The Quorum Sensing Volatile Molecule 2-Amino Acetophenon Modulates Host Immune Responses in a Manner that Promotes Life with Unwanted Guests

Bandyopadhaya, Arunava, Meenu Kesarwani, Yok-Ai Que, Jianxin He, Katie Padfield, Ronald Gary Tompkins, and Laurence G. Rahme. 2012. The quorum sensing volatile molecule 2-amino acetophenon modulates host immune responses in a manner that promotes life with unwanted guests. PLoS Pathogens 8(11): e1003024.

Published Version
doi:10.1371/journal.ppat.1003024

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:10579395

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
The Quorum Sensing Volatile Molecule 2-Amino Acetophenon Modulates Host Immune Responses in a Manner that Promotes Life with Unwanted Guests

Arunava Bandyopadhaya$^{1,2,3,*}$, Meenu Kesarwani$^{1,2,3,*}$, Yok-Ai Que$^{1,2,3}$, Jianxin He$^{1,2,3}$, Katie Padfield$^{1,3}$, Ronald Tompkins$^{1,3}$, Laurence G. Rahme$^{1,2,3,*}$

$1$ Department of Surgery, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts, United States of America, $2$ Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, United States of America, $3$ Shriners Hospitals for Children Boston, Boston, Massachusetts, United States of America

Abstract

Increasing evidence indicates that bacterial quorum sensing (QS) signals are important mediators of immunomodulation. However, whether microbes utilize these immunomodulatory signals to maintain infection remain unclear. Here, we show that the Pseudomonas aeruginosa QS-regulated molecule 2-amino acetophenone (2-AA) modulates host immune responses in a manner that increases host ability to cope with this pathogen. Mice treated with 2-AA prior to infection had a 90% survival compared to 10% survival rate observed in the non-pretreated infected mice. Whilst 2-AA stimulation activates key innate immune response pathways involving mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and pro-inflammatory cytokines, it attenuates immune response activation upon pretreatment, most likely by upregulating anti-inflammatory cytokines. 2-AA host pretreatment is characterized by a transcriptionally regulated block of c-JUN N-terminal kinase (JNK) and NF-κB activation, with relatively preserved activation of extracellular regulated kinase (ERK) 1/2. These kinase changes lead to CCAAT/enhancer-binding protein (c/EBP) $b$ activation and formation of the c/EBP$β$-p65 complex that prevents NF-κB activation. 2-AA’s aptitude for dampening the inflammatory processes while increasing host survival and pathogen persistence concurs with its ability to signal bacteria to switch to a chronic infection mode. Our results reveal a QS immunomodulatory signal that promotes original aspects of interkingdom communication. We propose that this communication facilitates pathogen persistence, while enabling host tolerance to infection.

Introduction

Host-pathogen interactions are characterized by an antagonistic interplay between bacterial and host factors. The overall success of a pathogen depends on the efficacy of its virulence factors, anti-immune weapons, and the immune status of the host. Secreted microbial products, which include many virulence factors, play a critical role in the outcome of this antagonistic interaction. Bacterial quorum sensing (QS) regulates many of these products [1], [2]. QS is a communication system widely utilized by bacteria to perceive and promote collective behaviors that depend on population density signaling. This cell density-dependent communication system is achieved through the production and regulation of low-molecular-weight, excreted signal molecules [1], [3] as a means for pathogens to activate virulence factors [4] critical for infecting mammals [4–6]. Several such signal molecules are becoming more appreciated recently as important anti-immune weapons and mediators of inter-kingdom antagonistic relations [7].

One of the best characterized QS systems is that of the recalcitrant Gram-negative bacterium Pseudomonas aeruginosa [8], [9]. This opportunistic pathogen defies eradication by antibiotics and is an exemplar of bacteria that produce clinically problematic acute and chronic infections. It causes particularly difficult to treat infections in people with cystic fibrosis (CF), burn wounds, trauma, and compromised immune systems [10], [11]. P. aeruginosa controls the gene expression of many virulence factors through three QS systems: LasR, RhlR, and MvfR (PqsR) [9]. LasR and RhlR are activated by the N-acyl-homoserine lactone (AHL) signaling molecules N-3-oxododecanoyl homoserine lactone (3-oxo-C$12\text{-HSL}$) and N-3-butanoyl-DL-homoserine lactone (C$4\text{-HSL}$) [9]. Meanwhile, MvfR (PqsR) is activated by the 4-hydroxy-2-alkyloxyquinolines (HAQs) signaling molecules PQS (2-heptyl-3,4-dihydroxyquinoline (Pseudomonas Quinolone Signal) and HHQ (4-hydroxy-2-heptyloxyquinoline) [5], [6], [8], [12]. In addition to their role as QS signal molecules, AHLs and HAQs also modulate inflammation and immune responses in mammals [7], [13]. The 3-oxo-C$12\text{-HSL}$ signal molecule inhibits dendritic cell and T-cell
activation [14], promotes apoptosis [15–17] and inhibits the ability of macrophages and monocytes to respond to a range of Toll-like receptor (TLR) agonists through disruption of nuclear factor (NF)-κB signaling [15]. 3-oxo-C12-HSL is a strong inducer of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 in airway epithelial cells, lung fibroblasts, and macrophages, and is an enhancer of neutrophil chemotaxis [18]. Although it is known that NF-κB limits the inflammatory response by balancing the secretion of pro- and anti-inflammatory mediators in vivo, as well as in vitro, while improving survival against P. aeruginosa infection. The ability of 2-AA to dampen inflammatory processes, while increasing host survival and pathogen persistence, suggests that this molecule promotes host tolerance to infection. Our findings provide novel insights into pathogen weapons and mechanisms used to enable long-term bacterial presence in infected tissues.

**Results**

2-AA pretreatment leads to increased survival of mice and bacterial persistence in infected tissues

To determine if 2-AA modulates host immune responses, we assessed the susceptibility of 2-AA pretreated mice to P. aeruginosa infection (strain PA14) using a burn and infection (BI) model [52]. We observed a time-interval dependent protective effect of host pre-exposure to 2-AA. Mice pretreated with 2-AA 6 h or 1 d pre-BI showed no protection; the animals died at the same rate as untreated BI controls (Fig. 1A). However, mice injected 4 d pre-BI had a survival rate of 90%, which was remarkably better than the 10% rate observed in untreated BI controls (Fig. 1A). Pretreatment with 2-AA 2 d, 8 d, or 30 d before BI had more modest benefits; these mice showed survival rates of 30%.

