The bacterial quorum-sensing signal molecule N-3-oxo-dodecanoyl-L-homoserine lactone reciprocally modulates pro- and anti-inflammatory cytokines in activated macrophages

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Abstract

The bacterial N-3-oxo-dodecanoyl-L-homoserine lactone (C12) has critical roles in both inter-bacterial communication and inter-kingdom signaling. The ability of C12 to down-regulate production of the key pro-inflammatory cytokine tumor necrosis factor α (TNFα) 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 TNFα suppression, C12 amplifies production of the major anti-inflammatory cytokine interleukin-10 (IL-10) in lipopolysaccharide (LPS)-stimulated murine RAW264.7 macrophages as well as peritoneal macrophages. Furthermore, C12 increases IL-10 mRNA level 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 NFκB DNA-binding level, and prolonged p38 MAP kinase phosphorylation in the RAW264.7 macrophages, suggesting that increased transcriptional activity of NFκB and/or p38-activated transcription factors serves to up-regulate 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 down-regulate immune responses in order to flourish and establish a chronic infection.

Introduction

Pseudomonas aeruginosa (*P. 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 immuno-compromised 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 (hereafter 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 has been 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 immuno-modulatory 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 somewhat inconsistent. While several studies suggest that C12 enhances a pro-inflammatory 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 have shown that LPS-induced production of the key pro-inflammatory cytokine TNFα is suppressed in the presence of C12 (13, 20, 29, 30). Yet, other studies have shown that C12 failed to affect TNFα production in resting cells (11, 16) or in LPS-stimulated cells (16). Immunization of mice with C12-protein conjugate reduces TNFα levels and increases survival during a subsequent challenge with *P. aeruginosa*, suggesting that C12 has a pro-inflammatory effect (14). However, the protocol of that particular experiment does not distinguish between the quorum sensing role of C12 (e.g. stimulation of virulence factors production) and its direct immuno-modulatory effects. The mechanism of C12 effects on host cells has been suggested to involve modulation of the transcriptional activity of either NFκB (13, 24, 25) or peroxisome proliferator-activated receptor gamma (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, has demonstrated C12-dependent reduction of TNFα and increase of IL-10 production in a co-culture of dendritic cells and T cells, but the secreted cytokine levels were at 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 TNFα and IL-10 in RAW264.7 macrophages. These experiments revealed that an inhibitory effect of C12 on LPS-induced production of TNFα positively correlated 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 NFκB and/or p38 rather than PPARγ.

Materials & Methods

Reagents and plasmid

Lipopolysaccharide (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 Gibco. BSA was purchased from Amresco (Solon, OH). ELISA reagents sets for TNFα 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. Smale (34). The IL-10 promoter reporter plasmid was amplified using DH10B bacteria (Invitrogen, Carlsbad, CA), and purified using Endofree Plasmid Maxi Kit (Qiagen, Hamburg, Germany). HD-fugene 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 (Hamburg, Germany). The synthesis and purification of C12 was carried out as previously described (35), and purity was confirmed by LC-MS and NMR 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 time of roughly 10 hours, which is far beyond the incubation time used in our experiments.

Cell culture

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

Animal care

Male C57BL/6 mice (12 weeks) were obtained from Harlan (Israel). Animal care and experimentation was carried out in accordance with TAU 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 $1.5 \cdot 10^5$ cells per well, in culture medium supplemented with 5% FBS, up to a confluence of 90%. The culture medium was replaced 2 h before treatment in order to avoid an artifact on signaling caused by medium replacement (36). The cells were stimulated with 10 ng/ml LPS and/or C12 (10 - 100 μM) at 37°C for 2-5 h. Upon termination, media was collected for cytokine concentration assessment and fresh media was added for measurement of cell viability by the XTT method, according to the manufacturer's instructions. IL-10 and TNFα 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, Vermont). The samples were stored at -80°C until used. All experiments were repeated as least three times.

