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Role of PPAR γ in COX-2 activation in mycobacterial pulmonary inflammation

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Abstract

Preliminary studies show that intranasal (i.n.) administration of BCG in mice induces M1 activation of alveolar macrophages (M ϕ) that increase TNF- α production and cyclooxygenase-2 (COX-2) expression but reduce constitutive peroxisome proliferator-activated receptor gamma (PPAR γ) expression. However, COX-2 is catalytically inactive for prostaglandin E₂ release, unlike COX-2 active in M1 activation *in vitro* by BCG. In this study, we determined the role of PPAR γ for BCG-induced M1 activation *in vivo* and *in vitro*. We found that treatment of mice with GW9662, a PPAR γ antagonist, prior to i.n. BCG, partially restored PPAR γ expression, and decreased TNF- α production and COX-2 expression. But COX-2 was still inactive. The decreased effects on TNF- α and COX-2 were also observed when alveolar M ϕ were treated *in vitro* with GW9662/BCG, but COX-2 was still active. Our results indicate that PPAR γ up-regulates M1 activation of alveolar M ϕ , but inactive COX-2 formation is independent of PPAR γ in mycobacterial pulmonary inflammation.

Keywords

alveolar macrophages; BCG; COX-2; PGE₂; PPAR γ ; M1

Introduction

Inducible cyclooxygenase 2 (COX-2), a marker for classically (M1) activated macrophages (M ϕ), is central to increased biosynthesis of immunoregulatory prostaglandins including prostaglandin E₂ (PGE₂) (1–3). When normal alveolar M ϕ are activated *in vitro* with heat-killed *Mycobacterium bovis* BCG, a human tuberculosis vaccine and Th1 adjuvant, COX-2 is induced; this COX-2 is subcellularly associated with the nuclear envelope (NE) and catalytically active (4). In sharp contrast, intranasal (i.n.) BCG administration induces only catalytically inactive and NE-dissociated COX-2 (and COX-1) that is accumulated and aggregated in the endoplasmic reticulum (ER) in alveolar M ϕ (4). Although PGE synthase, converting PGH₂ to PGE₂, appears to be active (3), these COX-2⁺ M ϕ release no PGE₂ (4). We use the designations active or inactive COX-2 interchangeably with NE associated or dissociated, respectively (4–6), but the exact *in vivo* mechanism regulating alveolar M ϕ activation in mycobacterial pulmonary inflammation is still unclear.

Mucosal PGE₂ down-regulates innate and Th1-mediated immune responses, such as mucosal granuloma formation and bactericidal M1 activation (7–10). Simultaneously, PGE₂

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up-regulates alternatively activated MØ (M2), Th2 immune responses and antibody production (1, 11–13), including mucosal anti-mycobacterial IgA antibodies (14). It is also reported that PGE₂ reduces necrotic death of bactericidal MØ, thereby inhibiting bacterial replication (15). Therefore, alveolar MØ, which are activated by i.n. BCG and release no PGE₂, are expected to promote Th1-mediated mucosal protection against pulmonary *M. tuberculosis* challenges (16), as well as the granuloma formation (17).

It should be noticed that the kinetics of inactive COX-2⁺ MØ formation is dependent on tissues and routes of BCG administration. To induce inactive COX-2 formation in alveolar MØ, intranasal route of BCG administration is required. Intranasal administration of BCG induces NE-dissociated COX-2 up to 28 days, whereas intraperitoneal administration of BCG does not induce COX-2 in alveolar MØ and only transiently induces NE-dissociated COX-2 in peritoneal and splenic MØ. Therefore, intraperitoneal administration of BCG is inappropriate for the induction of pulmonary inflammation (1, 3, 5, 6).

