Low Expression of T-cell Co-stimulatory Molecules in Bone Marrow-Derived Dendritic Cells in a Mouse Model of Chronic Respiratory Infection with *Pseudomonas Aeruginosa*

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Pseudomonas (*P.*) *aeruginosa* frequently colonizes the respiratory tract of patients with chronic respiratory tract infections such as diffuse panbronchiolitis (DPB). The number of dendritic cells (DCs) that play a central role in immune functions as antigen-presenting cells is reportedly increased in the bronchiolar tissues of patients with DPB. However, the functions of DCs in chronic *P. aeruginosa* respiratory tract infection have not been defined. Here, we assessed the functions of DCs and the effect of macrolide antibiotics that are therapeutic agents for DPB, in a murine model of DPB caused by *P. aeruginosa*. Mice were intubated with either *P. aeruginosa*- or saline-precoated tubes for 80 days. Thereafter, the expression of T-cell co-stimulatory molecules (CD40, CD80, and CD86) and cytokine secretion (interleukin (IL)-10, IL-6, IL-12p40, and tumor necrosis factor (TNF)-α) on bone marrow-derived DCs stimulated by lipopolysaccharide were examined by flow cytometry and enzyme-linked immunosorbent assays. The expression of co-stimulatory molecules was significantly decreased in mice infected with *P. aeruginosa* compared to the saline-treated control mice, but production of these cytokines did not significantly differ between the two groups. Pretreatment with clarithromycin *ex vivo* decreased CD40 expression on DCs obtained from *P. aeruginosa*-infected mice and also decreased the production of IL-6, IL-12p40 and TNF-α by DCs. These findings suggest that chronic *P. aeruginosa* infection alters DC functions and that macrolides function as anti-inflammatory agents by modulating the functions of DCs in chronic *P. aeruginosa* infection.

Dendritic cells (DCs) are antigen-presenting cells with the unique ability to prime naïve T cells. They play a central role in the initiation and regulation of immune responses and are preferentially located in the mucosal surfaces of virtually all organs except the brain (Lambrecht et al. 2001; Vermaelen and Pauwels 2005). After capturing antigens, DCs migrate to regional draining lymph nodes where they present sequestered antigen and mature. Respiratory tract DCs are relatively immature, and the maturation process results in the increased surface expression of many factors such as major histocompatibility molecules (MHC), T-cell costimulatory molecules, including CD40, CD80 and CD86 and chemokine receptors, as well as increased chemokine and cytokine secretion. Thereafter, DCs increase the ability to activate naïve T cells by presenting antigen fragments on MHC in combination with co-stimulatory molecules (Lambrecht et al. 2001; Vermaelen and Pauwels 2005). Airways are usually sterile, but cystic fibrosis (CF) and diffuse panbronchiolitis (DPB) are associated with chronic *Pseudomonas (P.) aeruginosa* infection. The numbers of DCs are increased in the bronchial tissues of patients with DPB (Todate et al. 2000), suggesting an association between DCs and immune modulation of the disease. However, the relationship between DCs and chronic *P. aeruginosa* respiratory tract infection remains obscure. We previously established a murine model of chronic *P. aerugi-*

nosa respiratory tract infection that mimics DPB to investigate the mechanisms of chronic infection (Yanagihara et al. 1997, 2000; Nagata et al. 2004). To clarify the effect of chronic *P. aeruginosa* respiratory tract infection on DCs, we examined the expression of co-stimulatory molecules and the production of several cytokines of bone marrow (BM)-derived DCs using our murine model. We also examined the effects of clarithromycin (CAM) and azithromycin (AZM) on DCs *ex vivo* because macrolide antibiotics have anti-inflammatory effects in patients with DPB (Kadota et al. 1993; Mukae et al. 1995; Tamaoki et al. 2004; Sugiyama et al. 2007).

Materials and Methods

Reagents

Lipopolysaccharide (LPS) derived from *P. aeruginosa* (lot 87F4009) was obtained from Sigma (St. Louis, MO). Recombinant mouse granulocyte-macrophage colony stimulating factor (rmGM-CSF) was purchased from R&D Systems (Oxford, UK). The macrolides CAM (Taisho Toyama, Tokyo, Japan) and AZM (Pfizer, Groton, CT) were donated by the respective companies. Both macrolides were dissolved in ethanol to a final ethanol concentration in culture of 0.02%, which our preliminary study showed does not affect the functions of DCs (Sugiyama et al. 2007).