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

Non-elicited C57BL/6 mouse peritoneal exudate cells were harvested, washed, re-suspended in culture medium supplemented with 10% FBS, and seeded in a 96-well culture plate (0.2 ml/well) at $5 \cdot 10^5$ cells/well. Following incubation of 24 h at 37°C, non-adherent 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 $3 \cdot 10^5$ cells per well, in culture medium supplemented with 10% FBS. The cells were then co-transfected for 24 h with 0.6 μg of the IL-10 promoter reporter plasmid and 0.2 μg of herpes simplex virus thymidine kinase promoter TK-renilla luciferase (for normalization), that were initially incubated with 2.4 μl HD-fugene 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 hr, 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 6-well culture plate at $8 \cdot 10^5$ cells per 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 of 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:

Gene	Forward primer	Reverse primer
Actin	CTTTGCAGCTCCTTCGTTGC	ACGATGGAGGGGAATACAGC
IL-10	CAGGGATCTTAGCTAACGGAAA	GCTCAGTGAATAAATAGAATGGGAAC

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 the data were analyzed using Student's t-test wherever applicable. In all cases, differences of $p < 0.05$ were considered to be significant. EC_{50} value was calculated by non-linear regression curve fitting, using SigmaPlot software.

Results

C12 inhibits LPS-induced TNFα secretion in RAW264.7 macrophages

The bacterial quorum sensing molecule C12 has been previously reported to reduce (13, 20, 29, 30) TNFα secretion by various cell types. To assess C12 activity in RAW264.7 macrophages, TNFα levels were measured following stimulation with LPS, and were found to decline by 40% in the presence of 50 μM C12 (Fig. 1). As 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 elevates 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 while 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 TNFα 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 co-stimulation of the macrophages with LPS and C12 (50 μM) elevated IL-10 production 5.4-fold, 3.4-fold 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 of IL-10 production was obtained with the lowest LPS concentration (1 ng/ml), which by itself only minimally induced IL-10. This result corroborates the synergistic nature of IL-10 production in macrophages co-stimulated by LPS and C12. The stimulation of IL-10 expression also reinforced the observation that suppression of TNFα production did not result from C12 cytotoxicity. We then used mouse peritoneal macrophages to verify this putative anti-inflammatory effect of C12 also in a more physiological setting of primary macrophages. Figure 3A shows indeed that non-elicited LPS-stimulated peritoneal macrophages produce and secrete 3.7-fold and 5.0-fold more IL-10 in response to C12 present at concentrations of 20 μM and 50 μM, respectively. Our results are the first indication, as far as we know, that C12 may limit inflammation by up-regulating 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, while the induction of IL-10 by the two TLR agonists alone differed, C12 elevated 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 immuno-suppressive activity through facilitating expression of the anti-inflammatory cytokine IL-10 in stimulated immune cells, independent of which specific TLR is activated.

C12 up-regulates IL-10 transcription in LPS-stimulated macrophages

Following the finding that C12 elevates IL-10 induction, we turned to examine the underlying mechanism of this activity, at 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 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 up-regulates IL-10 transcription, or down-regulates 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 elevated 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 NFκB in RAW264.7 macrophages

Previous reports have demonstrated that addition of C12 to a wide variety of cell types, including bone marrow-derived macrophages (BMDM), results in phosphorylation and subsequent modulation of activity of several key signaling proteins that are also regulated by TLR agonists, such as the mitogen-activated protein kinase (MAPK) p38 (11, 13) and the eukaryotic translation initiation factor 2α (eIF2α) (11). Furthermore, C12 addition to LPS- or TNFα-stimulated cells results in down-regulation of the inhibitor of NFκB alpha (IκBα), and subsequent disruption of NFκB signaling (13). We now 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. Figure 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 significantly stimulated p38 phosphorylation also over a prolonged time period of 2 hr. 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, but 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, while at the latter time point tested (2 hr) - 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 re-synthesis 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 NFκB.

The dramatic effect of C12 on IκBα re-synthesis in LPS-stimulated RAW264.7 macrophages (Fig. 5A), together with the reported role of the transcription factor NFκB in IL-10 expression by LPS-stimulated RAW264.7 macrophages (40, 41), prompted us to explore NFκB DNA-binding activity in cells co-stimulated with LPS and C12. A gel-shift analysis revealed that LPS stimulated the binding of a consensus NFκB sequence to at least two distinct protein complexes present in nuclear extracts from the macrophages, and that in RAW264.7 cells co-stimulated with LPS and C12 there was a substantial increase in the intensity of these DNA-bound NFκB complexes, in particular of the upper band (Fig. 5B). Specificity of binding to the NFκB sequence was demonstrated by competition with unlabeled oligonucleotides (data not shown). C12 alone only modestly stimulated DNA binding of NFκB 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 NFκB signaling.

