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The Bacterial Quorum-Sensing Signal Molecule *N*-3-Oxo-Dodecanoyl-L-Homoserine Lactone Reciprocally Modulates Pro- and Anti-Inflammatory Cytokines in Activated Macrophages

Yifat Glucksam-Galnoy,* Roy Sananes,* Nava Silberstein,* Pnina Krief,† Vladimir V. Kravchenko,‡ Michael M. Meijler,†,§ and Tsaffrir Zor*

The bacterial molecule *N*-3-oxo-dodecanoyl-L-homoserine lactone (C12) has critical roles in both interbacterial communication and interkingdom signaling. The ability of C12 to downregulate production of the key proinflammatory cytokine TNF-α in stimulated macrophages was suggested to contribute to the establishment of chronic infections by opportunistic Gram-negative bacteria, such as *Pseudomonas aeruginosa*. We show that, in contrast to TNF-α suppression, C12 amplifies production of the major anti-inflammatory cytokine IL-10 in LPS-stimulated murine RAW264.7 macrophages, as well as peritoneal macrophages. Furthermore, C12 increased IL-10 mRNA levels and IL-10 promoter reporter activity in LPS-stimulated RAW264.7 macrophages, indicating that C12 modulates IL-10 expression at the transcriptional level. Finally, C12 substantially potentiated LPS-stimulated NF-κB DNA-binding levels and prolonged p38 MAPK phosphorylation in RAW264.7 macrophages, suggesting that increased transcriptional activity of NF-κB and/or p38-activated transcription factors serves to upregulate IL-10 production in macrophages exposed to both LPS and C12. These findings reveal another part of the complex array of host transitions through which opportunistic bacteria downregulate immune responses to flourish and establish a chronic infection. *The Journal of Immunology*, 2013, 191: 000–000.

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that causes a wide range of acute and chronic infections, including sepsis, and wound and pulmonary infections, particularly in immunocompromised people (1, 2). Virulence factors, such as proteases and exotoxins, play a critical role in the infection, and their production is regulated by acyl homoserine lactones (AHLs), a repertoire of bacterial small molecules that vary in acyl chain length or oxidation state at the acyl C-3 position (3–5). AHLs are signaling molecules that mediate cell-to-cell communication among bacteria, known as quorum sensing. This phenomenon is enabled by interactions between small diffusible autoinducers (such as the AHLs) and receptors that act as transcriptional regulators, enabling bacteria to keep track of their cellular density and, thus, to regulate and synchronize their behavior as a group (3–5). For example, the AHL molecules were originally described in the marine bacteria *Photobacterium (vibrio) fischeri*, where they regulate bioluminescence (6). Following that discovery, AHLs have been identified in a multitude of Gram-negative pathogens that infect yeast, animals, or plants (7).

N-3-oxo-dodecanoyl-L-homoserine lactone (C12) is known as a key AHL, secreted and sensed by *P. aeruginosa* at sites of infection, regulating the expression of bacterial virulence factors (7, 8). An intact C12 quorum-sensing system is required for successful establishment of *P. aeruginosa* infection, highlighting the contribution of bacterial gene regulation by C12 to pathogenicity (9, 10). Interestingly, it was found that C12 also directly modulates host defense systems (7, 8) via TLR4-independent mechanisms (11). C12 was shown to modulate diverse activities of macrophages (11–14), fibroblasts (11, 13–16), epithelial cells (11, 13, 15, 17), mast cells (18), T lymphocytes (19), B lymphocytes (20), and neutrophils (21). Importantly, mutations at the bacterial C12 receptor gene (*lasR*), but not at the synthase gene (*lasI*), are frequently found during the chronic phase of *P. aeruginosa* infection (22). These findings suggest that the immunomodulatory activity of C12, rather than its quorum-sensing activity, is essential for an infection to become chronic.

Published data regarding the role of C12 in host immune response modulation are inconsistent. Although several studies suggest that C12 enhances a proinflammatory host response (14, 15, 23–27), others indicate that C12 imparts an anti-inflammatory effect and, thus, contributes to the establishment of persistent infection (13, 20, 28–30). A comparative meta-analysis of these reports suggests that the apparently contradicting effects of C12 may be dose dependent (7, 8). Multiple studies showed that LPS-induced production of the key proinflammatory cytokine TNF-α is suppressed in the presence of C12 (13, 20, 29, 30). Yet, other studies showed that C12 failed to affect TNF-α production in resting cells (11, 16) or in LPS-stimulated cells (16). Immunization of mice with C12–protein conjugate reduces TNF-α levels and increases survival

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Abbreviations used in this article: AHL, acyl homoserine lactone; C12, *N*-3-oxo-dodecanoyl-L-homoserine lactone; PPARγ, peroxisome proliferator–activated receptor γ.

during a subsequent challenge with *P. aeruginosa*, suggesting that C12 has a proinflammatory effect (14). However, the protocol of that particular experiment does not distinguish between the quorum-sensing role of C12 (e.g., stimulation of virulence factor production) and its direct immunomodulatory effects. The mechanism of C12 effects on host cells was suggested to involve modulation of the transcriptional activity of either NF-κB (13, 24, 25) or peroxisome proliferator-activated receptor γ (PPARγ) (15, 17) or stimulation of calcium signaling (23, 31, 32).