Alveolar MØ from humans and mice constitutively express relatively high levels of peroxisome proliferator-activated receptor gamma (PPAR γ) at normal steady state (18–20). Intranasal BCG administration reduced PPAR γ expression (Preliminary study). PPAR γ enhances protein degradation in the ER by inducing an ER stress response (21, 22), which could theoretically explain the accumulation of inactive COX-2 in the ER in alveolar MØ activated by BCG *in vivo*. Furthermore, acting in an anti-inflammatory role, PPAR γ antagonizes inflammatory transcription factors (e.g., NF- κ B and activator protein -1 [AP-1]) (23). Therefore, deletion of the PPAR γ gene in murine alveolar MØ results in persistent production of interferon- γ (IFN- γ) and expression of inducible nitric oxide synthase (iNOS) (24). On the other hand, PPAR γ agonist treatments *in vitro* of several MØ including thioglycollate-elicited peritoneal MØ, bone marrow-derived MØ, monocyte-derived MØ and J774 MØ result in inhibition of COX-2 and iNOS expression and M1 cytokine production (23, 25, 26). Consistent with this observation, an irreversible antagonist of PPAR γ , GW9662, enhanced COX-2 expression and PGE₂ release *in vitro* by LPS-stimulated J774.2 MØ, indicating that PPAR γ down-regulates active COX-2 expression (25). Based on these studies, it has been hypothesized that PPAR γ down-regulates COX-2 expression in our alveolar MØ.

The literature, however, does not always support the hypothesis that PPAR γ down-regulates COX-2 activity in MØ. Unlike LPS, PPAR γ agonist NSAID diclofenac increases COX-2 expression and PGE₂ release in J774.2 MØ cultures (25). GW9662 inhibits BCG-induced PGE₂ release by peritoneal MØ *in vitro* (27). In the present study, our goals were to determine using GW9662 whether PPAR γ down-regulates M1 activation and COX-2 expression and whether PPAR γ contributes to the accumulation of inactive COX-2 when alveolar MØ are activated by i.n. BCG. The data presented here have documented the role of PPAR γ on BCG-induced M1 activation with COX-2 expression in alveolar MØ *in vivo*. The *in vivo* results have also been compared with those from *in vitro* alveolar MØ activation with BCG.

Materials and Methods

Mice

Non-pregnant female C57Bl/6 mice, 8–10 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME). Experimental protocols employed in this study were approved by the IACUC at Florida Atlantic University.

Administration of BCG and GW9662

M. bovis BCG Tokyo 172 strain (Japan BCG Laboratory, Tokyo, Japan) was prepared (4). Five, 50 or 500 µg of BCG in 0.05 ml saline was administered i.n. to groups of 3 – 6 mice, as described (4). As a control, mice were given i.n. doses of 0.05 ml saline. To evaluate the role of the PPAR γ pathway, GW9662 (1 mg/kg in saline; Cayman Chemical, Ann Arbor, MI) was given twice intraperitoneally 50 min followed by i.n. 20 min before i.n. BCG administration. The schedule of GW9662 treatment was chosen based on previous studies (28, 29). Alveolar MØ were isolated 24 h after the BCG administration.

MØ preparations

To obtain alveolar MØ from mice, bronchoalveolar lavage (BAL) cells were obtained (4). Total cell counts were determined with a Coulter counter (Model Z1; Beckman Coulter, Hialeah, FL). To enrich plastic-adherent MØ, BAL cells at 10^6 cells/ml in RPMI 1640 containing 5% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B were incubated in culture dishes (Falcon, Oxnard, CA) for 1 h at 37°C. BAL MØ and other inflammatory cells were enumerated by differential counts following cytopspin preparations and staining with Diff-Quik (Baxter Healthcare, Miami, FL). Adherent BAL cell differentials from all animals used in experiments revealed >90% MØ. In some experiments, adherent MØ were further cultured with 20 or 100 µg/ml BCG for additional 24 h. GW9662 (10 µM) (30) was added 30 min before BCG addition.

Western blot

Washed MØ were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 4 mM EDTA, 1% NP-40, 10 mM NaF, 2 mM Na₃VO₄, 0.1% sodium deoxycholate, and 1:500 protease inhibitor cocktail [P8340; Sigma Aldrich]). After 10 min on ice, debris was eliminated by centrifugation (10 min at 10,000 g). Protein concentration in the lysate was measured with a bicinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein from each sample were separated by SDS-PAGE and then transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 7.5% nonfat dry milk and incubated with antibodies (anti-PPAR γ , 1:200 [Santa Cruz Biotechnology, Santa Cruz, CA]; anti-COX-2, 1:200 [Novus Biologicals, Littleton, CO]; anti-COX-1, 1:1000 [Cayman Chemical]; anti- β -actin, 1:5000 [Sigma Aldrich]) in 7.5% nonfat dry milk overnight at 4°C. Following incubation with peroxidase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch, West Grove, PA), proteins were detected by chemiluminescence (ECL plus, Amersham, Piscataway, NJ).