Animals pretreated with the 2-AA metabolite 3OH-2-AA [53] 4 d prior to BI (Fig. 1B) showed a lesser degree of protection (60% survival at 72 h) relative to animals pretreated with 2-AA, suggesting that 3OH-2-AA metabolite 3OH-2-AA protects mice from P. aeruginosa infection. The presence of 2-AA in infected human tissues together with its ability to signal bacterial changes that favor chronic infection [49] raise the question of whether this molecule modulates host immune responses, and whether such modulation may favor the long-term presence of the pathogen. Thus, in the present study, we investigated 2-AA’s possible immunomodulatory role. Animal and ex vivo studies were conducted to explore the impact of 2-AA on inflammatory processes as well as to assess 2-AA’s effects on the activation of immune effectors and on the ability of the mice to tolerate the long-term presence of P. aeruginosa.

**Author Summary**

*Pseudomonas aeruginosa*, a recalcitrant Gram-negative opportunistic pathogen, defies eradication by antibiotics and exemplifies current highly problematic pathogens that often cause untreatable acute or chronic infections. There is increasing evidence that small molecules excreted by bacterial pathogens may impact human health by affecting functions including immunity. Although such small molecules in favor of acute infections have been reported, small molecules that may impact immune responses in pathogens’ favor of maintaining a chronic infection have not. We recently published work showing that the *P. aeruginosa* small molecule 2-amino acetophenone (2-AA) promotes bacterial phenotypic and genetic changes associated with chronic infection. Here, we provide evidence that this diagnostically important small volatile molecule dampens the host inflammatory process triggered by infection, thus favoring chronic infection. 2-AA limits the inflammatory response by balancing the secretion of pro- and anti-inflammatory mediators in vivo, as well as in vitro, while improving survival against *P. aeruginosa* infection. The ability of 2-AA to dampen inflammatory processes, while increasing host survival and pathogen persistence, suggests that this molecule promotes host tolerance to infection. Our findings provide novel insights into pathogen weapons and mechanisms used to enable long-term bacterial presence in infected tissues.

P. aeruginosa's approach to its hosts is to adopt efficient survival and persistence tactics that obfuscate the inflammatory process, while increasing host survival and pathogen persistence. Thus, in the present study, we investigated 2-AA's possible immunomodulatory role. Animal and ex vivo studies were conducted to explore the impact of 2-AA on inflammatory processes as well as to assess 2-AA's effects on the activation of immune effectors and on the ability of the mice to tolerate the long-term presence of *P. aeruginosa*.
provided a moderate level of protection (20–50% survival when delivered 4 d before BI) relative to that observed with 2-AA (90%) (Fig. 1B). These findings show that 2-AA provides markedly stronger protection than structurally similar molecules, indicating that the 2-AA effect is relatively specific.

The survival benefit yielded by 2-AA pretreatment cannot be attributed to the reduced bacterial proliferation since 2-AA pretreatment significantly increased PA14 loads at the wound site 7 d after BI compared to untreated controls (Fig. 1C). Moreover using a chronic wound infection model [54], we showed that animals co-inoculated with 2-AA and bacteria maintained high PA14 loads (mean of ~1 × 10^4 colony forming units [CFU]/g of tissue) in infected tissues 11 d post-infection, whereas the untreated animals almost completely cleared the bacteria (Fig. 1D). These results suggest that 2-AA supports the long term presence of bacteria.

**2-AA modulates the inflammatory response in vivo**

We proceeded to examine the mechanism by which 2-AA can reduce mortality against *P. aeruginosa* without eliminating bacterial load in mice. We found that 2-AA treatment 4 d pre-BI reduced serum levels of the pro-inflammatory cytokines IL-1α, IL-1β, IL-4, interferon (IFN)γ, and tumor necrosis factor (TNF)-α, compared to untreated BI mice, while augmenting secretion of the anti-

---

**Figure 1. 2-AA enhances survival following BI.** (A) Mice were injected with 2-AA (6.75 mg/kg mice) or PBS 6 h (n = 20), 2 d (n = 20), 4 d (n = 20), 8 d (n = 20), or 30 d (n = 20) prior to BI with PA14. The data shown are averages of two independent experiments. Significance of survival rate differences was determined using the Kaplan-Meier method, with a hazard ratio of 1.8932 (95% CI, 1.0664–6.0718). Infection (−) drastically reduced survival relative to (2-AA W/O BI) controls (p = 0.03). Delivery of 2-AA 4 d before BI (red) had a particularly powerful influence on survival versus mice not pretreated with 2-AA (p = 0.03). A less remarkable, but still significant, survival benefit was also observed in BI mice pre-exposed to 2-AA 6 h, 2 d, 8 d, or 30 d before BI (all p = 0.03 vs. non-infected 2-AA exposed controls). (B) Relative to the effects observed with 2-AA (n = 20), 4 d pretreatment with the 2-AA analogs 4-AA (n = 8; p = 0.03), 2-NA (n = 8; p = 0.03), or MA (n = 8; p = 0.03), or the 2-AA metabolite 3OH-2-AA (n = 8; p = 0.03) prior to PA14 infection had weak, though still statistically significant, positive effects on survival after infection. Significance of survival rate differences was calculated as in A. (C) Bacterial loads in the local muscle 7 d post-BI were significantly higher in mice pretreated with 2-AA 4 d before BI (n = 7) than in control mice subjected to BI without 2-AA pretreatment (n = 7; p < 0.05, Kruskal-Wallis test). CFU data are presented on a log_10 scale. (D) CFU counts at the site of infection in mice 11 d postinfection. The 2-AA treated mice showed proliferation and higher counts than mice that were not treated with 2-AA. (n = 6; p < 0.001, Kruskal-Wallis test). CFU data are presented on a log_10 scale.

doi:10.1371/journal.ppat.1003024.g001
inflammatory cytokine IL-10 (Fig. 2). No effects of the pretreatment on IL-6, IL-2, and IL-12 were observed (data not shown). These results suggest that 2-AA pretreatment may reduce inflammation by maintaining a balance between pro- and anti-inflammatory processes.

Additionally, we found that pretreating mice with 2-AA 4 d prior to infection provided protection against severe inflammation in an acute lung infection model [55]. Lung histopathology was compared with untreated control mice (Fig. 3A). Untreated mice infected with PA14 rapidly developed extensive inflammation within the lungs, as evidenced by a red hepatization of the lung tissues 24 h after infection. After 48 h, there was extensive progression of the pneumonia with the formation of bacteria-filled necrotic foci throughout the lung parenchyma (Fig. 3B). In sharp contrast, lung inflammation was markedly attenuated in mice given 2-AA 4 d prior to being infected (Fig. 3C).

