulin (BMG) and IFN-γ. The primer sequences for BMG and IFN-γ have been described elsewhere [40]. The PCR was performed in a Minicycler (MJ Research) for either 25 cycles (60°C annealing temperature [BMG]) or 40 cycles (55°C annealing temperature [IFN-γ]). PCR products were analyzed on an ethidium bromide–stained 2% agarose gel.

Statistical analysis. Statistical analysis was performed with paired Student’s t test or 1-site analysis of variance.

Results

Effect of nicotine and EGCg on L. pneumophila growth in macrophages. The treatment of macrophages with 10 μg/mL nicotine after infection with bacteria significantly induced an enhancement of the replication of L. pneumophila in the cells at both 24 and 48 h after infection (figure 1). Because the higher concentrations of nicotine (100 μg/mL) showed cytotoxicity to MH-S macrophages, as determined by the trypan blue dye exclusion method (data not shown), we did not use nicotine concentrations >10 μg/mL in the present study. The lower concentrations of nicotine (1 μg/mL) showed only a minimal effect on the growth of L. pneumophila. To determine the possible therapeutic activity of EGCg on the nicotine-impaired antimicrobial activity, the macrophages were infected, were treated with nicotine, and then were incubated with various concentrations of EGCg. As shown in figure 1, the treatment of macrophages with
EGCg induced marked inhibition of *L. pneumophila* growth in the cells. When the nicotine-impaired macrophages were incubated with a concentration of as little as 0.5 μg/mL EGCg, the anti-*L. pneumophila* activity was readily recovered to the nontreated control group level. Treatment with higher concentrations of EGCg (5 and 50 μg/mL) strengthened the antimicrobial activity of the nicotine-impaired macrophages in a dose-dependent manner.

**Effect of EGCg on cytokine production of nicotine-impaired macrophages in response to *L. pneumophila* infection.** To determine the possible involvement of cytokines in EGCg-enhanced resistance of nicotine-impaired macrophages to *L. pneumophila* infection, the cytokine production of macrophages treated with or without nicotine/EGCg in response to the infection was assessed. The treatment of macrophages with nicotine alone markedly down-regulated IL-6, IL-12, and TNF-α production in response to *L. pneumophila* infection. The production of IL-10 was not affected by nicotine treatment (figure 2). In contrast, the effect of EGCg treatment on the cytokine production was notable. As shown in figure 2, the treatment of EGCg alone up-regulated IL-12 and TNF-α production by macrophages in response to *L. pneumophila* infection, even with a concentration as low as 0.5 μg/mL in the case of TNF-α. On the other hand, EGCg at a concentration of 50 μg/mL significantly down-regulated the production of IL-10 induced by bacterial infection. The treatment of macrophages with EGCg without any infection induces minimum production of the cytokines tested [40]. When the nicotine-impaired macrophages were treated with EGCg, the suppressed cytokine production was completely restored. The treatment with EGCg at the high concentration (50 μg/mL) enhanced even greater production of TNF-α. There was no detectable IFN-γ determined by ELISA assay (lower limit of detection, 32 pg/mL) in the culture supernatants of macrophages infected with bacteria and treated with or without nicotine and/or EGCg.

**Effect of exogenous TNF-α on *L. pneumophila* growth in nicotine-impaired macrophages.** To determine the involvement of nicotine-induced suppression of cytokines (TNF-α in particular, which is known to be a powerful activator for anti-*L. pneumophila* activity of macrophages [28]), in the susceptibility to bacterial infection, the nicotine-impaired macrophages were treated exogenously with recombinant TNF-α. This experiment also could possibly provide evidence as to whether the up-regulation of TNF-α production by EGCg is related to recovered antimicrobial activity of the nicotine-impaired macrophages. As shown in figure 3, when the nicotine-impaired macrophages were supplemented with exogenous TNF-α after infection, an enhanced inhibition of the bacterial growth in the cells was evident. The treatment of macrophages with 1 ng/mL of exogenous TNF-α almost completely recovered the antimicrobial activity suppressed by nicotine. The higher concentration of TNF-α (5 ng/mL) resulted in even more inhibition of bacterial replication in nicotine-suppressed macrophages.

**Involvement of enhanced cytokine production in the inhibition of *L. pneumophila* growth by EGCg.** Because both endogenous and exogenous TNF-α and IFN-γ are known to activate macrophages to inhibit *L. pneumophila* growth [26, 43, 45], and, in fact, the supplement of exogenous TNF-α to the nicotine-impaired macrophages restored the antimicrobial activity to *L. pneumophila* in the present study, involvement of enhanced TNF-α and IFN-γ in the EGCg-recovered *L. pneumophila* growth inhibition was examined by neutralization of these cytokines with specific antibodies. The macrophages infected and treated with nicotine and EGCg were incubated with either anti–TNF-α, anti–IFN-γ, both anti–TNF-α and anti–IFN-γ antibodies, or control IgG. The number of viable bacteria in the macrophages then was determined at 24 and 48 h after infection. As shown in figure 4, treatment of macrophages with anti–TNF-α antibody significantly reduced the EGCg-induced antimicrobial activity of the nicotine-impaired macrophages, but this was only partial. Anti–IFN-γ antibody treatment of macrophages also resulted in a partial abolishment of the EGCg-induced antimicrobial activity of nicotine-impaired macrophages. However, when
the macrophages were treated with both anti–TNF-α and anti–IFN-γ antibodies, the EGCg-induced antimicrobial activity was almost completely abolished at both 24 and 48 h after infection. The control IgG treated macrophages did not show any alteration of the EGCg-induced antimicrobial activity of nicotine-impaired macrophages.