C12 targets distinct intra-cellular pathways upstream to TNFα and IL-10 expression

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

Discussion

The bacterial quorum sensing signaling molecule C12 regulates production of virulence factors in a cell density-dependent manner. In addition, multiple reports have documented C12 effects on production of pro-inflammatory cytokines by host cells. We report here that in addition to its suppressive effect on production of the pro-inflammatory cytokine TNFα by LPS-stimulated macrophages, C12 also 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 the 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 and coworkers, 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 which does not involve physical interaction between C12 and a specific TLR.

Previous contradictory reports have claimed that C12 either promotes (14, 15, 23-27) or suppresses (13, 20, 28-30) production of pro-inflammatory 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 pro-inflammatory mediators by C12 has been demonstrated only in resting cells, whereas suppression was obviously observed only in stimulated cells (i.e. by TLR agonists such as LPS), as TNFα is not significantly produced in resting cells. We found that C12 suppresses LPS-stimulated TNFα 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 TNFα suppression (14). However, the suppressive effect of C12 on TNFα expression in that study is likely to be indirect, due to the inhibition of the quorum sensing activity of C12 and concomitant reduction of virulence factor secretion by the bacteria. It should be noted that the magnitude of TNFα suppression by C12 in LPS-stimulated RAW264.7 cells was at most 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 immortalized cells (31).

The physiological relevance of the pro-inflammatory 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 (CF) patient sputum (42), and 1-2 μM in a murine *P. aeruginosa* acute lung infection model (14). While a planktonic culture of *P. aeruginosa* secretes C12 at low μM concentrations (43), in biofilms C12 may reach concentrations as high as 600 μM (44). This is clinically relevant as biofilms are formed *in-vivo*, for example in the lungs of CF patients (45, 46). Taken together, C12 concentrations required for significant induction of a pro-inflammatory response may exist *in-vivo* only in the vicinity of a biofilm, and thus, it is likely that the host

cells that are exposed to these high C12 concentrations are also simultaneously stimulated by the bacterial LPS, and are therefore down-regulated by C12 rather than stimulated by it. The estimated EC_{50} 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 TNFα (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 evident from the occurrence of mutations at the bacterial receptor *LasR*, but not at the synthase gene *LasI* (22). It is therefore conceivable that C12 promotes establishment of a chronic infection by simultaneously up-regulating the anti-inflammatory cytokine IL-10 (Figs. 2-3) and down-regulating the pro-inflammatory cytokine TNFα (Fig. 1).

The similar time course for TNFα suppression and IL-10 enhancement by C12 (as early as these cytokines could be detected, i.e. 2 hr) suggests that these two effects occur independently. In contrast, IL-12, another critical pro-inflammatory cytokine, was stimulated by LPS and suppressed by C12 in dendritic cells at a more distant time point of 15 hr (28). It is therefore 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 down-regulating production 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 elevated IL-10 secretion from macrophages would suppress *in-vivo* expression of IL-12. Moreover, IL-10 down-regulates a multitude of other important pro-inflammatory cytokines (48), including, to name just a few, TNFα (49), IFNγ (50), IL-1α, IL-1β and IL-6 (51, 52). Therefore, by synergistically elevating LPS-stimulated IL-10 expression in macrophages, C12 is predicted to indirectly suppress the release of an array of major pro-inflammatory cytokines *in-vivo*.