2-AA pretreatment represses 2AA-induced NF-kB activation pathway in mouse macrophages

We examined how 2-AA affects the innate immune system ex vivo in a mouse macrophage cell line stably expressing a NF-kB-luciferase transcriptional fusion gene. 2-AA stimulation produced a dose-dependent, transient NF-kB activation that peaked at about 4 h (Figs. 4A–B, S1A). Interestingly, this NF-kB activation effect of 2-AA stimulation was dampened in macrophages pretreated with 2-AA for 48 h (Fig. 4B). This dampening effect was not due to 2-AA cytotoxicity (see data in Fig. S3). Furthermore, the effect was found to be related to 2-AA since pretreatment of macrophages with the 2-AA analogs 3-amino acetophenone (3-AA) (data not shown) and 4-AA (Fig. 4C) did not modify 2-AA induced NF-kB activation.

Stimulation of macrophages with 2-AA caused a rapid degradation of the NF-kB inhibitor I-kBa within 15 min, and cleavage of the NF-kB inhibitor I-kBβ by 60–120 min. In contrast, 2-AA-pretreated macrophages maintained high levels of I-kBz and I-kBβ upon stimulation (Fig. 4D). Western blot analysis further showed that, in 2-AA pretreated cells, phosphorylation of the p65 subunit of NF-kB, which enables I-kBz release and proteolysis [56], was reduced relative to that in non-pretreated cells (Fig. 4E & S5A) Additionally, a concentration-dependent increase in DNA binding of activated NF-kB p65 was observed in non-pretreated macrophages upon 2-AA stimulation, whereas this DNA binding was reduced in 2-AA pretreated cells (Fig. 4F).
contrast, p50 binding was increased in 2-AA pretreated cells (Fig. 4G). Taken together, these findings demonstrate that 2-AA pretreatment modulates MAPK signaling. Inflammatory cell infiltration with large areas of consolidation in lung parenchyma 48 h after infection with PA14 (Black arrows indicate the infiltration and necrotic foci). (C) Lack of infiltration 48 h after PA14 infection in the lungs of mice pretreated with 2-AA 4 d prior to BI.

2-AA pretreatment down-regulates pro-inflammatory cytokines while upregulating anti-inflammatory cytokine production in mouse macrophages

Since NF-κB regulates the production of pro-inflammatory mediators [57], we hypothesized that 2-AA pretreatment would reduce the production of pro-inflammatory cytokines, as seen in the in vivo experiments presented above (Fig. 2). Compared to 2-AA non-pretreated macrophages, 2-AA pretreated cells showed a decreased production of the pro-inflammatory cytokines TNF-α and IFN-γ (Fig. 3A and 3B), and an increased release of the anti-inflammatory cytokine TGF-β following stimulation with 0.2 or 0.4 mM 2-AA (Fig. 3C).

2-AA pretreatment modulates MAPK signaling components of innate immunity

We explored the effects of 2-AA stimulation and pretreatment on activation of ERK1/2, JNK, p38 MAPK, c-Jun, and c-Fos, which are essential components of innate immune signaling pathways in macrophages [58], [59]. A transient increase in phosphorylated p38 and JNK1/2 MAPKs was observed 5–15 min after 2-AA stimulation (Fig. 6A & B, S5B & C). This increase was blocked in 2-AA pretreated macrophages (Fig. 6A & B, S5B & C). 2-AA stimulation did not activate ERK1/2 in 2-AA naïve cells, but did induce ERK1/2 phosphorylation in 2-AA pretreated cells (Fig. 6C & S5D).

TransAM assays demonstrated that 2-AA stimulation of 2-AA naïve cells resulted in phosphorylation, and therefore activation, of the AP-1 family transcription factors c-Fos and c-Jun, enabling them to bind to the AP-1 promoter element. This 2-AA stimulation-induced binding of c-Fos and c-Jun was dampened in pretreated cells (Fig. 6D & E). These data fit with the aforementioned down-regulation of JNK and p38 phosphorylation observed in 2-AA pretreated macrophages. Pretreatment with the 2-AA analog 4-AA did not alter c-Fos and c-Jun binding to the AP-1 element (Fig. S6).

c/EBPβ over-expression and formation of the c/EBPβ-p65 complex supports the inhibition of p65 phosphorylation in 2-AA pre-treated cells

To investigate the possible role of ERK1/2 in dampening of inflammation after 2-AA pretreatment, we examined c/EBPβ and NF-κB activation [60]. We found that expression of the c/EBPβ, which functions downstream of the MEK-ERK1/2 pathway [60], was markedly increased in 2-AA pretreated macrophages following 2-AA stimulation relative to the expression observed in non-pretreated, stimulated macrophages (Fig. 7A). Moreover, in the presence of a MEK inhibitor, c/EBPβ expression was blocked and 2-AA pretreated cells showed the expected down-regulation of ERK1/2 and c/EBPβ (Fig. 7B).

We proceeded to examine whether increased c/EBPβ expression contributes to the prevention of NF-κB activation in 2-AA pretreated cells. As shown in Figure 7G, 2-AA stimulation resulted in phosphorylation of the NF-κB p65 subunit at ser 536 in the trans-activating domain (TAD)-1 in non-pretreated cells, but this phosphorylation was abolished in 2-AA pretreated cells. Moreover, co-immunoprecipitation (IP) studies supported the notion that c/EBPβ/p65 complex formation occurs in 2-AA pretreated cells only (Fig. 7C). Formation of the c/EBPβ/p65 complex prevents subsequent p65 activation.

2-AA-mediated silencing is controlled, at least in part, at the transcriptional level

Because 2-AA stimulation alone caused activation of NF-κB and transcriptional targets of NF-κB can inhibit JNK activation [59], we used NF-κB signaling inhibitors, at the time of 2-AA pretreatment, to examine the possibility that activation of NF-κB may account for 2-AA mediated silencing. We found that JNK phosphorylation was sustained after stimulation with 2-AA in the presence of NF-κB inhibitors (Fig. 8A & B). Further exposure of macrophages to the transcription inhibitor actinomycin D at the time of 2-AA pretreatment also partially restored phosphorylation of JNK in response to subsequent 2-AA stimulation (Fig. 8C),
Figure 4. 2-AA pretreatment modulates activation of the NF-κB pathway in mouse macrophages. (A) Schematic of 2-AA treatment. Macrophages were left untreated (No Pre) or pretreated with 0.8-mM 2-AA or 4-AA for 48 h (2-AA/4-AA Pre). The untreated and 2-AA pretreated cells were then stimulated with 0.2 mM, 0.4 mM, or 2.0 mM 2-AA (for experiment in B) or 4-AA (for experiment in C). (B) Pretreatment with 2-AA blocked bacterial QS mediated immunomodulation.
NF-κB activation relative to cells not pretreated with 2-AA (0.8 mM). (C) NF-κB was activated by 2-AA analog 4-AA in 4-AA pretreated and not pretreated cells. Mean values calculated from 2–4 replicate experiments are depicted with SD error bars. (D and E) Following stimulation with 2-AA (0.4 mM), cellular extracts prepared from not pretreated and 2-AA pretreated macrophages. Western blots of I-κBα and I-κBβ degradation (D) and phosphorylation of NF-κB subunit p65 (E). Loading was normalized relative to mouse β-actin. (F and G) A TransAM NF-κB assay showed binding of NF-κB p65 and p50 with the NF-κB promoter in not pretreated and 2-AA pretreated cells following stimulation with 2-AA. Mean values calculated from three replicate experiments are depicted with SD error bars. (p<0.05, Student’s t test).