Laboratory animals

The Nagasaki University School of Medicine committee on animal research approved the experimental protocol. Female BALB/c

mice (5-12 weeks old) purchased from Charles River (Yokohama, Japan) were housed in a specific pathogen-free facility at the Laboratory Animal Center for Biomedical Science at Nagasaki University and provided with sterile food and water *ad libitum*.

Bacterial strain

The *P. aeruginosa* strain PAO1 used in the present study has been characterized and studied in detail. The complete genome sequence has also been published (Stover et al. 2000). The bacteria were stored at –80°C in brain-heart infusion broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with 10% (v/v) glycerol and 5% (w/v) skim milk (Yukijirushi, Tokyo, Japan) until use.

Animal model of DPB

Thawed *P. aeruginosa* was cultured on Muller-Hinton agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) for 24 h, suspended in sterile saline and adjusted to a density of 1×10^8 colony-forming units (cfu)/ml determined by turbidimetry. The intubation tubes, comprising disposable sterile plastic intravenous catheters (3-French, 1.0-mm diameter; Atom, Tokyo, Japan) cut down to 3.0-mm lengths and with a few slits cut at the proximal end to prevent clogging with oral secretions, were immersed in bacterium-saline suspensions for 3 days at 37°C. The bacteria were detached from the tubes using a concussion machine for 5 min and then counted. The number of bacteria on the tubes at 3 days after incubation was 6.06 ± 0.19 $\log_{10}$ cfu/tube (mean $\pm$ s.d., $n = 10$). The mice were infected as described (Yanagihara et al. 1997). In brief, the mice were intubated under general anesthesia (pentobarbital sodium, 40 mg/kg i.p.). The blunted end of the inner needle of an intravenous catheter (Angiocath;
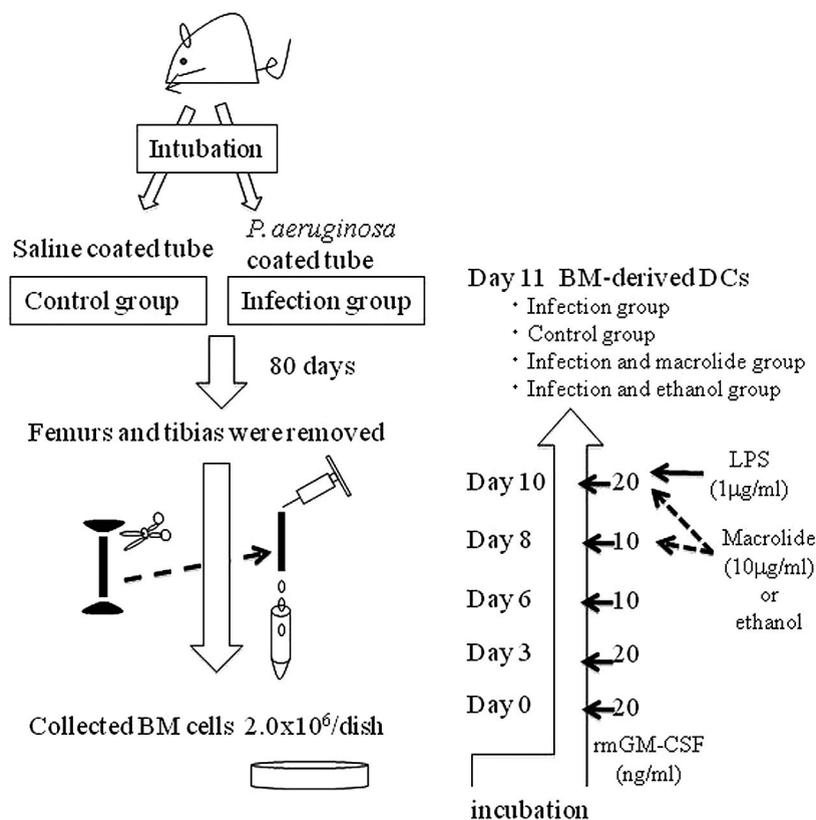

Fig. 1. Experimental protocol.

Becton Dickinson Vascular Access, Sandy, UT) was inserted through the oral cavity with the outer sheath and tube attached at the tip. The tube was advanced through the vocal cords into the trachea. The inner needle was then retracted and then the outer sheath was gently pushed to place the coated tube into the main bronchus. Both the *P. aeruginosa*-infected and control (saline) groups were observed for 80 days to assess the long-term effect of *P. aeruginosa* infection (Fig. 1) (Nagata et al. 2004).