Similar to opportunistic pathogens, such as *P. aeruginosa*, obligate pathogens would also 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*, that employ N-octanoyl homoserine lactone (53) and N-dodecanoyl homoserine lactone (54) as their quorum sensing signals, respectively. In both cases, however, it is not yet clear how exactly these pathogens use their quorum sensing systems to regulate virulence. Interestingly, N-dodecanoyl homoserine lactone, which is in particular reminiscent of *P. aeruginosa*–derived C12 (lacking the 3-oxo group present in C12), stimulates NFκB activity and modestly elevates TNFα production in resting RAW264.7 macrophages (12), consistent with the immuno-modulatory effects of C12 in the absence of LPS (as discussed above). It is therefore conceivable that bacteria that use N-dodecanoyl homoserine lactone for quorum sensing, such as the obligate pathogen *Brucella melitensis*, would also manipulate the host immune system as *P. aeruginosa* does via C12.

Two reports suggested that C12 modulates the activity of PPARγ in alveolar epithelial cell lines (15, 17). Jahoor et al showed that C12 at high concentrations (50-100 μM and above) acts as an antagonist in a PPARγ reporter assay, and that a PPARγ agonist blocked the pro-inflammatory effects of C12 (15). Cooley at al demonstrated that at low μM concentrations, C12 acts as a weak partial agonist of PPARγ (17). As PPARγ agonists suppress TNFα 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 while a PPARγ agonist was more efficient than C12 at TNFα 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 only of TNFα 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 have identified the transcription factor NFκB as a central mediator of C12 effects on host cells. Kravchenko et al showed that C12 inhibits the LPS-induced expression of NFκB-responsive genes, such as TNFα, in macrophages (13). Notably, the rapid LPS-induced degradation of IκBα which enables NFκB nuclear translocation was not inhibited by C12 (Fig. 5A). Furthermore, C12 blocked IκBα re-synthesis via two possible mechanisms: i) by stimulating phosphorylation of eIF2α, as we have demonstrated in both RAW264.7 macrophages (Fig. 5A) and BMDM (13), leading to translation attenuation of cellular proteins in general (58), and of IκBα specifically (59); and/or ii) by inhibition of IκBα transcription, as was previously demonstrated in cells co-stimulated with C12 and either LPS or TNFα (13). In accordance with these two mechanisms – IκBα protein expression is completely blocked following 2 h of co-stimulation with C12 and LPS (Fig. 5A). The time-course of the dramatic down-regulation of IκBα re-synthesis (Fig. 5A) and the concomitant increase in DNA binding activity of NFκB complexes in nuclear extracts of C12-stimulated macrophages (Fig. 5B), indicates that the duration of LPS-stimulated nuclear localization and DNA-binding of NFκB was prolonged by C12. Thus, if C12 suppresses production of pro-inflammatory mediators such as TNFα via inhibition of the stimulatory NFκB 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 NFκB p50 homodimer may also be the mechanism for down-regulation of TNFα transcription (40, 60, 61). Consistently with the above conclusions, data from the groups of Phipps and Iglewski apparently indicate that C12 activates NFκB in various non-immune cell lines (24-26). In two of these reports it has been shown that C12 stimulates the nuclear translocation of the NFκB p65 subunit (24, 25). The third report showed that induction of cyclooxygenase (COX)-2 by C12 was blocked by SN50, a peptide inhibitor of NFκB p50 subunit nuclear translocation (26). Of note, NFκB p50 homodimers have been shown to bind the NFκB enhancer at the mouse IL-10 promoter, and to positively regulate transcription (41). Consistently, we recently demonstrated that IL-10 transcription is blocked by SN50 and additional specific inhibitors of NFκB p50 (but not p65) nuclear translocation (40). Taken together, it is possible that the amplification of LPS-induced IL-10 expression reported here is mediated by C12 prolonging the activity of NFκB p50 homodimers. Moreover, this may also be a mechanism for TNFα suppression by C12, as NFκB p50 homodimers repress TNFα 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). It is therefore 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, while LPS transiently stimulates the phosphorylation-dependent activation of p38, the addition of C12 to LPS-stimulated macrophages, be that either primary (13) or cultured RAW264.7 (Fig. 5A), significantly prolonged p38 activation. Thus, the extended period of p38 activation may also be accountable for enhanced IL-10 production in macrophages co-stimulated 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 showed that the reported cellular response to C12 exposure was mediated by a decrease in calcium concentration in the ER and a subsequent store-operated cAMP production, rather than by a C12-triggered increase in intra-cellular calcium (32). To date, it has not been investigated whether C12 can elicit a similar response in macrophages. Yet, we note that an increase in the intra-cellular level of cAMP leads to synergistic IL-10

expression in LPS-stimulated macrophages (63), as well as to suppression of the pro-inflammatory cytokine TNFα (62, 64-67), echoing C12 effects described in this report.