Subcellular localization of COX-1 and COX-2 by confocal microscopy

To identify the NE-localization of COX-2 (and COX-1), MØ were fixed in 4% paraformaldehyde in PBS, pH 7.5, for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 7 min and incubated in blocking buffer (PBS with 10% FBS) overnight at 4°C prior to incubation overnight at 4°C with anti-COX-2 (1:500) or anti-COX-1 antibody (1:500) in blocking buffer. Subsequently, cells were washed with PBS three times and incubated for 1 h at 22°C with Alexa Fluor 488 goat anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) and 10 µg/ml propidium iodide (PI; Sigma Aldrich) for detection of nuclei and BCG (6). After washing three times, cells were examined with a laser scanning confocal microscope (LSM700, ZEISS, Thornwood, NY) with ZEN Software.

Measurement of PGE₂ and TNF- α concentrations

Levels of PGE₂ from the cell cultures and TNF- α in supernatants from the BAL and cell cultures were measured by ELISA, as previously described (4).

Statistical analysis

The statistical significance for differences between values was determined by one way analysis of variance followed by the Scheffé post-hoc test for multiple comparisons. All statistical analyses were performed with StatView Version 5.0 (SAS Institute Inc., Cary, NC). Results are expressed as mean $\pm$ SEM. $P < 0.05$ is considered statistically significant.

Results

PPAR γ mediates COX-2 expression in alveolar M ϕ when activated *in vivo* by i.n. BCG

Immunoreactive PPAR γ consisting of 53-kDa PPAR γ 1 and 57-kDa PPAR γ 2 as well as a relatively low level of COX-1 were present in alveolar M ϕ isolated from saline-treated control mice (Fig. 1). PPAR γ 2 has not been previously reported in alveolar M ϕ ; it is thought to be selectively expressed in adipose and to induce adipose differentiation (31, 32). BCG reduced both PPAR γ 1 and PPAR γ 2 levels, increased COX-1 expression, and induced COX-2 expression in a dose-dependent manner (Fig. 1).

To determine whether PPAR γ participates in these M ϕ responses, mice were treated with GW9662 prior to i.n. BCG administration. GW9662 is an irreversible PPAR γ antagonist that covalently modifies a cysteine residue in the ligand binding site of PPAR γ , resulting in the loss of ligand binding (33). GW9662 is highly selective for the inhibition of PPAR γ , since 10- and 600-fold more are required for the inhibition of PPAR α and PPAR δ , respectively (33). As shown in Fig. 2, GW9662 alone had no effect on the constitutive levels of COX-1 and induced no COX-2, but slightly reduced PPAR γ . GW9662/BCG treatment reduced the BCG-increased COX-1 and COX-2 levels. PPAR γ expression was reduced by BCG alone at 3% of control, and recovered by GW9662/BCG at 41% of control (Fig. 2). These results indicate that BCG suppression of PPAR γ was associated with enhanced expression of COX-1 and COX-2.

As reported previously (4), over 95% of alveolar M ϕ isolated from mice given i.n. 500 μ g dose BCG exhibit NE-dissociated COX-1 and COX-2, detected by immunofluorescent staining and confocal microscopy (Fig. 3). Despite of less expression of COX-2 expressed M ϕ at 5 and 50 μ g doses than those at 500 μ g BCG dose, over 90% of these COX-2⁺ M ϕ showed NE-dissociated forms (data not shown). As shown in Fig. 3, more than 95% of COX-1⁺ and COX-2⁺ alveolar M ϕ in the group of GW9662/BCG still showed NE-dissociated forms. Alveolar M ϕ isolated from untreated and BCG-treated mice both release around 300 pg PGE₂/10⁶ cells (4); alveolar M ϕ isolated from any of the four groups in this study released comparable ranges of PGE₂ (data not shown). Taken together, our results indicate that PPAR γ enhances COX-2 expression but does not contribute to the subcellular localization of COX-2 in BCG-activated alveolar M ϕ .

GW9662 is known to reduce phagocytosis of apoptotic neutrophils by human monocyte-derived M ϕ (34). To confirm that the effects of PPAR γ are not being mediated by impairment of BCG phagocytosis, we examined the percent of cells that contained intracellular BCG. We found that in both the BCG and the GW9662/BCG treatment groups, 95% M ϕ phagocytosed BCG (data not shown). We conclude that PPAR γ had no effect on phagocytosis of BCG.