The immune balance is critically regulated by anti-inflammatory cytokines, such as IL-10. Yet, the effect of the bacterial-signaling molecule C12 on IL-10 production in LPS-stimulated macrophages remains unclear. Interestingly, C12-producing bacteria did not affect IL-10 production in murine bone marrow–derived dendritic cells stimulated by LPS (28). In contrast, another study that used C12 directly, rather than C12-producing bacteria, demonstrated a C12-dependent reduction in TNF-α and increase in IL-10 production in a coculture of dendritic cells and T cells; however, the secreted cytokine levels were in the low pg/ml range, putting in question the physiological relevance (33). To address this open question, we examined the effect of C12 on LPS-induced expression of TNF-α and IL-10 in RAW264.7 macrophages. These experiments revealed that an inhibitory effect of C12 on LPS-induced production of TNF-α correlated positively with increased expression of IL-10 in LPS-stimulated macrophages. Our data also suggest that the increase in transcription of IL-10 occurred through a mechanism involving NF-κB and/or p38, rather than PPARγ.

Materials and Methods

Reagents and plasmid

LPS (*Escherichia coli* serotype 055:B5) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). XTT, L-glutamine, and penicillin-streptomycin-nystatin were purchased from Biological Industries (Beit Haemek, Israel). DMEM and FBS were purchased from Life Technologies (Carlsbad, CA). BSA was purchased from Amresco (Solon, OH). ELISA reagent sets for TNF-α and IL-10 were purchased from R&D Systems (Minneapolis, MN). Ciglitazone was purchased from A.G. Scientific (San Diego, CA). The full-length (−1538/+64) mouse IL-10 promoter luciferase reporter gene construct was a kind gift from Dr. S.T. Smale (University of California Los Angeles, Los Angeles, CA) (34). The IL-10 promoter reporter plasmid was amplified using DH10B bacteria (Invitrogen, Carlsbad, CA), and purified using an EndoFree Plasmid Maxi Kit (QIAGEN, Hamburg, Germany). FuGENE HD Transfection Reagent was purchased from Roche (Mannheim, Germany). Dual-luciferase reporter assay kit was from Promega (Madison, WI). MasterPure RNA purification kit was from Epicentre Biotechnologies (Madison, WI), the Verso cDNA synthesis kit was from Thermo Scientific (Waltham, MA), and the SYBR green reagent was purchased from QIAGEN. The synthesis and purification of C12 were carried out as previously described (35), and purity was confirmed by liquid chromatography mass spectra and nuclear magnetic resonance analysis. C12 is stable for years when stored as a powder or as a stock solution in dry DMSO at 4°C. In aqueous media at neutral pH, C12 will undergo hydrolysis to the inactive open ring derivative, with a half-life of roughly 10 h, which is far beyond the incubation time used in our experiments.

Cell culture

Mouse RAW264.7 macrophage cells, obtained from the American Type Culture Collection (Rockville, MD), were grown to 80–90% confluence in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1250 U/ml nystatin (hereafter culture medium), as well as with 10% FBS, at 37°C in a humidified incubator with 5% CO_2.

Animal care

Male C57BL/6 mice (12 wk) were obtained from Harlan (Rehovot, Israel). Animal care and experimentation were carried out in accordance with Tel Aviv University guidelines.

Cytokine production and cytotoxicity assays in RAW264.7 macrophages

RAW264.7 macrophages were maintained for 48 h prior to the experiment in 96-well plates, at 1.5×10^5 cells/well, in culture medium supplemented with 5% FBS, up to a confluence of 90%. The culture medium was replaced 2 h before treatment to avoid an artifact on signaling caused by medium replacement (36). The cells were stimulated with LPS (10 ng/ml) and/or C12 (10–100 μM) at 37°C for 2–5 h. Upon termination, media were collected for cytokine concentration assessment, and fresh media were added for measurement of cell viability using the XTT method, according to the manufacturer's instructions. IL-10 and TNF-α secretion to the medium were measured with commercially available ELISA reagents sets, according to the manufacturer's instructions, using a microplate reader (Bio-Tek, Winooski, VT). The samples were stored at −80°C until use. All experiments were repeated as least three times.

Isolation, culture, and ex vivo activation of mouse peritoneal macrophages

Nonelicited C57BL/6 mouse peritoneal exudate cells were harvested, washed, resuspended in culture medium supplemented with 10% FBS, and seeded in a 96-well culture plate (0.2 ml/well) at 5×10^5 cells/well. Following incubation for 24 h at 37°C, nonadherent cells were removed, and fresh medium was added to the adherent cells (~98% homogenous by appearance). Stimulation was then performed with LPS (100 ng/ml) in the presence or absence of C12 (20–50 μM) at 37°C for 24 h. Cytokine levels in the culture medium were determined by ELISA. All incubations were carried out in a humidified incubator with 5% CO_2.

Transfection and reporter gene assay

RAW264.7 macrophages were grown for 24 h in 12-well plates, at 3×10^5 cells/well, in culture medium supplemented with 10% FBS. The cells were then cotransfected for 24 h with 0.6 μg of the IL-10 promoter reporter plasmid and 0.2 μg HSV thymidine kinase promoter TK-*Renilla* luciferase (for normalization), which were initially incubated with 2.4 μl FuGENE HD Transfection Reagent in culture medium for 15 min at room temperature. Following transfection, the cells were washed and stimulated with LPS (10 ng/ml) and/or C12 at 37°C for 5 h, after which luciferase activity in cell extracts was determined following the manufacturer's instructions. Data were expressed as a ratio of IL-10 promoter–driven luciferase activity divided by the *Renilla* luciferase activity, relative to the respective ratio in unstimulated cells. Transfection with the empty reporter vector (pGL2B) yielded no detectable activity.