**Effect of EGCg on IFN-γ mRNA expression of nicotine-treated macrophages in response to *L. pneumophila* infection.** Although detectable levels of IFN-γ were not found by the ELISA assay in the culture supernatants of macrophages infected with bacteria and treated with or without nicotine and/or EGCg, the anti–IFN-γ antibody treatment experiments indicated possible involvement of IFN-γ in the EGCg-induced recovery of antimicrobial activity by the nicotine-impaired macrophages. Therefore, the steady-state levels of IFN-γ mRNA in macrophages were analyzed by RT-PCR. The relative expression levels of IFN-γ messages to the levels of housekeeping gene BMG mRNA were compared between experimental groups (figure 5). Noninfected control macrophages did not show any message expression, but the *L. pneumophila* infection induced a marked elevation of the expression levels. The nicotine treatment significantly depressed the induction of IFN-γ messages. In contrast, EGCg treatment of nicotine-impaired macrophages clearly enhanced the expression levels of IFN-γ mRNA. EGCg alone did not induce any detectable IFN-γ mRNA in macrophages without infection at both 6 and 24 h after treatment (data not shown).

**Effect of cytokine neutralization on other cytokine production by macrophages.** To evaluate the effect of TNF-α and IFN-γ neutralization treatment on other cytokine production, IL-10, IL-12, and TNF-α production in antibody-treated macrophages was analyzed (figure 6). The treatment of macrophages with anti–TNF-α antibody almost completely neutralized production of TNF-α that was detected only minimally by the ELISA assay. However, both the up-regulation of IL-12 and down-regulation of IL-10 by EGCg were not affected by the anti–TNF-α antibody treatment. The anti–IFN-γ antibody treatment did not affect the levels of other cytokines, such as IL-10, IL-12, and TNF-α. The combination treatment with anti–TNF-α and anti–IFN-γ antibodies also did not affect the production of cytokines tested except TNF-α, which was markedly inhibited by the combination treatment.

**Discussion**

The health issues presented by tobacco smoke are complex and include the development of cancer, cardiovascular disease,
and infection. Although smokers develop a wide variety of pulmonary infections, including viral, fungal, and, more commonly, pneumonia caused by bacteria, smokers have the added burden of developing bacterial infections that can be relatively recalcitrant to traditional antibiotic therapy. In fact, smoking accelerates pneumonia caused by *Streptococcus pneumoniae* [13], one of the most common causative pathogens of community-acquired pneumonia. Moreover, pneumonia caused by other bacteria, such as *Chlamydia, Legionella,* and *Mycobacteria* species, are also known to occur frequently in smokers [3, 10, 12]. Bacterial pneumonia in immunocompromised patients, as well as in persons who are heavy tobacco smokers, often becomes a life-threatening disease, despite the extensive use of traditional antibiotic treatment. In fact, smoking accelerates pneumonia caused by *Streptococcus pneumoniae* [13], one of the most common causative pathogens of community-acquired pneumonia. Moreover, pneumonia caused by other bacteria, such as *Chlamydia, Legionella,* and *Mycobacteria* species, are also known to occur frequently in smokers [3, 10, 12]. Bacterial pneumonia in immunocompromised patients, as well as in persons who are heavy tobacco smokers, often becomes a life-threatening disease, despite the extensive use of traditional antibiotic treatment. In addition, the emergence of antibiotic-resistant bacteria in immunocompromised patients requires additional types of therapeutics to supplant traditional treatment with antibiotics. In this regard, development of a new approach to treat or care for patients who are susceptible to respiratory infections is an urgent clinical matter.