doi:10.1371/journal.ppat.1003024.g004

suggesting that the pretreatment-induced molecular silencing effect observed is controlled, at least in part, at the transcriptional level.

Discussion

This study demonstrates the contribution of the low-molecular-weight QS molecule 2-AA in the modulation of mammalian innate-immune signaling pathways. This is the first study to show that this long-known [61], but scarcely studied, P. aeruginosa molecule modulates host responses. Unlike other immune-suppressing QS molecules, which promote activation of virulence functions leading to acute infection [8], [62], herein we showed that 2-AA minimizes activation of immune effectors and increases survival of infected animals, while permitting a long-term presence of the pathogen in vivo. Our cytokine profiling results suggest that 2-AA pretreatment limits inflammation by dampening pro-inflammatory cytokine activation. These data support the notion that 2-AA pretreatment protects host animals by balancing pro- and anti-inflammatory cytokine levels. 2-AA’s ability to dampen host inflammation may be critical for both host survival and long-term survival of 2-AA secreting bacteria in host tissues.

Chronic infection is normally established after an acute infection period involving activation of both the innate and acquired immune systems. The ability of a host to tolerate a bacterial presence without negatively affecting the pathogen’s fitness is particularly important since 2-AA eventually decreases the expression of many acute P. aeruginosa virulence related genes [49], making the pathogen vulnerable to clearance. Importantly, 2-AA not only silences the MvfR virulence regulon [49], but also promotes bacterial changes that favor long-term survival in a dynamic host environment. Thus, considered together with our prior published findings, the present results strongly suggest that 2-AA may serve a dual purpose: (1) to promote bacterial changes that favor chronic adaptability of the pathogen and (2) to suppress innate immune responses, allowing successful bacterial maintenance in host tissues.

The 2-AA mediated concomitant regulation of pro-inflammatory and anti-inflammatory cytokines resembles the endotoxin tolerance promoted by lipopolysaccharide (LPS) [63], [64]. The decreased mortality observed in LPS-pretreated animals, however, is accompanied by a more efficient bacterial clearance [65], [66], rather than a long-lasting bacterial presence as occurred in our 2-AA-pretreated animals. Recently, Khajanchi et al. showed that animals pretreated with the QS molecule 3-oxo-C12-HSL had reduced levels of pro-inflammatory cytokines and cleared Aeronas hydrophila bacteria without tissue damage [67]. Conversely, we found that 2-AA pretreatment not only did not clear P. aeruginosa bacteria or affect its growth, but rather resulted in a higher bacterial load relative to that in non-pretreated mice. These findings strongly suggest that 2-AA’s effect on host responses may enable the long-term presence of P. aeruginosa. A similar phenomenon was observed in plant infections involving the virulence factor XopD, which promotes Xanthomonas campestris pathovar vesicatoria growth in planta, while reducing host defense responses and delaying the development of disease symptoms [68].

Several recent studies have shown down-regulation of NF-κB, ERK1/2, JNK, and p38 MAPK activation in endotoxin-tolerant mouse macrophages [63], [69]. Although clarifying the exact molecular mechanism mediating the 2-AA pretreatment response will require further investigation, our findings show that 2-AA negatively impacts activation of NF-κB, JNK, and p38 MAPK, but increases ERK activation in pretreated cells. Based on the presented findings, we propose the model depicted in Figure 9. Briefly, 2-AA pretreatment may trigger a tolerance phenomenon to subsequent 2-AA challenges, leading to a dramatically increased survival rate of P. aeruginosa infected mice. Our findings further suggest that these effects are achieved by a block in MAPK and NF-κB activation, and activation of ERK1/2, leading to c/EBPβ activation and formation of the c/EBPβ-p65 complex that
Figure 6. 2-AA pretreatment alters activation of the MAPKs and AP-1 in macrophages upon 2-AA stimulation. Western blotting of cellular extracts with phospho-specific antibodies after 48 h pretreatment with 2-AA (0.8 mM) followed by stimulation with 0.4 mM 2-AA (A) p38 MAPK, (B) JNK1/2 and (C) ERK1/2. One representative experiment (out of three) is shown. Loading was normalized relative to mouse β-actin. A TransAM AP-1 transcription factor assay after 48-h pretreatment with 2-AA (0.8 mM) followed by stimulation with 2-AA, showing the binding of c-Fos (D) and c-Jun (E) to AP-1 promoter. Mean values calculated from three replicate experiments are depicted with SD error bars. (p<0.05, Student’s t test).

doi:10.1371/journal.ppat.1003024.g006
prevents NF-κB activation. In support of this model, we found that 2-AA strongly increased p65 phosphorylation in non-pretreated macrophages, but not in 2-AA pre-treated cells. It is well known that pro-inflammatory stimuli induce phosphorylation of NF-κB subunit p65, which is thought to increase the transactivation potential of p65 [70–72] and that suppression of p65 phosphorylation coincides with inhibition of IκBα degradation [56]. Moreover, we showed that pretreatment with 2-AA induces the expression of ERK1/2 and in turn c/EBPβ, which binds directly to p65, resulting in c/EBPβ-p65 complex formation. ERK1/2 induction down-regulates NF-κB mediated transcription [29] and initiates post-transcriptional modification of c/EBPβ. This modification leads to a conformational change in c/EBPβ that unmasks its bZIP heterodimerization domain [30], thereby enabling formation of the c/EBPβ-p65 complex [73], [74]. Formation of the c/EBPβ-p65 complex may mechanically alter interactions between NF-κB p65 and its inhibitors by blocking specific phosphorylation sites [75], [76], thereby enabling c/EBPβ to form an inhibitory box [77] in 2-AA pretreated cells. Since c/EBPβ is involved in immune modulation [31], c/EBPβ-p65