Quantitative real-time PCR

The mRNA levels of IL-10 and actin in RAW264.7 cells were quantified by real-time PCR. The cells were seeded in a six-well culture plate at 8×10^5 cells/well and cultured for 48 h in culture medium supplemented with 10% FBS. The cells were then treated with LPS (10 ng/ml) in the presence or absence of C12 for 5 h at 37°C. Total RNA was isolated using the MasterPure RNA purification kit, and 1 μg RNA from each sample was reverse transcribed into cDNA using the Verso cDNA synthesis kit. Quantification was performed with 5 ng cDNA on the ABI PRISM one step Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR green. The sequences of primers were as follows: actin, forward primer 5′-CTTTGCAGCTCCTTCGTTGC-3′ and reverse primer 5′-ACGATGGAGGGGAATACAGC-3′; and IL-10, forward primer 5′-CAGGGATCTTAGCTAACGGAAA-3′ and reverse primer 5′-GCTCAGTGAATAAATAGAATGGGAAC-3′.

Western blot analysis

Whole-cell lysates were prepared and used for Western blot assays, as previously described (37).

EMSA

Nuclear extracts were prepared and used for EMSA, as previously described (38).

Statistical analysis

All data were analyzed using the Student *t* test wherever applicable. In all cases, differences of $p < 0.05$ were considered significant. The EC_{50} value was calculated by nonlinear regression curve fitting, using SigmaPlot software.

Results

C12 inhibits LPS-induced TNF-α secretion in RAW264.7 macrophages

The bacterial quorum-sensing molecule C12 was reported to reduce TNF-α secretion by various cell types (13, 20, 29, 30). To assess C12 activity in RAW264.7 macrophages, TNF-α levels were

measured following stimulation with LPS and were found to decline by 40% in the presence of 50 μM C12 (Fig. 1). Because C12 may cause apoptosis at that concentration (11, 14, 18), we examined whether C12 was cytotoxic for the RAW264.7 cell line and found that cell viability was not affected by C12 during the experimental time-frame (data not shown).

C12 increases LPS-induced IL-10 secretion in cultured and primary macrophages

We found that LPS-induced production of IL-10 was substantially increased in the presence of C12 (50 μM) over a 5 h incubation (Fig. 2A). A titration experiment demonstrated that, although C12 alone did not affect IL-10 production in resting macrophages (data not shown), it dose dependently enhanced LPS-induced production of IL-10 up to 3.2-fold at 50 μM and 4.4-fold at 100 μM (Fig. 2B). The apparent EC_{50} of 20 μM for IL-10 stimulation (Fig. 2B) is identical to the EC_{50} reported for suppression of TNF-α production by C12 in whole blood (13). We then explored the magnitude of synergism between LPS and C12 by measuring the effect of increasing C12 concentrations on IL-10 levels, simultaneously induced by increasing LPS concentrations. Fig. 2C shows that costimulation of the macrophages with LPS and C12 (50 μM) increased IL-10 production 5.4-, 3.4-, and 3.0-fold relative to LPS alone at concentrations of 1, 10, and 100 ng/ml, respectively. Thus, the maximal C12-dependent fold increase in IL-10 production was obtained with the lowest LPS concentration (1 ng/ml), which, by itself, induced IL-10 only minimally. This result corroborates the synergistic nature of IL-10 production in macrophages costimulated by LPS and C12. The stimulation of IL-10 expression also reinforced the observation that suppression of TNF-α production did not result from C12 cytotoxicity. We then used mouse peritoneal macrophages to verify this putative anti-inflammatory effect of C12 in a more physiological setting of primary macrophages. Fig. 3A shows that nonelicited LPS-stimulated peritoneal macrophages produce and secrete 3.7- and 5.0-fold more IL-10 in response to C12 present at concentrations of 20 and 50 μM, respectively. Our results are the first indication, to our knowledge, that C12 may limit inflammation by upregulating the anti-inflammatory cytokine IL-10.

C12 modulates TLR4- and TLR7-stimulated cytokine production

Our next objective was to determine whether C12 specifically affects LPS signaling or generally imparts an anti-inflammatory effect on stimulated macrophages, regardless of the identity of the TLR agonist. To this end, we compared the effects of C12 on macrophages stimulated by either the TLR4 agonist LPS or the synthetic TLR7 agonist imiquimod. As shown in Fig. 3B, although

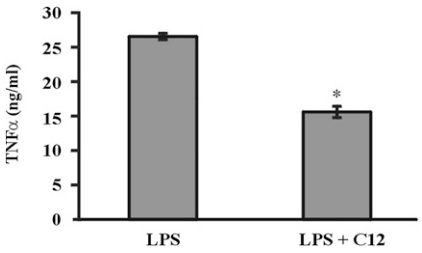

FIGURE 1. C12 reduces LPS-induced TNF-α secretion. Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with LPS (10 ng/ml) in the presence or absence of C12 (50 μM). TNF-α release to the medium was measured by ELISA. Values represent the mean ± SD ($n = 6$). C12 alone had no effect on background TNF-α level in unstimulated cells (<0.6 ng/ml). The experiment was carried out three times with similar results. *$p < 0.005$ versus treatment with LPS alone.