C12 has also been 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), and therefore AP-2 may be considered as 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 also directly inhibits inflammation via TNFα suppression and IL-10 induction in LPS-stimulated macrophages, as shown here. The BALB/c mice strain is resistant to *P. aeruginosa* lung infection due to a harsh inflammatory response which culminates in bacterial clearance. In contrast, the susceptible DBA/2 mice strain is defective in TNFα production and therefore mounts a milder inflammatory response that does not suffice for bacterial clearance (69). It has further been shown that TNFα 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 to a decrease in pro-inflammatory cytokines, such as TNFα (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 TNFα 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 will therefore enable the host to overcome an infection before it becomes chronic.

Acknowledgments

Abbreviations used in the article

AHL	acyl homoserine lactone
BMDM	bone marrow-derived macrophages
C12	N-3-oxo-dodecanoyl-L-homoserine lactone
P. aeruginosa	Pseudomonas aeruginosa

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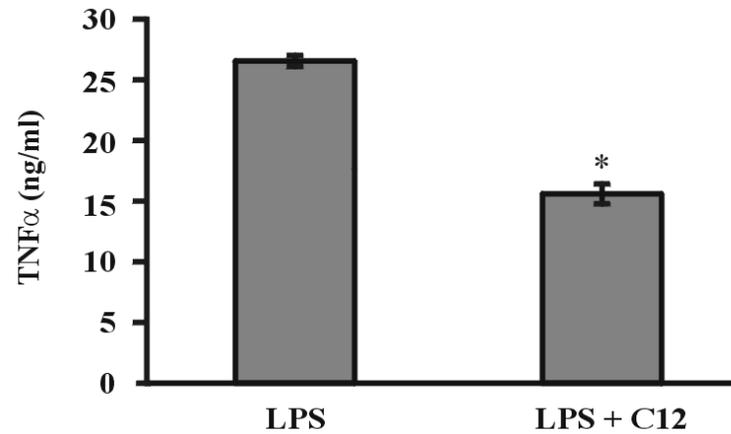
Figure 1. C12 reduces LPS-induced TNFα secretion
Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with LPS (10 ng/ml) in the presence or absence of C12 (50 μM). TNFα release to the medium was measured by ELISA. Each data point represents the mean ± S.D. (n=6). C12 alone had no effect on background TNFα level in unstimulated cells (< 0.6 ng/ml). * $p < 0.005$ relative to treatment with LPS alone. The experiment was carried out 3 times with similar results.
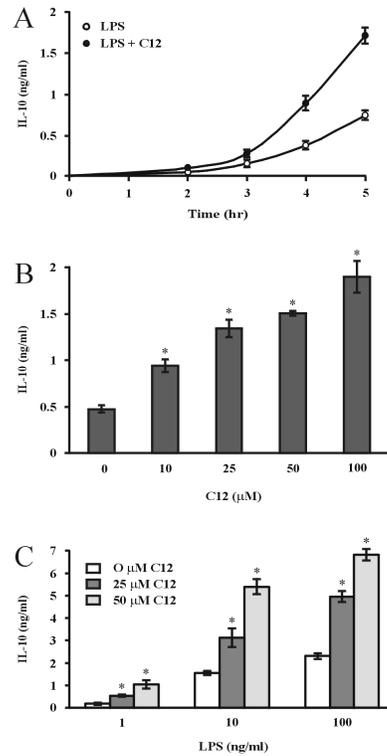
Figure 2. C12 increases LPS-induced IL-10 secretion in RAW264.7 macrophages
Mouse macrophage RAW264.7 cells were incubated at 37°C during a period of: **(A)** 2-5 h
with LPS (10 ng/ml) in the presence or absence of C12 (50 μM). *p* < 0.02 for cells treated
with LPS + C12 vs. LPS alone, at each time point **(B)** 5 h with LPS (10 ng/ml) and C12
(10-100 μM). * *p* < 0.002 relative to treatment with LPS alone. **(C)** 5 h with LPS (1-100
ng/ml) and C12 (25-50 μM). * *p* < 0.02 relative to treatment with LPS (at the same
concentration) alone. **(A-C)** IL-10 release to the medium was measured by ELISA. Each
data point represents the mean ± S.D. (n=6). IL-10 production in resting cells or by C12
alone was undetectable (< 40 pg/ml). All experiments were carried out 3 times with similar
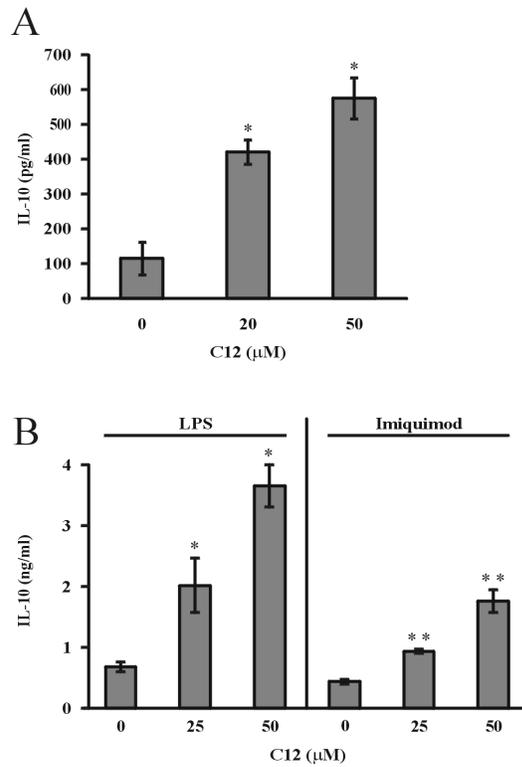results.