Figure 7. Inhibition of p65 phosphorylation in 2-AA pretreated cells is accompanied by de novo formation of c/EBPβ-p65 complexes. (A) Western blots of cellular extracts incubated with c/EBPβ from macrophages that had been incubated for 48 h with 0.8 mM 2-AA (2-AA Pre) or plain medium (No Pre) and subsequently stimulated with 0.4 mM 2-AA for the indicated time periods. (B) Western blot showing inhibition of ERK1/2 and c/EBPβ in 2-AA pretreated cells in the presence of MEK1 inhibitor PD98059 (1 μM, 5 μM, or 10 μM). Loading was normalized relative to mouse β-actin. (C) In cells treated as above, c/EBPβ-p65 complex formation monitored by IP followed by immunoblotting with anti-c/EBPβ or anti-p65 antibodies.

doi:10.1371/journal.ppat.1003024.g007
Figure 8. **Transcription and NF-κB inhibitors can block the effects of 2-AA pretreatment.** Western blots showing phosphorylation of JNK1/2 in 2-AA pretreated or untreated cells along with CAPE (1.5 μM) (A), MG-132 (1 μM) (B), and actinomycin D (1 μM) (C) following 0.2 mM or 0.4 mM 2-AA stimulation. Loading was normalized relative to mouse β-actin. One representative experiment (of three) is shown for JNK1/2. doi:10.1371/journal.ppat.1003024.g008

Figure 9. **Proposed model for 2-AA immunomodulatory mechanisms.** In naïve cells (left), stimulation with 2-AA induces activation of NF-κB, which leads to the phosphorylation and degradation of IκBα, releasing the NF-κB dimers p65 and p50. 2-AA also induces the p38 MAPK and JNK pathways to stimulate c-Jun and c-Fos. Activation of MAPK and NF-κB pathway upregulates pro-inflammatory genes. In contrast, in 2-AA pretreated cells (right) over-expression of ERK1/2 activates C/EBPβ, which binds directly to p65, resulting in c/EBPβ-p65 complex formation, and preventing 2-AA induced phosphorylation of p65 upon 2-AA stimulation. This interaction inhibits NF-κB mediated transactivation. The activation of JNK and p38 MAPK are repressed in 2-AA pretreated cells. All together, repression of the p38 MAPK, JNK, and NF-κB pathways abrogates the activation of pro-inflammatory mediators.

doi:10.1371/journal.ppat.1003024.g009
association in 2-AA pretreated cells could result in reduced expression of pro-inflammatory cytokines.

Interestingly, LPS pretreated cells also exhibit reduced NF-kB activation and binding activity, accompanied by decreased degradation of IkBα and IkBβ [63]. Thus, as shown in Figure 9, a 2-AA induced tolerance in murine macrophages ex vivo may be achieved by inhibiting activation of both NF-kB and AP-1 factors (i.e., c-Jun and c-Fos). Additionally, our observations that actinomycin D and NF-kB inhibitors can block 2-AA pretreatment effects on JNK activation are consistent with the idea that 2-AA works at the transcriptional level and requires de novo protein synthesis. We also showed that 2-AA pretreated macrophages were not fully responsive to other pathogen-associated molecules, such as LPS and peptidoglycan (PGN) (Fig. S4). Therefore dampening of immune signaling pathways by other pathogen associated molecules [78], [79] or endogenous danger signals—such as pro-inflammatory cytokines [80], heat shock proteins [81], [82], and hanyuran [83]—might also play a role in infection-induced immunosuppression and the establishment of chronic/persistent bacterial infections. It remains to be determined whether these other agents also improve host survival in vivo and modulate defense mechanisms in the context of an active infection.

Several mechanisms may collectively contribute to 2-AA mediated tolerance in vivo and ex vivo. Firstly, genes encoding pro-inflammatory cytokines are inactivated to limit tissue damage. Meanwhile, anti-inflammatory mediators that do not disrupt host tissue physiology provide protection from inflammation and pathogen-induced damage. Our data indicate that selective and transient inactivation of immune mediators at the intracellular signaling level may play an important role in 2-AA induced host tolerance.

In conclusion, our results strongly support the notion that 2-AA produces immunomodulatory effects that enhance the host’s ability to tolerate a pathogen presence. Meanwhile, 2-AA silences bacterial acute virulence functions [49] and dramatically increases survival rates among infected mice (Fig. 1A). From an evolutionary perspective, it is intriguing that P. aeruginosa would produce a QS molecule that decreases its own virulence. However, this seemingly counter-intuitive effect can ultimately be adaptive if it enables the pathogen to secure long-term survival within its host. This hypothesis is supported by data showing that 2-AA pretreatment ameliorated the inflammatory response upon subsequent infection, allowing infected mice to survive, and thus increasing the pathogen’s fitness. 2-AA’s ability to render immune cells tolerant, as observed in the present study, may mirror the mechanism by which this pathogen avoids elimination and persists in chronic infections. Elucidation of the molecular mechanisms involved in enabling host organisms to tolerate persistent/chronic infections may open new avenues for the development of diagnostics and therapeutic strategies to treat chronic, persistent, and relapsing infections.

**Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments at Massachusetts General Hospital ( Permit Number: 2006N000093/2, for burn wound infection and 2005N000111, for open wound infection). All procedures were performed under ketamine/xylazine anesthesia, and every effort was made to minimize suffering.

**Bacterial strains and growth conditions**

A P. aeruginosa strain known as Rif-resistant human clinical isolate UCBPP-PA14 (commonly referred to as PA14) was used in the present experiments [84], [85]. The bacteria were grown at 37°C in Luria-Bertani (LB) broth under shaking and aeration or on plates of LB agar containing appropriate antibiotics, unless otherwise indicated. Overnight PA14 cultures were grown in LB+rifampicin (50 mg/L) and diluted the following day in fresh LB media.

**Mouse mortality and CFU assays**

A thermal injury mouse model [52] was used as described previously [85] to assess bacterial pathogenicity in 6-wk-old CD1 mice (Charles River; Boston, MA). Following administration of anaesthesia, a full-thickness thermal burn injury involving 5–8% of the total body surface area [52] was produced on the dorsum of the shaved mouse abdomen, and an inoculum of 5.0×10^5 PA14 cells in 100 μl of saline was injected intradermally into the burn eschar. Mouse survival was subsequently assessed over the course of 10 d.

2-AA treated mice received a single intravenous (IV) injection of 2-AA (6.75 mg/kg mice) 6 h, 2 d, 4 d, 8 d, and 30 d prior to BI. Mice treated with 4-AA (6.75 mg/kg mice), 2-NA (8.25 mg/kg mice), MA (7.55 mg/kg mice), 3OH-2-AA (7.6 mg/kg mice), and/or phosphate buffered saline (PBS; vehicle control) also received a single IV injection, but 4 d prior to BI. All injected compounds were purchased from Sigma-Aldrich (Saint Louis, MO).