Generation of bone marrow-derived DCs

Fig. 1 shows the preparation of DCs from BM progenitor cells as described (Lutz et al. 1999; Sugiyama et al. 2007). BALB/c mice were sacrificed at 80 days after intubation and both lungs were homogenized and separately cultured. The bacterial density in mice infected with *P. aeruginosa* was $> 1 \times 10^4$ cfu/ml. The ends of the femurs and tibias of the mice dissected out from surrounding muscle tissues were cut and then the marrow was flushed out and washed with phosphate buffer saline (PBS). Leukocytes (2×10^6/ml) were suspended in cRPMI medium comprising RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (GIBCO BRL Products, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen) and 50 μM 2-mercaptoethanol (Sigma). The cells were cultured with 10 ml of 20 ng/ml rmGM-CSF in 100-mm dishes for 3 days, and then another 10 ml of cRPMI containing 20 ng/ml rmGM-CSF was added. On days 6 and 8, half of the culture supernatant was replaced with fresh cRPMI containing 20 ng/ml rmGM-CSF. On day 10, non-adherent cells were also collected and resuspended in fresh cRPMI containing 20 ng/ml rmGM-CSF, and then incubated with 1 μg/ml of LPS for 24 h to maturate the DCs. Supernatants were collected on day 11 and then the DCs (about 1-2 $\times 10^5$ cells/ml) were washed twice with PBS and used for FACS analysis. The ratio (%) of the DC marker CD11c$^+$ was > 90%. We assessed the effects of CAM or AZM added on days 8 and 10 to a final concentration of 10 μg/ml, which is the physiological concentration of macrolides in lung tissues (Morris et al. 1991; McCarty 2000) determined from preliminary experiments and our report (Sugiyama et al. 2007). The control con-

tained an equivalent amount of ethanol without macrolides. All plates were incubated in a humidified 5% atmosphere at 37°C and supernatants were stored at −80°C.

Surface marker expression determined by flow cytometry

We examined DCs by direct immunofluorescence staining using fluorescein isothiocyanate-labeled antibodies to I-A/I-E (MHC II) or CD11c and phycoerythrin-labeled antibodies to CD11c, CD40, CD80 or CD86 (all from BD PharMingen, San Diego, CA). The stained cells were analyzed by flow cytometry (FACScan; Becton Dickinson). Data are presented as mean fluorescence intensity (MFI).

ELISA measurements

The concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 or IL-12p40 in the culture supernatants from DCs were measured in duplicate using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as means ± SE of 5 experiments. Differences between multiple groups were compared by one-way analysis of variance (ANOVA). The post hoc test for multiple comparisons was Fisher's PLSD test. Significance was assumed at $P < 0.05$.

Results

Expression of surface markers and production of cytokines

We initially examined the expression of co-stimulatory molecules such as CD40, CD86 or CD80 on DCs derived from the BM of mice. The intensity of CD40, CD86 and CD80 in stimulated DCs from *P. aeruginosa*-infected mice was decreased compared to that of control mice (Fig. 2). In contrast, MHC II and CD11c expression (data not shown) and IL-6, IL-12p40, IL-10 and TNF-α production by DCs
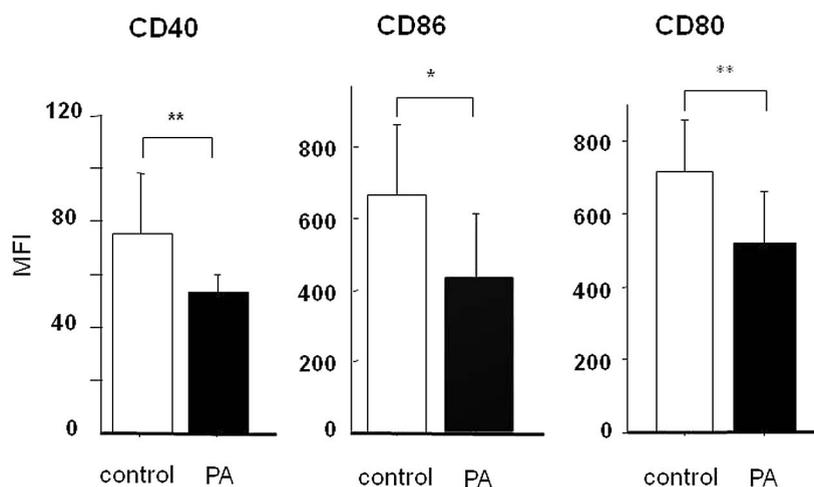

Fig. 2. Effect of chronic *P. aeruginosa* respiratory tract infection on surface markers of co-stimulatory molecules expressed by murine bone marrow-derived dendritic cells (DCs).
Mice were inoculated with either *P. aeruginosa* PAO1 (PA) or sterile saline (control). After 80 days, bone marrow-derived DCs were incubated with 1 μg/ml lipopolysaccharide. CD40, CD86 or CD80 expression on DCs was analyzed by flow cytometry. Values of mean fluorescence intensity (MFI) are expressed as means ± SE of 5 experiments; *$P < 0.05$, ** $P < 0.01$ compared to control.