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. Each data point represents the mean ± S.D. (n=6). IL-10 production in resting cells was undetectable (< 40 pg/ml). * $p < 0.008$ relative to 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. Each data point represents the mean ± S.D. (n=6). IL-10 production in resting cells or by C12 (100 μM) alone was undetectable (< 40 pg/ml). * $p < 0.03$ relative to treatment with LPS alone, ** $p < 0.004$, relative to treatment with imiquimod alone. The experiment was carried out 3 times with similar results.
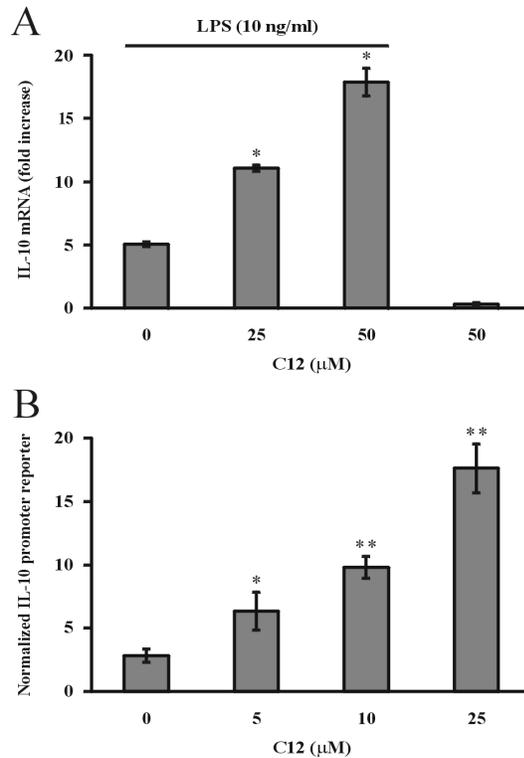
Figure 4. C12 up-regulates LPS-induced IL-10 transcription
RAW264.7 macrophages were stimulated with LPS (10 ng/ml) in the presence of the
indicated C12 concentrations for 5 h at 37°C. **(A)** Total RNA was isolated from the cells,
and IL-10 mRNA levels were assessed by real time PCR. The intensity of IL-10 mRNA in
unstimulated cells, normalized by actin mRNA, was set to 1. Each data point represents the
mean ± S.D. (n=3). * $p < 0.001$ relative to treatment with LPS alone. The results are
representative of 3 independent experiments. **(B)** RAW264.7 macrophages were transiently
transfected for 24 h at 37°C with a reporter gene construct which codes for firefly luciferase
under regulation of the mouse IL-10 promoter, and with a renilla luciferase construct for
normalization. Following treatment with LPS and C12 as described above, luciferase
activity assay was performed. Each data point represents mean ± S.D. (n=3) of values
normalized against renilla luciferase activity, relative to unstimulated control cells and to
endogenous IL-10 (measured by ELISA), multiplied x1000. * $p < 0.02$ or ** $p < 0.002$
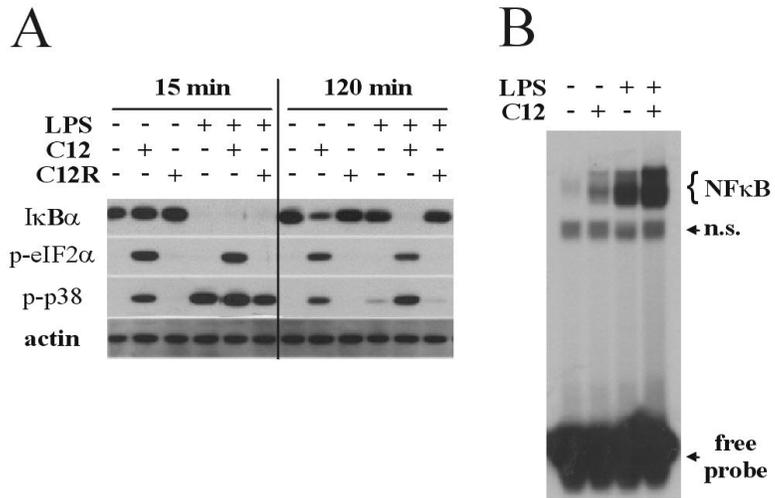relative to treatment with LPS alone. The experiment was carried out 3 times with similar
results.