To allow examination of bacterial persistence in the BI model, we inoculated mice with 2×10^5 PA14 cells to avoid mortality of the control set. Animals were injected with 2-AA (6.75 mg/kg mice) 4 d prior to BI. After 4 d, 2×10^4 PA14 cells, in 100 μl of saline, were injected as and described above. Seven days post-BI, CFU counts were assessed in 5–6 mice per group from muscle samples obtained from underneath the burn. Samples were homogenized in 1 ml of 1× PBS. The samples were diluted and plated on LB-agar plates containing rifampicin (50 mg/L).

**Open wound model and CFU assays**

The open wound mouse model was used as described in detail elsewhere [54] to examine the long-term bacterial presence at the chronic wound site. Briefly, 6-wk-old CD1 male mice (Charles River; Boston, MA) were anesthetized by intraperitoneal injection and their backs were shaved. Under aseptic conditions, a patch of 1.5×1.5 cm skin was surgically removed from the shaved back of each mouse, exposing an area of collagen connective tissue above the panniculus carnosus muscle. On the top of the surgical wound, 7×10^4 bacterial cells were inoculated with 10 μl of 2-AA (0.22 mg/kg mice) or an equal volume of vehicle. The surface of the wound was dressed with a transparent and semipermeable Tegaderm film to provide protection from non-experimental bacterial contamination and to allow for daily inspection of the wound. The mice were monitored for 11 d postinfection and CFU counts were performed in 6 mice per group. Tissue samples from the infected area of each mouse were homogenized in 1 ml of PBS, diluted, and plated on LB-agar plates containing rifampicin (50 mg/L).

**Lung infection model and lung biopsy**

We assessed the effects of 2-AA in a neonatal mouse lung infection model [55] relevant to CF. A minimum inoculum of 1.5×10^5 PA14 cells/animal is 100% lethal in this model. Mice were pretreated with 2-AA 4 d before being infected and then sacrificed 24 h, 48 h, or 72 h postinfection. The specimens were fixed in 10% buffered formalin overnight and then, following a
dehydrated, embedded in paraffin blocks and sectioned into 6-µm-thick sections. The sections were stained with hematoxylin & eosin (H-E) and evaluated by light microscopy. Lung histopathology was assessed and the cytoarchitecture of the infected animals was compared to that of controls.

In vivo cytokine analysis
Blood was collected 24 h post-BI from 4 mice in each group by cardiac heart puncture. Serum was isolated and assayed by multiplex sandwich enzyme-linked immunosorbent assay (ELISA) (Quansys Biosciences, UT) containing nine antibodies (against IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12/23p70, TNF-α, and IFN-γ). The plate was imaged by cooled CCD camera and integrated density values of the standard spots were used to generate standard curves for the assayed proteins, and density values of the unknown samples were calculated. This service was provided by Quansys Biosciences, USA.

Cell culture
A Raw 264.7 murine macrophage cell line (American type culture collection, Bethesda, MD) was maintained in Iscove’s modified Dulbecco’s medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (Gibco) and penicillin/streptomycin (Gibco) in the presence of 5% CO2 at 37°C. The cells were seeded in T-75 tissue culture flasks (Falcon, USA) and used between passages 2 and 3.

Preparation of stably transfected cells with luciferase reporter plasmid
RAW 264.7 cells (5 × 10^5 cells) were seeded in 60-mm dishes; 24 h later, a mixture of Lipofectamine LTX and Plus reagent (Invitrogen, Grand Island, NY) and 2.5 µg of pGL4.77-kb luciferase plasmid (a gift from Dr. Xavier’s Lab, MGH, Boston) were added and then incubated for 6 h in serum-free medium. The cells were then cultured in medium supplemented with serum for an additional 2 h prior to being subjected to further analysis. Medium containing 250 µg/ml hygromycin B (Roche Applied Science, Basel, Switzerland) was used to select stable transfecants.

Measurement of TNF-α, IFN-γ, and TGF-β by ELISA
TNF-α, IFN-γ, and TGF-β protein levels in culture supernatants were measured by ELISA using the Quantikine mouse TNF-α and TGF-β kits (R & D Systems, Minneapolis, MN) and an OptEIA mouse IFN-γ kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturers’ instructions. Briefly, the culture supernatants were added to anti-human TNF-α monoclonal antibody-coated ELISA plates and incubated for 2 h at room temperature. Following four washes, the detector molecules (HRP-conjugated streptavidin and biotinylated anti-human TNF-α) were added to plates and incubated for 2 h at room temperature. The assay was developed with tetramethyl benzidine (TMB) substrate reagent. Following a 20-min incubation at room temperature, the absorbance at 450 nm was determined using a Sunrise plate reader (Tecan Group Ltd, Mannendorf, Switzerland). For the TGF-β and IFN-γ assays, we followed the same procedure as described above.
**Supporting Information**

**Figure S1** 2-AA activates NF-κB pathways and pro-inflammatory cytokines in mouse macrophages. (A) Mouse macrophages were incubated with 0.2 mM, 0.4 mM, or 2 mM 2-AA for the indicated time periods, and NF-κB activation was monitored by luciferase assays. The results are expressed as fold change compared to control cells. Mean values calculated from three replicate experiments are depicted with SD error bars. Macrophages were stimulated with 0.2-mM or 0.4-mM 2-AA at the indicated time points. (B) IFN-γ and (C) TNF-α secretion was measured in cell supernatants by ELISA. Mean values calculated from three replicate experiments are depicted with SD error bars. *p<0.05 vs. naïve (Student’s t test). (TIF)

**Figure S2** Structures of 2-AA, the 2-AA metabolite 3OH-2-AA, and the 2-AA analogs 4-AA, 2NA, and MA. (TIF)

**Figure S3** Effects of 2-AA on viability of mouse macrophages. MTT assay measuring cell viability in mouse macrophage cells after treatment with 0.2 mM, 0.4 mM or 0.8 mM 2-AA for different time points, as indicated in the figure. SDs (vertical bars) were calculated from three replicate experiments. (TIF)

**Figure S4** 2-AA pretreated macrophages are broadly hyporesponsive to pathogen associated molecules. Macrophages were pretreated with 2-AA (0.8 mM), LPS (100 ng/ml), or PGN (100 ng/ml) for 48 h and then stimulated with LPS (1 ng/ml) or PGN (10 ng/ml) for 2 h. Activation of NF-κB (expressed as fold change over background) upon stimulation with LPS or PGN is shown. Mean values calculated from three replicate experiments are depicted with SD error bars. (TIF)