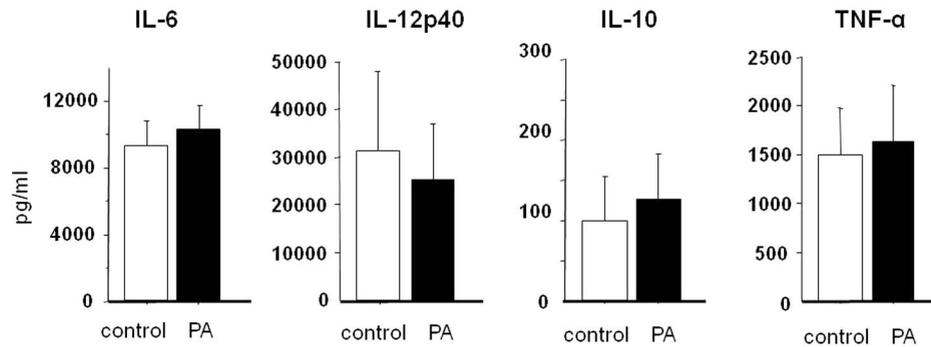IL-6   IL-12p40   IL-10   TNF-α

Fig. 3. Effect of chronic *P. aeruginosa* respiratory tract infection on cytokine production by murine bone marrow-derived dendritic cells (DCs).
Mice were inoculated with either *P. aeruginosa* PAO1 (PA) or sterile saline (control). After 80 days, bone marrow-derived DCs were incubated with 1 μg/ml lipopolysaccharide. Production of IL-6, IL-10, IL-12p40 and TNF-α (e) by DCs was measured by ELISA. Values are expressed as means ± SE of 5 experiments.

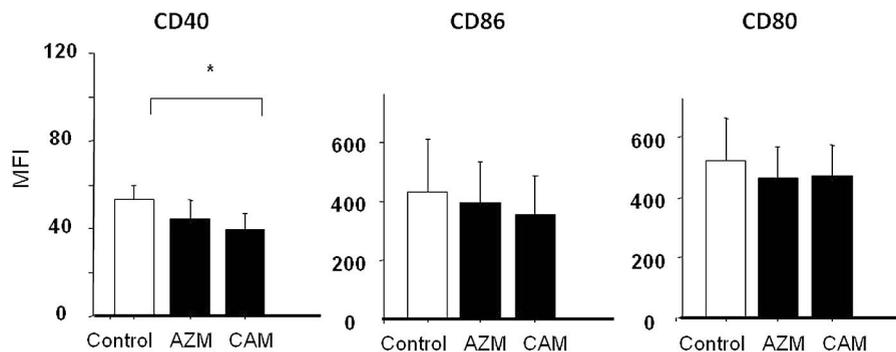CD40   CD86   CD80

Fig. 4. Effect of macrolides on surface markers of co-stimulatory molecules of murine bone marrow-derived dendritic cells (DCs).
Mice were inoculated with *P. aeruginosa* PAO1 (PA). After 80 days, bone marrow-derived DCs were incubated with 1 μg/ml lipopolysaccharide (LPS) alone (control) or LPS with azithromycin (AZM) or clarithromycin (CAM) and then CD40, CD86 or CD80 expression on DCs was analyzed by flow cytometry. Values of mean fluorescence intensity (MFI) are expressed as means ± SE of 5 experiments; *$P < 0.05$ compared to control.

(Fig. 3) did not significantly differ between the two groups.

Effect of macrolides on DCs

Fig. 4 shows the effect of AZM and CAM on the expression of co-stimulatory molecules on DCs derived from mice infected with *P. aeruginosa*. Only CAM significantly decreased the intensity of CD40 on DCs compared to control (without macrolides), although AZM and CAM tended to decrease the intensity of CD86 and CD80 on DCs derived from infected mice compared to controls. On the other hand, neither AZM nor CAM affected MHC II expression (data not shown). Fig. 5 shows that AZM and CAM significantly decreased IL-6 production and that CAM also significantly decreased the production of IL-12p40 and TNF-α in culture supernatants from DCs. Both CAM and AZM increased IL-10 production to a similar extent between both groups.