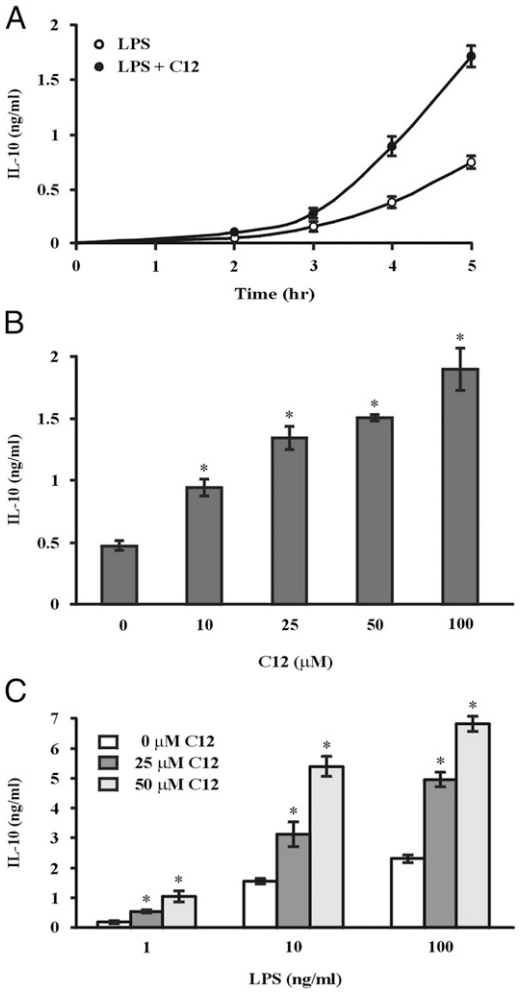

FIGURE 2. C12 increases LPS-induced IL-10 secretion in RAW264.7 macrophages. (**A**) Mouse macrophage RAW264.7 cells were incubated at 37°C for 2–5 h with LPS (10 ng/ml) in the presence or absence of C12 (50 μM). $p < 0.02$, cells treated with LPS + C12 versus LPS alone, at each time point. (**B**) Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with LPS (10 ng/ml) and C12 (10–100 μM). *$p < 0.002$ versus treatment with LPS alone. (**C**) Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with LPS (1–100 ng/ml) and C12 (25–50 μM). *$p < 0.02$ versus treatment with LPS (at the same concentration) alone. (A–C) IL-10 release to the medium was measured by ELISA. Values represent the mean ± SD ($n = 6$). IL-10 production in resting cells or by C12 alone was undetectable (<40 pg/ml). All experiments were carried out three times with similar results.

the induction of IL-10 by the two TLR agonists alone differed, C12 increased IL-10 production stimulated by LPS or by imiquimod with similar relative efficacy (5–6-fold) and potency. These results suggest that C12 can manifest its immunosuppressive activity through facilitating the expression of the anti-inflammatory cytokine IL-10 in stimulated immune cells, independent of which specific TLR is activated.

C12 upregulates IL-10 transcription in LPS-stimulated macrophages

Following the finding that C12 increases IL-10 induction, we examined the underlying mechanism of this activity, first by measuring IL-10 mRNA levels using quantitative real-time PCR. We found that C12 (50 μM) increased IL-10 mRNA levels in LPS-stimulated cells up to 3.5-fold, but it had no effect on resting cells (Fig. 4A). These mRNA data are in good agreement with our data from the IL-10 protein analysis (Fig. 2) and suggest that C12 either

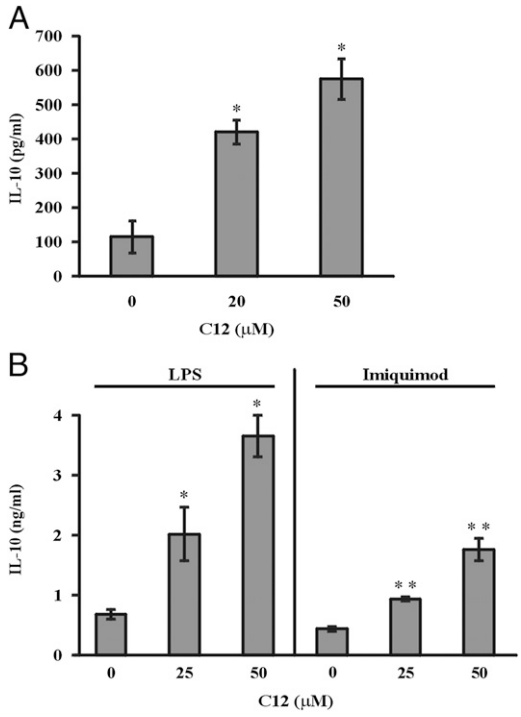

FIGURE 3. C12 increases TLR-dependent IL-10 secretion in cultured and primary macrophages. (**A**) C57BL/6 mouse peritoneal macrophages were incubated at 37°C for 24 h with LPS (100 ng/ml) in the presence or absence of C12 (20–50 μM). IL-10 release to the medium was measured by ELISA. Values represent the mean ± SD (n = 6). IL-10 production in resting cells was undetectable (<40 pg/ml). *$p < 0.008$, versus treatment with LPS alone. (**B**) Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with the TLR4 agonist LPS (10 ng/ml) or the TLR7 agonist imiquimod (10 μM), in the presence or absence of C12 (25–50 μM). IL-10 release to the medium was measured by ELISA. Values represent the mean ± SD (n = 6). IL-10 production in resting cells or by C12 (100 μM) alone was undetectable (<40 pg/ml). The experiment was carried out three times with similar results. *$p < 0.03$ versus treatment with LPS alone, **$p < 0.004$ versus treatment with imiquimod alone.