**Figure S5** 2-AA modulates NF-κBp65, p38, JNK, and ERK phosphorylation in 2-AA pretreated mouse macrophages. Cells were pretreated with 2-AA (2-AA Pre) or medium only (No Pre) for 48 h and subsequently stimulated with 2 mM 2-AA for the indicated time periods. Western blotting of cellular extracts with phospho-specific antibodies was used to reveal the effects of 2-AA pretreatment on phosphorylation of (A) NF-κBp65, (B) p38, (C) JNK1/2, and (D) ERK1/2 following 2-AA (2 mM) stimulation. Loading was normalized relative to mouse β-actin. (TIF)

**Figure S6** 4-AA pretreatment does not alter activation of AP-1 in macrophages upon 4-AA stimulation. A TransAM AP-1 transcription factor assay after a 48 h pretreatment with 4-AA (0.8 mM) followed by stimulation with 4-AA, showing binding of c-Fos (A) and c-Jun (B) to the AP-1 promoter. Mean values calculated from three replicate experiments are depicted with SD error bars (p<0.05, Student’s t test). (TIF)

**Acknowledgments**

We acknowledge Dr. Ramnik Xavier, M.D MGH, Boston for the kind gift of pGL4-NF-κB luciferase plasmid and Dr. Ruslan M. Medzhitov, Yale School of Medicine for his insightful comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: AB MK LGR. Performed the experiments: AB MK YQ JH KP. Analyzed the data: AB MK LGR. Contributed reagents/materials/analysis tools: LGR RT. Wrote the paper: AB MK LGR.
References

1. Ng WL, Basler BL (2009) Bacterial quorum-sensing network architectures. Annu Rev Genet 43: 197–222.

2. Antunes LC, Ferreira RB, Buckner MM, Finlay BB (2010) Quorum sensing in bacteria: a paradigm shift in virulence. Annu Rev Microbiol 63: 2271–2292.

3. Keller L, Suzuki MM (2006) Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 4: 249–258.

4. Parker CT, Sperandeo V (2009) Cell-to-cell signalling during pathogenesis. Cell Microbiol 11: 363–369.

5. Desai E, Gopalas S, Tampakaki AP, Lepine F, Padfield KE, et al. (2005) The contribution of MvfR to Pseudomonas aeruginosa pathogenesis and quorum sensing circuitry regulation: multiple quorum-sensing-regulated genes are modulated without affecting laR, rhlR or the production of N-3-oxododecanoyl homoserine lactones. Mol Microbiol 55: 998–1014.

6. Gao X, Desiel E, He J, Lepine F, Leste B, et al. (2006) MvrR, a key Pseudomonas aeruginosa pathogenicity-lTRR-class regulatory protein, has dual ligands. Mol Microbiol 62: 1689–1699.

7. Rumbaugh KP, Kauffman GF (2011) Exploitation of host signaling pathways by microbial quorum sensing signals. Curr Opin Microbiol 14: 162–8.

8. Williams P, Camara M (2009) Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol 12: 182–191.

9. Nadal Jimenez P, Koch G, Thompson JA, Xavier KB, Cool RH, et al. (2012) Analysis on the target genes of PQS and HHQ in J774A.1 monocyte/macrophage cell line. Immunology 129: 578–588.

10. Imamura Y, Yanagihara K, Mizuta Y, Seki M, Ohno H, et al. (2004) Azithromycin inhibits MUC5AC production induced by the Pseudomonas aeruginosa autoinducer N-(3-oxododecanoyl) homoserine lactone. Infect Immun 72: 5687–5692.

11. Kerr KG, Snelling AM (2009) Pseudomonas aeruginosa: a formidable and ever-present adversary. J Hosp Infect 73: 338–344.

12. Diggle SP, Cornelis P, Williams P, Camara M (2008) β-quimoline signalling in Pseudomonas aeruginosa: old molecules, new perspectives. Int J Med Microbiol 296: 83–91.

13. Kravchenko VV, Kauffman GF, Mathison JC, Scott DA, Katz AZ, et al. (2008) Modulation of gene expression via disruption of NF-kappaB signalling by a bacterial small molecule. Science 321: 259–263.

14. Boomham P, Robins A, Chandran P, Pritchard D, Camara M, et al. (2008) Significant immunomodulatory effects of Pseudomonas aeruginosa quorum-sensing signal molecules: possible link in human sepsis. Clin Sci (Lond) 115: 343–351.

15. Kerr KG, Shin YM, Kakarla S, Matsuoka M, Mustafi R, et al. (2003) The Pseudomonas aeruginosa autoinducer N-3-oxodecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils. Infect Immun 71: 5705–5710.

16. Li H, Wang L, Ye L, Mao Y, Xie X, et al. (2009) Influence of Pseudomonas aeruginosa quorum sensing signal molecule N-3-oxodecanoyl homoserine lactone on mast cells. Med Microbiol Immunol 198: 113–121.

17. Jacoby CA, Schiffer F, Henkel M, Wajbel M, Stork B, et al. (2009) Effects of bacterial N-acyl homoserine lactones on human Jurkat T lymphocytes: OdDH1 induces apoptosis via the mitochondrial pathway. Int J Med Microbiol 299: 515–519.

18. Zimmermann S, Wagner C, Muller W, Brenner-Weiss G, Hug F, et al. (2006) Induction of neutrophil chemotaxis by the quorum-sensing molecule N-3-oxodecanoyl-L-homoserine lactone. Infect Immun 74: 5607–5612.

19. Smith RS, Fedly ER, Springer TA, Makaain B, Iglesiasi BH, et al. (2001) IL-8 production in human lung fibroblasts and epithelial cells activated by the Pseudomonas aeruginosa autoinducer N-3-oxodecanoyl homoserine lactone is transcriptionally regulated by NF-kappaB and activator protein-2. J Immunol 167: 366–374.

20. Imamura Y, Yanagihara K, Minuta Y, Seki M, Ohno H, et al. (2004) Azithromycin inhibits MUC5AC production induced by the Pseudomonas aeruginosa autoinducer N-3-oxodecanoyl homoserine lactone in NCI-H292 cells. Antimicrob Agents Chemother 48: 3457–3461.

21. Vikstrom E, Magnum KE, Pivoriunas A (2005) The Pseudomonas aeruginosa quorum-sensing molecule N-3-oxodecanoyl-L-homoserine lactone stimulates phagocytic activity in human macrophages through the p38 MAPK pathway. Microbes Infect 7: 1512–1518.