It is known that smoking damages lung defenses by impairing mucociliary flow, increasing the permeability of the respiratory epithelium, reducing humoral responses to inhaled antigens, and increasing susceptibility of the host against respiratory pathogens [46]. In addition, alveolar macrophages obtained from smoker’s bronchoalveolar lavage fluid secrete significantly fewer proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, compared with those of nonsmokers [47–49]. Although these observations may account for the increased susceptibility of smokers to respiratory infections, how smoking components directly alter the host local lung defense against respiratory pathogens and, in particular, affect immune responses of alveolar macrophages, which are critical effector cells in host defense, is not well understood. In this regard, the present study concerning the effect of nicotine, which is one of the major components of tobacco, on the susceptibility of alveolar macrophages to *L. pneumophila* infection revealed that nicotine directly causes suppression of immune responses of alveolar macrophages to infection and leads to an enhancement of bacterial replication in the macrophages. In particular, the finding of the selective inhibition of cytokine production, such as IL-6, TNF-α, and IFN-γ, by nicotine indicates how such immunomodulation contributes to the...
susceptibility of the cells to infections. The precise mechanism of nicotine-induced impairment of antimicrobial activity of macrophages is still unclear. However, from the results of the selective inhibition of cytokine production by nicotine and the recovery of cytokine production, as well as antimicrobial activity, by EGCg treatment, it seems likely that impaired TNF-\(\alpha\) and IFN-\(\gamma\) production may be a major mechanism responsible for the nicotine-induced impairment of antimicrobial activity against \(L.\) pneumophila infection. This hypothesis can be supported by reports published elsewhere that both TNF-\(\alpha\) and IFN-\(\gamma\) are strong activators for macrophages to induce anti–\(L.\) pneumophila activity [25, 26, 43, 45]. The results of exogenous TNF-\(\alpha\) treatment were consistent with this hypothesis.

The production of IFN-\(\gamma\) in the culture supernatants of nicotine-treated macrophages infected with \(L.\) pneumophila and stimulated with or without EGCg could not be detected by ELISA. However, the reduced expression levels of IFN-\(\gamma\) mRNA in the nicotine-treated macrophages and the recovery of the expression levels by treatment with EGCg were observed. In addition, the treatment of macrophages with anti–IFN-\(\gamma\) antibody inhibited the EGCg-induced anti–\(L.\) pneumophila activity. From these results, it is likely that the amount of IFN-\(\gamma\) in culture supernatants of macrophages stimulated by EGCg in response to \(L.\) pneumophila infection, although it was low and could not be detected by ELISA, may be still involved in the EGCg-induced anti–\(L.\) pneumophila activity. This conclusion is consistent with recent studies that have indicated the production of IFN-\(\gamma\) by macrophages [50–53], even though it is generally considered that NK cells and activated T lymphocytes are the major source for IFN-\(\gamma\). On the other hand, the role of reduced IL-6 production in nicotine-impaired antimicrobial activity is not clear, but this cytokine may not have a direct role, given the absence of IL-6 modulatory effects on macrophages with respect to anti–\(L.\) pneumophila activity [54].

The cytokine IL-10 has been shown to exhibit important deactivating effects on macrophages in murine models of legionella [31], leishmanial [55], and mycobacterial [56] infections. Moreover, it is known that IL-10 is secreted by \(L.\) pneumophila–infected monocytes and alveolar macrophages, enhances bacterial
growth, reverses the protective effect of IFN-γ, and blocks the secretion of TNF-α by infected cells [31]. However, modulation of IL-10 may not be directly involved in the EGCg-induced anti- L. pneumophila activity of macrophages in vitro, because the diminution of anti- L. pneumophila activity by neutralization treatment was not associated with change of IL-10 production. IL-12 plays a key role in the development of Th1 responses, leading to IFN-γ production [57]. Therefore, down-regulation of IL-12 production by nicotine and up-regulation of nicotine-suppressed IL-12 production by EGCg may be beneficial in the host defense against L. pneumophila infection.

The recovery of nicotine-induced impairment of anti- L. pneumophila activity of macrophages by EGCg is notable. The bioavailability of tea catechins after oral administration of tea extracts is high because of excellent absorption characteristics. For example, consumption of 5 capsules of green tea extracts (~10 cups of green tea) results in a plasma level of ~2 μg EGCg/mL 90 min after ingestion [38, 39]. Furthermore, it is evident that radio-labeled EGCg orally administered to mice is distributed to various organs of the body, including skin [58]. In fact, it has been shown that the oral administration of tea extracts induces protection against UV-induced photocarcinogenesis of skin in mice [37]. These observations indicate that the bioavailability of tea catechins in tissues after oral administration, including daily consumption of tea, can be predicted at certain levels. Therefore, daily consumption of tea may be useful somewhat for restoring tobacco-suppressed host lung defenses against infection in smokers. Furthermore, a relatively high concentration of EGCg (50 μg/mL) strengthened the antimicrobial activity of nicotine-impaired macrophages in vitro. Practically, such high concentrations of EGCg in respiratory areas may be possible by inhalation treatment with EGCg. In fact, the study of inhalation treatment with tea catechins, including EGCg, on intractable respiratory infections showed a clinical improvement [59]. Thus, the results of present study indicate that EGCg achieves not only restoration but also activation of nicotine-impaired macrophages with respect to resistance to bacterial infection, as well as production of certain cytokines that are critical in the host defense against infections. Therefore, tea catechin EGCg may be one of the novel agents for treating patients who are susceptible to respiratory infections, because of its potential immunomodulatory, as well as antimicrobial, activity.

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References