PPAR γ mediates BCG-induced COX-2 expression in alveolar M ϕ when activated *in vitro* with BCG

Incubation of alveolar M ϕ with BCG *in vitro* resulted in slightly but significantly reduced PPAR γ (PPAR γ 1) levels along with dose-dependent increases in levels of COX-2 (Fig. 4); all COX-2⁺ M ϕ showed NE-associated COX-2 (data not shown). Interestingly, expression

of PPAR γ 2 in alveolar M ϕ was lost after 24 h *in vitro* without BCG (Fig. 4), but not after 1 h incubation (Fig. 1 and 2). As we found previously (4), levels of COX-1 were not increased by BCG treatment *in vitro* (Fig. 4).

Next, we determined the role of PPAR γ in BCG-induced NE-associated COX-2 expression *in vitro*. The reduction of PPAR γ (g1) following exposure to BCG was not prevented by GW9662 (Fig. 5a). Moreover, COX-1 in alveolar M ϕ was not induced by BCG or GW9662/BCG. In contrast, BCG-induced COX-2 expression and PGE₂ release were both significantly reduced by GW9662 (Fig. 5a, b and c). However, GW9662/BCG did not impair the localization of active COX-2 in the NE since over 99% of COX-2⁺ M ϕ showed NE-localization (Fig. 5d). Taken together, the induction of COX-2 expression and increased PGE₂ release by BCG are dependent on PPAR γ signaling; PPAR γ does not alter the localization of COX-2 *in vitro*.

PPAR γ up-regulates BCG-induced TNF- α production *in vivo* and *in vitro*

Animals treated intranasally with BCG alone had significant increase of TNF- α production in bronchoalveolar lavage (BAL) than that in saline-treated controls (Fig. 6a). This increase was completely reversed by GW9662/BCG together. An inhibitory effect on TNF- α production was also detected in alveolar M ϕ cultures with BCG and GW9662 (Fig. 6b). The results indicate that PPAR γ enhances TNF- α production, another M1 phenotype.

Discussion

Using GW9662, a highly selective and irreversible PPAR γ antagonist (33), we have shown for the first time that PPAR γ up-regulates M1 activation and COX-2 expression in alveolar M ϕ when activated by BCG *in vivo* (Fig. 2) or *in vitro* (Fig. 5). However, PPAR γ does not contribute to the formation of NE-dissociated/inactive COX-2 (Fig. 3) or the NE-associated/active COX-2 (Fig. 5), respectively. Table 1 summarizes the effects of GW9662 on the expression of COX-2 and PGE₂ release. Malur *et al.* (24) demonstrated that lack of PPAR γ in alveolar M ϕ in PPAR γ ^{-/-} mice disrupts lung homeostasis and results in an M1-like inflammatory response. Taken together, these results suggest that constitutive PPAR γ down-regulates M1 activation in the normal steady state, whereas PPAR γ up-regulates M1 activation in mycobacterial pulmonary inflammation.

A provocative finding is that when alveolar M ϕ are activated by BCG, PPAR γ expression is reduced (Fig. 1) in association with induction of TNF- α , COX-1 and COX-2. The literature suggests that the PPAR γ deficiency is comparable to that observed in sarcoidosis, pulmonary alveolar proteinosis, and asthma in humans, as well as in several animal models of lung inflammation (35–40). Furthermore, activation of PPAR γ by IFN- γ , thiazolidinediones, or endogenous ligands in adipocytes results in PPAR γ degradation mediated by ubiquitin-proteasome and TNF- α -activated caspase-1 (31, 32). Thus, a GW9662-seinstive mechanism for reducing PPAR γ levels could include endogenous and exogenous PPAR γ agonists generated by BCG treatments.

The PPAR γ pathway has been reported to mediate bovine serum albumin-induced ER stress in renal proximal tubule cells (22) and 15d-PGJ₂-induced ER stress, which attenuates IL-1 and IFN- γ signaling in pancreatic β -cells (21). COX-2 can be degraded in the ER via the ER-associated degradation pathway in response to an ER stress (41, 42). It is possible, therefore, that the accumulation of inactive COX-2 formation in response to i.n. BCG is mediated by an ER stress, but independent of PPAR γ signaling.