Discussion

The respiratory tract of patients with DPB and CF is often colonized by *P. aeruginosa*. In addition, the numbers

of DCs in the bronchiolar tissues and of activated CD8+ cells in bronchoalveolar lavage fluid (BALF) are increased in patients with DPB (Mukae et al. 1995; Todate et al. 2000). Dendritic cells are dedicated antigen-presenting cells with the unique ability to prime naïve T cells and play a central role in the initiation and regulation of immune responses (Lambrecht et al. 2001; Vermaelen and Pauwels 2005). Therefore, DCs might be closely associated with the immune responses of patients with DPB. However, because the relationship between DCs and DPB is obscure, we investigated the effect of chronic respiratory tract infection by *P. aeruginosa* on DCs in a murine model of DPB (Yanagihara et al. 2000; Kaneko et al. 2003; Nagata et al. 2004). In this context, airway DCs originate from BM precursors that arrive in the airway mucosa via the circulation. Lambrecht et al. demonstrated in a rat model of ovalbumin-induced airway inflammation that exposure to inhaled antigen leads to a profound increase in the DC network, not only at the site of antigen exposure but also at the BM precursor stage (Lambrecht et al. 1999). Pène et al. also showed that polymicrobial sepsis increased long-term sus-
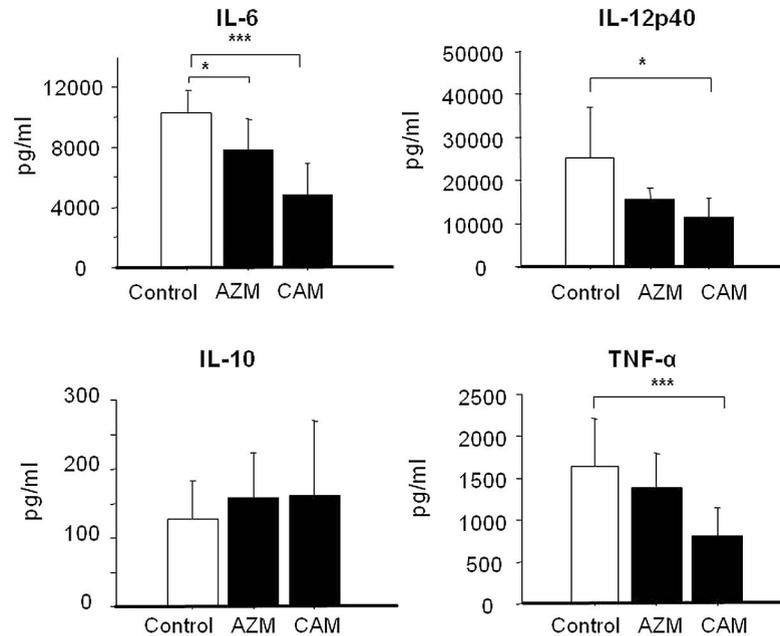
Fig. 5. Effect of macrolides on cytokine production by murine bone marrow-derived dendritic cells (DCs). Mice were inoculated with *P. aeruginosa* PAO1 (PA). After 80 days, bone marrow-derived DCs were incubated with 1 μg/ml lipopolysaccharide (LPS) alone (control) or LPS with azithromycin (AZM) or clarithromycin (CAM) and then IL-6, IL-10, IL-12p40 and TNF-*α* (e) production by DCs was measured by ELISA. Values are expressed as means ± SE of 5 experiments; *$P < 0.05$ and ***$P < 0.001$ compared to control.

ceptibility to secondary *P. aeruginosa* pneumonia through persistent functional abnormalities of BM-derived DCs (Pène et al. 2008). Because these findings suggest that BM-derived DCs are as important as lung DCs, the present study focused on these cells.