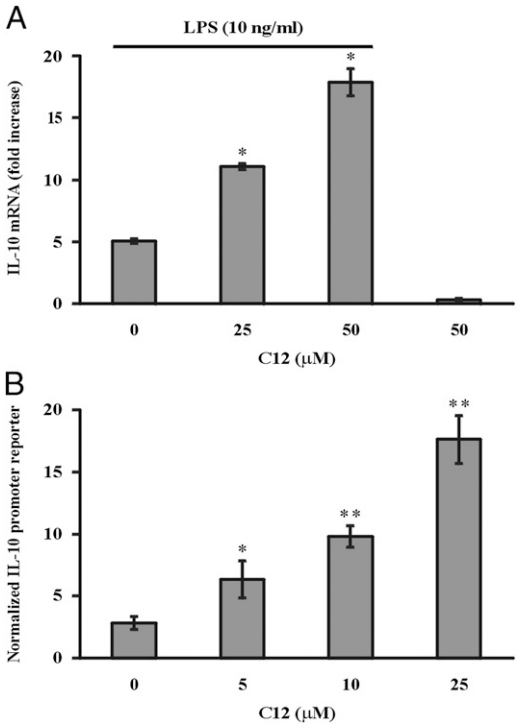

FIGURE 4. C12 upregulates LPS-induced IL-10 transcription. RAW264.7 macrophages were stimulated with LPS (10 ng/ml) in the presence of the indicated concentrations of C12 for 5 h at 37°C. (**A**) Total RNA was isolated from the cells, and IL-10 mRNA levels were assessed by real-time PCR. The intensity of IL-10 mRNA in unstimulated cells, normalized by actin mRNA, was set to 1. Values represent the mean ± SD (n = 3). The results are representative of three independent experiments. *$p < 0.001$, versus treatment with LPS alone. (**B**) RAW264.7 macrophages were transiently transfected for 24 h at 37°C with a reporter gene construct that codes for firefly luciferase under regulation of the mouse IL-10 promoter, as well as with a *Renilla* luciferase construct for normalization. Following treatment with LPS and C12, as described above, luciferase activity assay was performed. Values represent the mean ± SD (n = 3) of values normalized against *Renilla* luciferase activity, relative to unstimulated control cells and to endogenous IL-10 (measured by ELISA), multiplied by 1000. The experiment was carried out three times with similar results. *$p < 0.02$, **$p < 0.002$ versus treatment with LPS alone.

upregulates IL-10 transcription or downregulates IL-10 mRNA degradation. To distinguish between the two alternatives, we measured the effect of C12 in an IL-10 promoter luciferase reporter assay. We found that C12 dramatically increased LPS-induced IL-10 reporter activity of the full IL-10 promoter up to 6-fold at 25 μM (Fig. 4B). These results indicate that C12 synergizes with TLR signaling in transcription of the IL-10 gene.

C12 modulates activity of p38, eIF2α, and NF-κB in RAW264.7 macrophages

Previous reports demonstrated that addition of C12 to a wide variety of cell types, including bone marrow–derived macrophages, results in phosphorylation and subsequent modulation of activity of several key signaling proteins that are also regulated by TLR agonists, such as MAPK p38 (11, 13) and eIF2α (11). Furthermore, C12 addition to LPS- or TNF-α–stimulated cells results in downregulation of IκBα and subsequent disruption of NF-κB signaling (13). We examined whether these signaling proteins are modulated by C12 (50 μM) and/or LPS (100 ng/ml) during a 15-min or 2 h incubation, also in RAW264.7 macrophages. Fig. 5A demonstrates distinct temporal patterns of regulation of the examined signaling proteins. Both C12 alone and LPS alone stimulated rapid (15 min) phosphorylation of p38, but only C12 also significantly stimulated p38 phosphorylation over a prolonged time period of 2 h. The phosphorylation of eIF2α on serine 51, which is known to result in attenuation of protein synthesis (39), was observed at both time points only in cells exposed to C12; it was not observed at all in cells exposed to LPS alone. In contrast to the rapid effect of C12 on phosphorylation of p38 and eIF2α, IκBα levels were not modulated by C12 at the 15 min time point, whereas at the later time point tested (2 h) C12 partially decreased IκBα levels in resting cells and even completely eliminated it in LPS-stimulated cells. These effects of C12 on IκBα turnover could theoretically result either from enhanced degradation or from inhibition of its expression. The lack of effect of C12 alone in 15 min, together with its more dramatic effect at 2 h in the presence of LPS (relative to resting cells), suggests that C12 blocked the resynthesis of IκBα following the rapid LPS-stimulated degradation. The unnatural stereoisomer control, C12R, had no effect in these assays. These findings raise the possibility that C12 enhances LPS-induced IL-10 transcription in macrophages by extending the duration and extent of activation of p38-stimulated transcription factors and/or NF-κB.

The dramatic effect of C12 on IκBα resynthesis in LPS-stimulated RAW264.7 macrophages (Fig. 5A), together with the reported role of the transcription factor NF-κB in IL-10 expression by LPS-stimulated RAW264.7 macrophages (40, 41), prompted us to explore NF-κB DNA-binding activity in cells costimulated with LPS and C12. A gel-

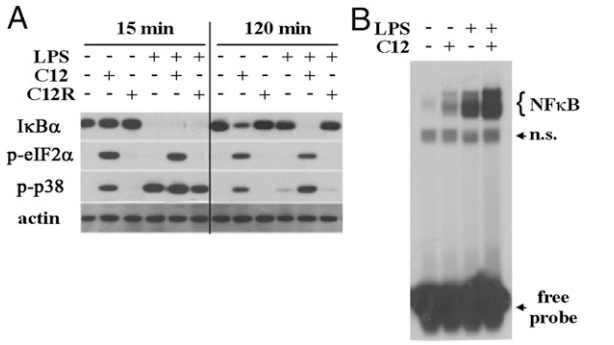

FIGURE 5. C12 modulates NF-κB and p38 signaling in RAW264.7 macrophages. (**A**) Western blot analysis of IκBα and phosphorylated forms of eIF2α (p-eIF2α) and p38 (p-p38) in whole-cell lysates prepared from RAW264.7 cells following treatment at 37°C for 15 or 120 min with LPS (100 ng/ml), C12 (50 μM), its unnatural stereoisomer control C12R (50 μM), or their combination, as indicated. Western blot analysis for actin was used as a loading control. The results are representative of at least three independent experiments for each analyzed protein. (**B**) In parallel, nuclear extracts were prepared after 2 h of treatment and analyzed by EMSA for binding activity to a consensus NF-κB sequence probe. A typical autoradiograph representing one of three independent experiments is shown.