In addition to COX-2 and PPAR γ , COX-1 expression by alveolar M ϕ is distinct between *in vivo* and *in vitro* BCG treatments; COX-1 is increased by *in vivo*, but not *in vitro*, treatment

with BCG (Fig. 1 and 4). COX-1 is detected by western blot with 5 µg or less protein/lane when samples were prepared from normal peritoneal MØ; the levels are almost unchanged among the control and treatments with BCG *in vivo* and *in vitro* (4, 5). The differential expression of COX-1 between *in vivo* and *in vitro* conditions, therefore, is somewhat more notable in alveolar MØ than peritoneal MØ. Our results clearly show that PPARγ up-regulates COX-1 expression *in vivo* (Fig. 2), although it is NE-dissociated (Fig. 3). Additional tissue-dependent cells and humoral factors, lacking *in vitro* MØ cultures, would contribute to the *in vivo* MØ activation. Identifying such limiting cells/factors for these phenotypes remains to be studied.

Almeida *et al.* (27) demonstrated that when peritoneal MØ are treated *in vitro* with BCG, increased PPARγ enhances PGE₂ release. In our preliminary study, we found that GW9662 inhibited PGE₂ release but COX-1 and COX-2 levels were unchanged when normal peritoneal MØ were treated *in vitro* with GW9662 and BCG (data not shown). These COX-1 and COX-2 were all NE-associated (data not shown). Therefore, it is possible that PPARγ up-regulates phospholipase A₂ and/or PGE synthase or down-regulates 15-prostaglandin dehydrogenase (which converts PGE₂ to DHK- PGE₂) in BCG-activated peritoneal MØ. It further implies that PPARγ has multiple and tissue-dependent roles for the regulation of MØ PGE₂ biosynthesis.

It is unlikely that over-expression of COX-2 (and COX-1) at relatively higher doses of BCG *in vivo* would result in NE-dissociation. Our results indicate that (i) COX-2 levels are comparable between *in vivo* and *in vitro* BCG treatments (Figs. 1 and 4), and (ii) the expressions of COX-1 and COX-2 are reduced by *in vivo* GW9662 plus BCG, but still NE-dissociated forms (Figs 2 and 3). However, factors regulating the COX-2 phenotypes and distinct between *in vivo* and *in vitro* conditions (4–6) are still unknown. The difference between MØ exposed to BCG *in vivo* and those exposed *in vitro* would be reduction/oxidation (redox) states (43, 44), monocyte-infiltrated MØ (45), and additional cell populations such as mast cells that are activated by BCG *in vivo* and modify the MØ activation (46). Prior to the present study, we conducted studies to test if the above factors significantly contribute to the regulation of COX-2 NE-localization. In the preliminary studies, we used (i) mice deficient in methionine sulfoxide reductase A, an enzyme protecting hosts from oxidative damages (47), (ii) mice deficient in Flt-1-tyrosine kinase, an enzyme regulating monocyte infiltration (48), (iii) mice deficient in CCR2 (chemoattractant receptor-homologous molecule expressed on Th2 cells), a receptor of PDG₂ derived from activated mast cells (49). We found, however, that alveolar MØ isolated from these animals still showed NE-dissociated COX-2 when activated *in vivo* with BCG and NE-associated COX-2 when activated *in vitro* (data not shown).

We conclude that PPARγ in alveolar MØ enhances M1 activation including COX-2 expression, but the subcellular localization of COX-2 is regulated by a PPARγ-independent pathway in mycobacterial pulmonary inflammation. Antagonism of PPARγ may contribute to the suppression of excessive mucosal M1 activation without changing mucosal PGE₂ level. Finally, further studies are necessary to elucidate the mechanisms regulating NE-dissociated/inactive COX-2 formation.

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Fig. 1. PPAR γ is reduced by BCG treatment *in vivo*

Alveolar M ϕ were isolated from groups of mice 24 h after i.n. administration of 0 (saline), 5, 50, and 500 μ g BCG. PPAR γ , COX-1, COX-2 and β -actin levels were determined by Western blot with 5 μ g protein. β -actin bands show equivalent loading of samples. **a** Representative western blot. **b** The ratio of each molecule to β -actin obtained by densitometric analysis is expressed as arbitrary units (A.U.). Values are mean $\pm$ SEM of three separate experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$ compared with control saline group. .