22. Kim K, Kim YU, Koh BH, Hwang SS, Kim SH, et al. (2010) HHQ and PQS, two Pseudomonas aeruginosa quorum-sensing molecules, down-regulate the innate immune responses through the nuclear factor-kappaB pathway. Microbiol Immunol 54: 351–359.

23. Kim K, Kim SH, Lepine F, Cho YH, Lee GR (2010) Global gene expression analysis on the target genes of PQS and HHQ in J774A1 monocyte/macrophage cells. Microb Pathog 49: 174–180.

24. Medzhikov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol 1: 135–145.

25. Brodsky IE, Medzhikov R (2009) Targeting of immune signalling networks by bacterial pathogens. Nat Cell Biol 11: 521–526.

26. Ikeda R (2007) Signaling to NF-kappaB by Toll-like receptors. Trends Mol Med 13: 460–469.

27. Schulze-Lueffelmann J, Ghosh S (2006) Antigen-receptor signaling to nuclear factor kappa B. Immunity 25: 701–715.
58. DeFranco AL, Crowley MT, Finan A, Hambleton J, Weinstein SL (1998) The role of tyrosine kinases and map kinases in LPS-induced signaling. Prog Clin Biol Res 397: 119–136.

59. Nakano H (2004) Signaling crosstalk between NF-kappaB and JNK. Trends Immunol 25: 402–405.

60. Hu J, Roy SK, Shapiro PS, Rodig SR, Reddy SP, et al. (2001) ERK1 and ERK2 activate CCAAT/enhancer-binding protein-beta-dependent gene transcription in response to interferon-gamma. J Biol Chem 276: 287–297.

61. Mann S (1966) Uber den Geruchsstoff von Pseudomonas aeruginosa. Archiv fur Mikrobiologie 34: 184–190.

62. Smith RS, Iglewski BH (2003) P. aeruginosa quorum-sensing systems and virulence. Curr Opin Microbiol 6: 36–60.

63. Medvedev AE, Kopydlowski KM, Vogel SN (2000) Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. J Immunol 164: 5364–5374.

64. Foster SL, Medzhitov R (2009) Gene-specific control of the TLR-induced inflammatory response. Clin Immunol 130: 7–15.

65. Murphey ED, Fang G, Varma TK, Shermood ER (2007) Improved bacterial clearance and decreased mortality can be induced by LPS tolerance and is not dependent upon IFN-gamma. Shock 27: 289–295.

66. Wheeler DS, Lahn PM, Denenberg AG, Poynter SE, Wong HR, et al. (2008) Induction of endotoxin tolerance enhances bacterial clearance and survival in marine polymicrobial sepsis. Shock 30: 267–273.

67. Kharanchi BK, Kirtley ML, Brackman SM, Chopra AK (2011) Immunomodulatory and Protective Roles of Quorum-Sensing Signaling Molecules N-Acyl Homoserine Lactones during Infection of Mice with Aeromonas hydrophila. Infect Immun 79: 2646–2657.

68. Kim JG, Taylor KW, Hotson A, Keegan M, Schmelz EA, et al. (2008) XopD SUMO protease affects host transcription, promotes pathogen growth, and delays symptom development in xanthomonas-infected tomato leaves. Plant Cell 20: 1915–1929.

69. Fan H, Cook JA (2004) Molecular mechanisms of endotoxin tolerance. J Endotoxin Res 10: 71–74.

70. Baud V, Karin M (2001) Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 11: 372–377.

71. Ghosh S, May MJ, Kopp EB (1998) NF-kappaB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 16: 225–266.

72. Schmitz ML, Maraldi I, Boss H, Kracht M (2004) NF-kappaB: a multifaceted transcription factor regulated at several levels. ChemBiochem 5: 1348–1358.

73. Puisieux G, MacDougald O, Schwartz J (2002) Dual regulation of phosphorylation and dephosphorylation of C/EBPbeta modulate its transcriptional activation and DNA binding in response to growth hormone. J Biol Chem 277: 43537–43563.

74. Stein B, Cogswell PC, Baldwin AS, Jr. (1993) Functional and physical associations between NF-kappaB and C/EBP family members: a Rel domain-bZIP interaction. Mol Cell Biol 13: 3964–3974.

75. Sakurai H, Chiba H, Miyoshi H, Sugita T, Tomimi W (1999) IkappaB kinase phosphatase: NF-kappaB p65 subunit on site 536 in the transcription domain. J Biol Chem 274: 30353–30356.

76. Wang D, Westerheide SD, Hanson JL, Baldwin AS, Jr. (2000) Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser536 is controlled by casein kinase II. J Biol Chem 275: 32392–32397.

77. Buck M, Poli V, Hunter T, Choquer M (2001) C/EBPbeta phosphorylation by RSK creates a functional NEXD caspase inhibitory box critical for cell survival. Mol Cell 8: 807–816.

78. Wang JH, Doyle M, Manning BJ, Blankson S, Wu QD, et al. (2003) Cutting edge: bacterial lipoprotein induces endotoxin-independent tolerance to septic shock. J Immunol 170: 14–18.

79. O'Brien GC, Wang JH, Redmond HP (2005) Bacterial lipoprotein induces resistance to Gram-negative sepsis in TLR4-deficient mice via enhanced bacterial clearance. J Immunol 174: 1029–1026.

80. Alves-Rosa F, Vulcano M, Beijer-Bompard M, Fernandez G, Palermo M, et al. (2002) Interleukin-1beta induces in vivo tolerance to lipopolysaccharide in mice. Clin Exp Immunol 128: 221–225.

81. Aneja R, Olenos K, Dunmore K, Stanley TP, Wong HR (2006) Extracellular heat shock protein-70 induces endotoxin tolerance in THP-1 cells. J Immunol 177: 7184–7192.

82. Kilmartin B, Reen DJ (2004) HSP60 induces self-tolerance to repeated HSP60 stimulation and cross-tolerance to other pro-inflammatory stimuli. Eur J Immunol 34: 2041–2051.

83. del Fresno C, Otero K, Gomez-Garcia L, Gonzalez-Leon MC, Soler-Ranger L, et al. (2003) Tumor cells deactivate human monocytes by up-regulating IL-1 receptor associated kinase-M expression via CD44 and TLR4. J Immunol 174: 3032–3040.

84. Rahme LG, Ausubel FM, Cao H, Devenard E, Goununver BC, et al. (2000) Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci U S A 97: 8815–8821.

85. Rahme LG, Stevens EJ, Wofford SF, Shao J, Tompkins RG, et al. (1995) Common virulence factors for bacterial pathogenicity in plants and animals. Science 268: 1899–1902.