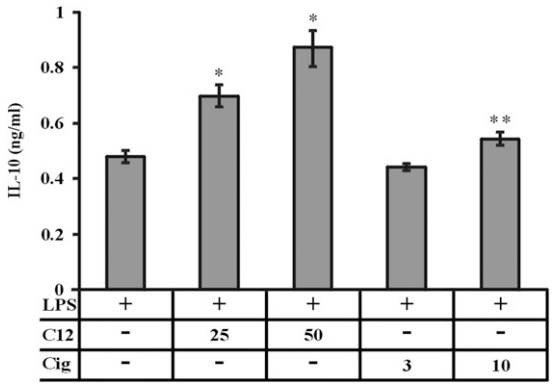

FIGURE 6. C12 increases IL-10 production in a PPARγ-independent mechanism. Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with LPS (10 ng/ml), in the presence or absence of either C12 (25–50 μM) or the PPARγ agonist ciglitazone (Cig; 3-10 μM). IL-10 release to the medium was measured by ELISA. Values represent the mean ± SD ($n = 6$). IL-10 production in the absence of LPS was undetectable (<40 pg/ml). The experiment was carried out three times with similar results. *$p < 0.0002$, **$p < 0.003$ versus treatment with LPS alone.

shift analysis revealed that LPS stimulated the binding of a consensus NF-κB sequence to at least two distinct protein complexes present in nuclear extracts from the macrophages and that, in RAW264.7 cells costimulated with LPS and C12, there was a substantial increase in the intensity of these DNA-bound NF-κB complexes, in particular of the upper band (Fig. 5B). Specificity of binding to the NF-κB sequence was demonstrated by competition with unlabeled oligonucleotides (data not shown). C12 alone only modestly stimulated DNA binding of NF-κB complexes (Fig. 5B), consistent with its lack of effect on IL-10 expression in resting cells (data not shown) and with its relatively modest effect on IκBα levels in resting cells (Fig. 5A). Thus, these findings suggest that C12 amplifies TLR4-mediated induction of IL-10 expression in LPS-stimulated macrophages through a transcriptional mechanism regulated by NF-κB signaling.

C12 targets distinct intracellular pathways upstream of TNF-α and IL-10 expression

C12 was suggested to act via the intracellular lipid receptor PPARγ (15, 17). Therefore, we examined whether the activities of C12 could be imitated by the PPARγ agonist ciglitazone. We found that C12, but not ciglitazone, was able to increase LPS-stimulated IL-10 release (Fig. 6), in contrast to the observation that both C12 and ciglitazone inhibited LPS-stimulated TNF-α release (data not shown). These results suggest that although the inhibition of TNF-α expression by C12 may occur via PPARγ, the increase in IL-10 expression occurs via a PPARγ-independent mechanism.

Discussion

The bacterial quorum-sensing signaling molecule C12 regulates the production of virulence factors in a cell density–dependent manner. In addition, multiple reports documented C12's effects on the production of proinflammatory cytokines by host cells. We report in this article that, in addition to its suppressive effect on the production of the proinflammatory cytokine TNF-α by LPS-stimulated macrophages, C12 amplified IL-10 mRNA and protein production via a transcriptional mechanism. C12 did not increase IL-10 expression in resting cells, and its positive effect was synergistic with both the TLR4 ligand LPS and the TLR7 ligand imiquimod. The finding that amplification of TLR-induced IL-10 expression by LPS does not depend on the type of TLR ligand is consistent with previous reports by Kravchenko et al., showing that TLRs are not required for both the agonistic (11) and the anti-inflammatory activities (13) of C12 in macrophages and other cell types. Thus, the data suggest that C12 boosts the minimal IL-10 expression occurring in cells stimulated by TLR ligands through a complementary mechanism that does not involve physical interaction between C12 and a specific TLR.

Previous contradictory reports claimed that C12 either promotes (14, 15, 23–27) or suppresses (13, 20, 28–30) the production of proinflammatory mediators. A recent review suggested that the positive or negative immune response depends on C12 concentration being high or low, respectively (7). Alternatively, we note that induction of proinflammatory mediators by C12 was demonstrated only in resting cells, whereas suppression was obviously observed only in stimulated cells (i.e., by TLR agonists, such as LPS), because TNF-α is not significantly produced in resting cells. We found that C12 suppresses LPS-stimulated TNF-α production in RAW264.7 macrophages (Fig. 1). This anti-inflammatory activity of C12 is consistent with multiple previous reports (13, 20, 29, 30). In contrast, in vivo neutralization of C12 during *P. aeruginosa* infection by prior immunization resulted in TNF-α suppression (14). However, the suppressive effect of C12 on TNF-α expression in that study is likely to be indirect, as a result of the inhibition of the quorum-sensing activity of C12 and concomitant reduction in virulence factor secretion by the bacteria. It should be noted that the maximum magnitude of TNF-α suppression by C12 in LPS-stimulated RAW264.7 cells was 40% (Fig. 1), significantly lower than the quantitative suppression in primary cells (13, 20, 29, 30). This is consistent with the report that primary cells are more sensitive to C12 than are immortalized cells (31).