Figure 5. C12 modulates NFκB 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 RAW274.7 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 NFκB sequence probe. A typical autoradiograph representing one of three independent experiments is shown.
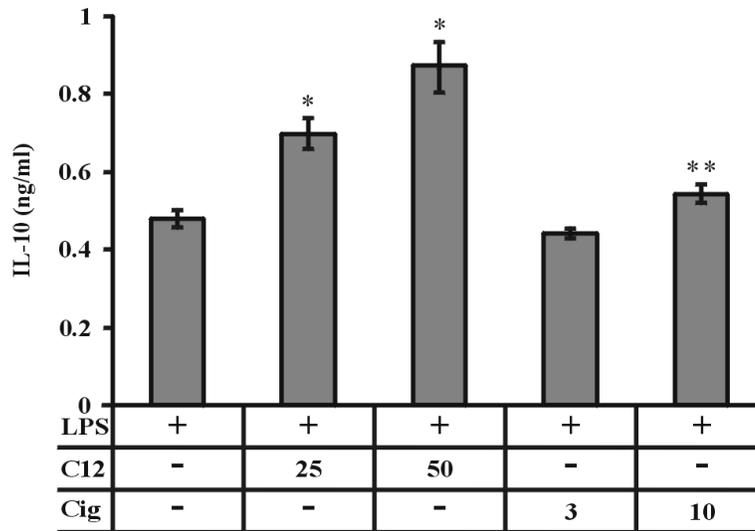
Figure 6. C12 elevates IL-10 production in a PPARγ-independent mechanism
Mouse macrophage RAW264.7 cells were incubated at 37°C for 5 h with LPS (10 ng/ml), in the presence or absence of either C12 (25-50 μM) or the PPARγ agonist ciglitazone (Cig, 3-10 μM). IL-10 release to the medium was measured by ELISA. Each data point represents the mean ± S.D. (n=6). IL-10 production in the absence of LPS was undetectable (< 40 pg/ml). * $p < 0.0002$ or ** $p < 0.003$ relative to treatment with LPS alone. The experiment was carried out 3 times with similar results.