Fig. 2. GW9662 slightly restores PPAR γ but reduces COX-1 and COX-2 expression by alveolar M ϕ activated by i.n. BCG

Mice received 1 mg/kg of a PPAR γ antagonist GW9662 (GW) i.p. and 1 mg/kg of GW9662 i.n., 50 min and 20 min, respectively, before 500 μ g of i.n. BCG administration. After 24 h, alveolar M ϕ were isolated from groups of mice. PPAR γ , COX-1, COX-2 and β -actin were determined by Western blot with 5 μ g protein. β -actin bands show equivalent loading of samples. **a** Representative western blot. **b** The ratio of each molecule to β -actin obtained by densitometric analysis is expressed as arbitrary units (A.U.). Values are mean $\pm$ SEM of three separate experiments. *, $p < 0.005$; **, $p < 0.0001$ compared with control saline group. †, $p < 0.05$; ††, $p < 0.0001$ compared with the BCG group.

Fig. 3. Subcellular localization of COX-1 and COX-2 in alveolar MØ after GW9662/BCG treatments *in vivo*

Mice were treated as described in Fig. 2. Cells were examined by confocal microscopy following immunofluorescent staining with anti-COX-1 or anti-COX-2 (green) and PI (red) for the nucleus and BCG. Bar = 5 μ m. Results are representative of three separate experiments.

Fig. 4. Alveolar MØ cultures with BCG reduce PPAR γ and induce COX-2

Alveolar MØ isolated from normal mice were incubated with 0 (saline), 20 or 100 μ g/ml BCG for 24 h, and cells were harvested. PPAR γ , COX-1, COX-2 and β -actin levels were determined by Western blot with 5 μ g protein. β -actin bands show equivalent loading of samples. **a** Representative western blot. **b** The ratio of each molecule to β -actin obtained by densitometric analysis is expressed as arbitrary units (A.U.). Values are mean $\pm$ SEM of three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p = 0.0001$ compared with control saline group.

Fig. 5. Effects of GW9662 on PPAR γ , COX-1 and COX-2 expression and PGE₂ production in alveolar MØ cultures

Normal alveolar MØ were pre-incubated with 10 μ M GW9662 for 30 min and then incubated with 100 μ g/ml BCG for 24 h. PPAR γ , COX-1, COX-2 and β -actin levels were examined by Western blot with 5 μ g protein. β -actin bands show equivalent loading of samples. **a** Representative western blot. **b** The ratio of each molecule to β -actin obtained by densitometric analysis is expressed as arbitrary units (A.U.). Values are mean $\pm$ SEM of three separate experiments. *, $p < 0.05$ compared with control saline group. †, $p < 0.05$ compared with the BCG group. ‡, $p < 0.005$ compared with the GW9662 group. **c** To measure PGE₂ release, adherent MØ were resuspended in serum-free RPMI 1640 and cultured for 2 h. PGE₂ was assayed by ELISA. Values are mean $\pm$ SEM, $n=3$. *, $p < 0.005$ compared with the saline group. †, $p < 0.005$ compared with the BCG group. **d** Subcellular localization of COX-2 in MØ was examined by confocal microscopy following immunofluorescent

staining with anti-COX-2 (green) and PI (red) for the nucleus and BCG. Bar = 5 μ m. Results are representative of three separate experiments.

Fig. 6. TNF- α levels in bronchoalveolar lavage (BAL) from GW9662/BCG-treated mice and supernatants of alveolar MØ cultures with GW9662/BCG

a TNF- α levels were measured by ELISA in BAL obtained by a 1 ml saline-lavage from mice, treated as in Fig. 2. **b** Normal alveolar MØ were treated as indicated in Fig. 5. TNF- α in the culture supernatants were measured by ELISA. Values are mean $\pm$ SEM; n=3. *, $p < 0.0001$ compared with control saline group. †, $p < 0.0001$ compared with BCG group. Results are representative of three separate experiments.

Table 1Summary of COX-2 expression and PGE₂ release by BCG-activated alveolar MØ

Treatment		COX-2 expression	COX-2 activity*	PGE ₂ release
<i>In vivo</i>	no	–	N/A	+/-
	BCG	+++	Inactive	+/-
	BCG/GW9662	+	Inactive	+/-
<i>In vitro</i>	no	–	N/A	+/-
	BCG	+++	Active	+++
	BCG/GW9662	+	Active	++

* Active COX-2, NE-associated form; inactive COX-2, NE-dissociated form –, negative; + ~ +++, positive; +/-, base line level; N/A, not applicable