The physiological relevance of the proinflammatory effects of C12 on resting cells (14, 15, 23–27) is not obvious. These effects are typically observed in vitro at 50–100 μM, whereas C12 has been detected in vivo at a concentration of 1–20 nM in cystic fibrosis patient sputum (42) and 1–2 μM in a murine *P. aeruginosa* model of acute lung infection (14). Although a planktonic culture of *P. aeruginosa* secretes C12 at low micromolar concentrations (43), C12 may reach concentrations as high as 600 μM in biofilms (44). This is clinically relevant because biofilms are formed in vivo (e.g., in the lungs of cystic fibrosis patients) (45, 46). Taken together, C12 concentrations required for significant induction of a proinflammatory response may exist in vivo only in the vicinity of a biofilm; thus, it is likely that the host cells that are exposed to these high C12 con-

centrations are also simultaneously stimulated by the bacterial LPS and, therefore, are downregulated by C12 rather than stimulated by it. The estimated EC_{50} that we measured for the increase in LPS-induced production of IL-10 is 20 μM (Fig. 2B), as was also measured for the suppression of LPS-induced production of TNF-α (13, 20, 29, 30) and IL-12 (28). C12 is required during the chronic phase of *P. aeruginosa* infection, although its quorum-sensing activity is dispensable at that stage, as is evident from the occurrence of mutations at the bacterial receptor *LasR* but not at the synthase gene *LasI* (22). Therefore, it is conceivable that C12 promotes establishment of a chronic infection by simultaneously upregulating the anti-inflammatory cytokine IL-10 (Figs. 2, 3) and downregulating the proinflammatory cytokine TNF-α (Fig. 1).

The similar time course for TNF-α suppression and IL-10 enhancement by C12 (as early as these cytokines could be detected, i.e., 2 h) suggests that these two effects occur independently. In contrast, IL-12, another critical proinflammatory cytokine, was stimulated by LPS and suppressed by C12 in dendritic cells at a more distant time point (15 h) (28). Therefore, it is possible that the late IL-12 modulation is secondary to C12-related modulation of an early secreted cytokine. IL-10 is instrumental in the resolution of inflammation, and it acts, in part, by downregulating the production of IL-12 (47). Although in the above report, C12 suppressed IL-12 without altering IL-10 secretion from the dendritic cells (28), it is conceivable that the increased IL-10 secretion from macrophages would suppress in vivo expression of IL-12. Moreover, IL-10 downregulates a multitude of other important proinflammatory cytokines (48), including, to name just a few, TNF-α (49), IFN-γ (50), IL-1α, IL-1β, and IL-6 (51, 52). Therefore, by synergistically increasing LPS-stimulated IL-10 expression in macrophages, C12 is predicted to indirectly suppress the release of an array of major proinflammatory cytokines in vivo.

Similar to opportunistic pathogens, such as *P. aeruginosa*, obligate pathogens also would benefit from information regarding their population density and regulate their virulence accordingly. While the quorum-sensing systems of opportunistic pathogens have been studied extensively, obligate pathogens have been less studied. Two obligate pathogens that show similarity to *P. aeruginosa* in terms of signaling molecule and receptor homology are *Burkholdeira mallei* and *Brucella melitensis*; they use *N*-octanoyl homoserine lactone (53) and *N*-dodecanoyl homoserine lactone (54) as their quorum-sensing signals, respectively. However, in both cases it is not clear exactly how these pathogens use their quorum-sensing systems to regulate virulence. Interestingly, *N*-dodecanoyl homoserine lactone, which is particularly reminiscent of *P. aeruginosa*–derived C12 (lacking the 3-oxo group present in C12), stimulates NF-κB activity and modestly increases TNF-α production in resting RAW264.7 macrophages (12), consistent with the immunomodulatory effects of C12 in the absence of LPS (as discussed above). Therefore, it is conceivable that bacteria that use *N*-dodecanoyl homoserine lactone for quorum sensing, such as the obligate pathogen *B. melitensis*, would also manipulate the host immune system as does *P. aeruginosa* via C12.

Two reports suggested that C12 modulates the activity of PPARγ in alveolar epithelial cell lines (15, 17). Jahoor et al. (15) showed that, at high concentrations (≥50–100 μM), C12 acted as an antagonist in a PPARγ reporter assay, and that a PPARγ agonist blocked the proinflammatory effects of C12. Cooley et al. (17) demonstrated that, at low μM concentrations, C12 acts as a weak partial agonist of PPARγ. Because PPARγ agonists suppress TNF-α production (55), it is possible that PPARγ mediates the anti-inflammatory effect of C12. Notably, a mechanism involving the nuclear receptor PPARγ would be reminiscent of the quorum-sensing activity of C12 in *P. aeruginosa*, mediated by the direct binding of C12 to the bacterial transcriptional activator LasR (56). However, we found that, although a PPARγ agonist was more efficient than was C12 at TNF-α suppression (data not shown), IL-10 production was stimulated by C12 but not significantly by the PPARγ agonist (Fig. 6). The high efficacy of PPARγ in transcription modulation of TNF-α, but not of IL-10, is consistent with a previous report (57). These results indicate that C12 increases LPS-induced IL-10 production in a PPARγ-independent mechanism.