We found that the intensity of co-stimulatory molecules such as CD40, CD80 and CD86 on BM-derived DCs after LPS stimulation was significantly lower in *P. aeruginosa*-infected, than in control mice. However, the production of cytokine by DCs did not differ between the two groups. These results suggest that chronic *P. aeruginosa* infection alters the phenotype of BM-derived DCs, which is the first description of an effect of chronic *P. aeruginosa* respiratory infection on BM precursors for DCs as far as we are aware. In this context, toll-like receptors (TLR) 2 and 4 might be the signaling receptors for LPS, and MyD88 is also a general adaptor protein that plays an important role in TLR signaling (Kaisho and Akira 2006). Using MyD88-deficient mice, Kawai et al. demonstrated that TLR4 signaling can activate NF-κB and mitogen-activated protein kinases in MyD88-deficient macrophages, albeit with delayed kinetics (Kawai et al. 1999). These delayed responses do not induce gene expression of inflammatory cytokines but are sufficient for DC maturation. Thus, the difference between cytokine production and expression of co-stimulatory molecules determined herein might be due, at least in part, to the diversity of the TLR signaling pathway.

Murine BM-derived DCs interact with and are activated by *P. aeruginosa in vitro* and the expression of co-stimu-

latory molecules as well as the secretion of cytokines is increased in activated DCs (Worgall et al. 2001). However, we demonstrated decreased expression of costimulatory molecules in chronic *P. aeruginosa* infection *ex vivo* in the present study, suggesting that the immune status of BM-derived DCs differs between acute and chronic infection. Worgall et al. (2001) also demonstrated that BM-derived DCs pulsed with *P. aeruginosa* administered to syngeneic mice induce a CD4+ T cell proliferative response and prolong survival after a lethal intrapulmonary challenge with *P. aeruginosa* in a process that is dependent on the presence of CD4+ cells. This shows that CD4+ cells are required for DCs pulsed with *P. aeruginosa* to induce protective immunity. However, CD8+, but not CD4+ cells are dominant in BALF from patients with DPB (Mukae et al. 1995). These findings suggest that chronic *P. aeruginosa* respiratory tract infection affects DC maturation and function, which might alter the process of inflammation (CD8+ cell dominant) in the respiratory mucosa, thus inducing some type of tolerance (West and Heagy 2002). One feature of chronic obstructive pulmonary disease (COPD) comprises continuous episodes of respiratory tract infection. Our findings are consistent with those of Robbins et al., who demonstrated that chronic exposure to smoke dramatically reduces the ratio (%) of DCs expressing CD80. In addition, chronic smoke exposure impairs the immune response against secondary viral infections by preventing the specific expansion and maximal activation of CD4+ cells (Robbins et al. 2004).

Sputum samples from patients with COPD and CF, as well as human neutrophil elastase (NE), downregulate

CD40, CD80 and CD86 expression on DCs *in vitro* (Roghanian et al. 2006). Human neutrophil elastase (NE) also inhibits the antigen-presenting ability of DCs. These findings also suggest that the downregulation of co-stimulatory molecules in the present study is least partly due to NE. Levels of NE are also notably elevated in BALF from patients with DPB (Yasuoka et al. 1992).

The anti-inflammatory and immunomodulatory activities of macrolide antibiotics have been established since the discovery that DPB could be treated with erythromycin over the long term (Kudoh et al. 1998). Levels of inflammatory cytokines are elevated in the lungs of patients with DPB and of the mouse model, and macrolides inhibit the production of these cytokines in the lung, ultimately reducing inflammatory cell accumulation (Kadota et al. 1993; Mukae et al. 1995; Sakito et al. 1996; Yanagihara et al. 2000). We previously demonstrated that AZM and CAM significantly increase the intensity of CD80 on mouse BM-derived DCs *in vitro* (Sugiyama et al. 2007). In addition, AZM significantly increases IL-10 production and CAM significantly inhibits the production of IL-6 by DCs (Sugiyama et al. 2007). The present study showed that CAM significantly decreased the intensity of CD40 on DCs from mice chronically infected with PAO1. Both AZM and CAM also significantly decreased IL-6 production and CAM significantly decreased IL-12p40 and TNF-α production by DCs. These cytokines are important in the pathogenesis of chronic *P. aeruginosa* respiratory tract infection (Sakito et al. 1996; Nixon et al. 1998; Taggart et al. 2000; Moser et al. 2002). Thus macrolides, especially CAM, might exert anti-inflammatory effects through reducing the amount of CD40, IL-12p40, TNF-α and IL-6 produced by DCs in chronic *P. aeruginosa* respiratory tract infection. Here, we did not demonstrate an association between these macrolides and TLR, but one study has shown that CAM downregulates LPS-induced TLR4 expression (Park et al. 2005). Thus, the anti-inflammatory effects of macrolides might be explained in part by the ability to suppress TLR including TLR4.

Acknowledgments

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