Several reports identified the transcription factor NF-κB as a central mediator of C12 effects on host cells. Kravchenko et al. (13) showed that C12 inhibits the LPS-induced expression of NF-κB–responsive genes, such as TNF-α, in macrophages. Notably, the rapid LPS-induced degradation of IκBα, which enables NF-κB nuclear translocation, was not inhibited by C12 (Fig. 5A). Furthermore, C12 blocked IκBα resynthesis via two possible mechanisms: by stimulating phosphorylation of eIF2α, as we demonstrated in both RAW264.7 macrophages (Fig. 5A) and bone marrow–derived macrophages (13), leading to translation attenuation of cellular proteins in general (58) and of IκBα specifically (59) and/or by inhibition of IκBα transcription, as demonstrated previously in cells costimulated with C12 and either LPS or TNF-α (13). In accordance with these two mechanisms, IκBα protein expression is completely blocked following 2 h of costimulation with C12 and LPS (Fig. 5A). The time-course of the dramatic downregulation of IκBα resynthesis (Fig. 5A) and the concomitant increase in DNA-binding activity of NF-κB complexes in nuclear extracts of C12-stimulated macrophages (Fig. 5B) indicate that the duration of LPS-stimulated nuclear localization and DNA-binding of NF-κB was prolonged by C12. Thus, if C12 suppresses production of proinflammatory mediators, such as TNF-α, via inhibition of the stimulatory NF-κB p65, it likely occurs at the *trans*-activation level rather than at the nuclear translocation or DNA-binding steps. Alternatively, increased nuclear localization of the inhibitory NF-κB p50 homodimer may also be the mechanism for downregulation of TNF-α transcription (40, 60, 61). Consistently with the above conclusions, data from the groups of Phipps and Iglewski (24–26) indicate that C12 activates NF-κB in various nonimmune cell lines. In two of these reports (24, 25), it was shown that C12 stimulates the nuclear translocation of the NF-κB p65 subunit. The third report (26) showed that induction of cyclooxygenase-2 by C12 was blocked by SN50, a peptide inhibitor of NF-κB p50 subunit nuclear translocation. Of note, NF-κB p50 homodimers were shown to bind the NF-κB enhancer at the mouse IL-10 promoter and to positively regulate transcription (41). Consistent with this, we recently demonstrated that IL-10 transcription is blocked by SN50 and additional specific inhibitors of NF-κB p50 (but not p65) nuclear translocation (40). Taken together, it is possible that the amplification of LPS-induced IL-10 expression reported in this study is mediated by C12 prolonging the activity of NF-κB p50 homodimers. Moreover, this may also be a mechanism for TNF-α suppression by C12, because NF-κB p50 homodimers repress TNF-α transcription during LPS tolerance (40, 60, 61).

Stimulation of the p38 pathway is critical for LPS-induced IL-10 expression in RAW264.7 macrophages (62). Therefore, it is of relevance that C12 activates the p38 kinase in RAW264.7 macrophages (Fig. 5A), as well as in primary macrophages and other cell types (11, 13, 27). Even more importantly, although LPS transiently stimulates the phosphorylation-dependent activation of p38, the addition of C12 to LPS-stimulated macrophages, either primary (13) or cultured RAW264.7 (Fig. 5A), significantly prolonged p38 activation. Thus, the extended period of p38 activation may also be responsible for enhanced IL-10 production in macrophages costimulated with LPS and C12.

Some of the effects of C12 on epithelial cells are mediated by the calcium pathway (23, 31, 32). Schwarzer et al. (32) showed that the reported cellular response to C12 exposure was mediated by a decrease in calcium concentration in the endoplasmic reticulum and a subsequent store-operated cAMP production, rather than by a C12-triggered increase in intracellular calcium. To the best of our knowledge, it has not been investigated whether C12 can elicit a similar response in macrophages. Yet, we note that an increase in the intracellular level of cAMP leads to synergistic IL-10 expression in LPS-stimulated macrophages (63), as well as to suppression of the proinflammatory cytokine TNF-α (62, 64–67), echoing C12's effects described in this report.

C12 was also reported to induce DNA-binding activity of the transcription factor AP-2 in an epithelial cell line (24). The human IL-10 promoter includes four AP-2 enhancers (68); therefore, AP-2 may be considered another candidate for mediating the effect of C12 on IL-10 transcription in LPS-stimulated macrophages.

Upon secretion from the bacteria, C12 indirectly promotes inflammation by stimulating the production of virulence factors (56), but it also directly inhibits inflammation via TNF-α suppression and IL-10 induction in LPS-stimulated macrophages, as shown in this study. The BALB/c mice strain is resistant to *P. aeruginosa* lung infection because of a harsh inflammatory response that culminates in bacterial clearance. In contrast, the susceptible DBA/2 mice strain is defective in TNF-α production; therefore, it mounts a milder inflammatory response that does not suffice for bacterial clearance (69). Further, it was shown that TNF-α aids in bacterial clearance by enhancing the recruitment of neutrophils (69) and stimulating their phagocytic activity (70). In contrast, IL-10 suppresses the phagocytic activity of neutrophils (71). Furthermore, C12 at low concentrations causes apoptosis of neutrophils (72); subsequent phagocytosis of apoptotic neutrophils by macrophages imposes an anti-inflammatory phenotype, which is highlighted by an increase in the anti-inflammatory cytokine IL-10, in parallel with a decrease in proinflammatory cytokines, such as TNF-α (73). Therefore, in vivo C12 may increase IL-10 production in macrophages by both direct and indirect mechanisms. Further research into the mechanism by which C12 diverts macrophages to an anti-inflammatory phenotype, highlighted by reduced TNF-α production and enhanced IL-10 production, may lead to the design of selective C12 antagonists that would specifically block the activity of C12 in eukaryotic cells and, therefore, would enable the host to overcome an infection before it becomes chronic.

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Disclosures

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