

# Innate Immune Response to the Bacterial Quorum Sensing Molecule N-(3-oxododecanoyl) Homoserine Lactone

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## Introduction

### Background Information

- Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterial pathogen whose ability to establish persistent infections is tightly linked to its quorum sensing (QS) system<sup>1</sup>.
- The QS system relies heavily on N-acyl homoserine lactones (HSLs) which are small, diffusible signaling molecules that coordinate population-level behaviors, including virulence factor production and immune evasion<sup>1</sup>.
- In *P. aeruginosa*, QS circuits are activated by HSLs 3-oxo-C12-HSL (C12) and C4-HSL, which regulate the expression of genes critical for pathogenicity and host interactions<sup>1</sup>.
- The chronic and fatal lung infections in individuals with Cystic Fibrosis are often caused by *P. aeruginosa* and are made worse by a buildup of biofilm<sup>2</sup>. Biofilm production is dependent on C12 and facilitates pathogen persistence and the development of antibiotic resistance<sup>2</sup>.
- Host-microbe interactions rely heavily on innate immunity, where host cells detect invading microorganisms through pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), such as flagellin, enabling the immune system to distinguish microbial invaders from self and mount an early response<sup>1</sup>.
- Cyclic GMP-AMP synthase (cGAS) is a PRR that was originally characterized for sensing aberrant DNA species in the cytosolic compartment and initiates the cGAS-STING pathway, which is the Stimulator of Interferon Genes<sup>3</sup>.
- New studies have further revealed cGAS to function as an innate immune sensor to play an essential role in mounting type I interferon cytokines (IFN-I), controlling inflammation, and enhancing host resistance during *P. aeruginosa*-induced pulmonary infection<sup>3</sup>.

### Objectives

- Here we investigate the immunomodulatory impact of *P. aeruginosa*-derived HSLs on host pattern recognition pathways, with a focus on the cGAS PRR and the C12 HSL.
- By understanding how bacterial QS signals modulate DNA-sensing immunity, we aim to uncover novel mechanisms of host-pathogen communication that may inform therapeutic strategies targeting chronic and antibiotic-resistant infections.

## Methodology

### Cell Culture, Reagents, and Antibodies

- RAW 264.7 murine macrophages were cultured in DMEM supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%) in 5% CO<sub>2</sub> at 37° C.
- Primary antibodies used in this study were TBK1, p-TBK1, IRF3, p-IRF3, and RSAD2.

### Immunoblot Analysis

- For immunoblot analysis, cells were harvested in ice cold NP-40 lysis buffer supplemented with complete EDTA-free protease inhibitors.
- Protein concentrations were determined via BCA protein assay.
- Samples were boiled at 95°C for 5 min followed by SDS PAGE and immunoblotting.
- Proteins were detected via enhanced chemiluminescence using the Amersham Imager 600.

### RNA Isolation and Quantitative Real-Time PCR

- RNA was isolated using TRIzol reagent and converted to cDNA using ABScript III RT mix.
- Quantitative PCR (q-PCR) was performed using SYBR green in a CFX96 thermocycler.
- Transcript abundance was first normalized to that of mRNA encoding the ribosomal protein L32 for murine mRNA transcripts, then normalized against values for unstimulated controls calculated via the 2<sup>-ΔΔCt</sup> method.

### Statistical Analysis

- Quantitative data are expressed as mean-fold increase ± S.E. relative to control levels from a representative experiment performed 2-3 times. Statistical significance was determined using student's t-test (\*\*P<0.001, \*P<0.01, and \*P<0.05).

## Results

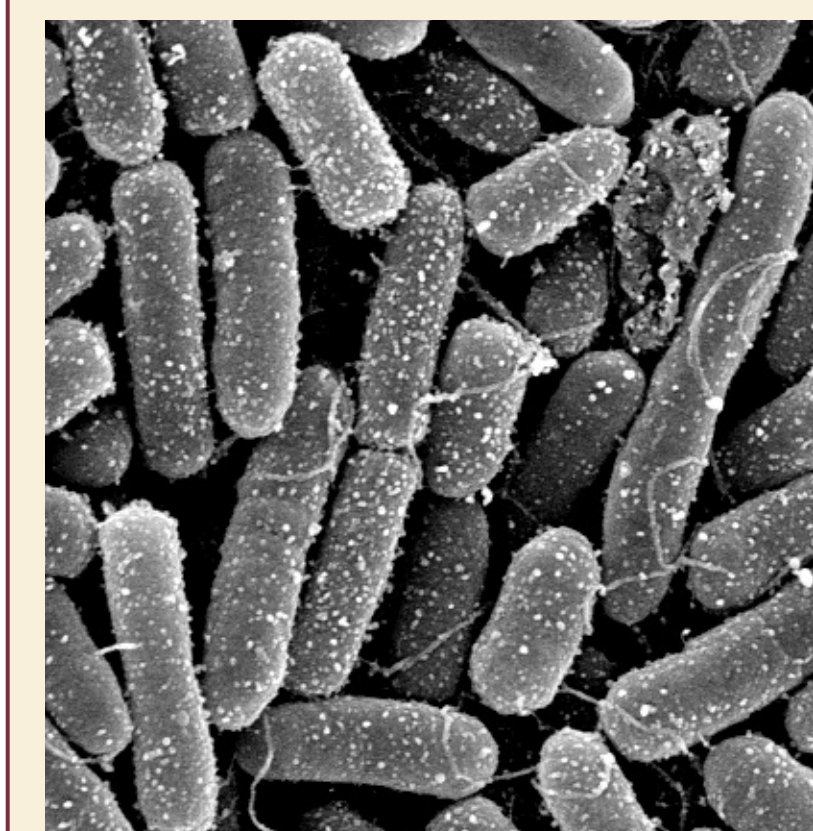


Figure 1. *P. aeruginosa*<sup>4</sup>

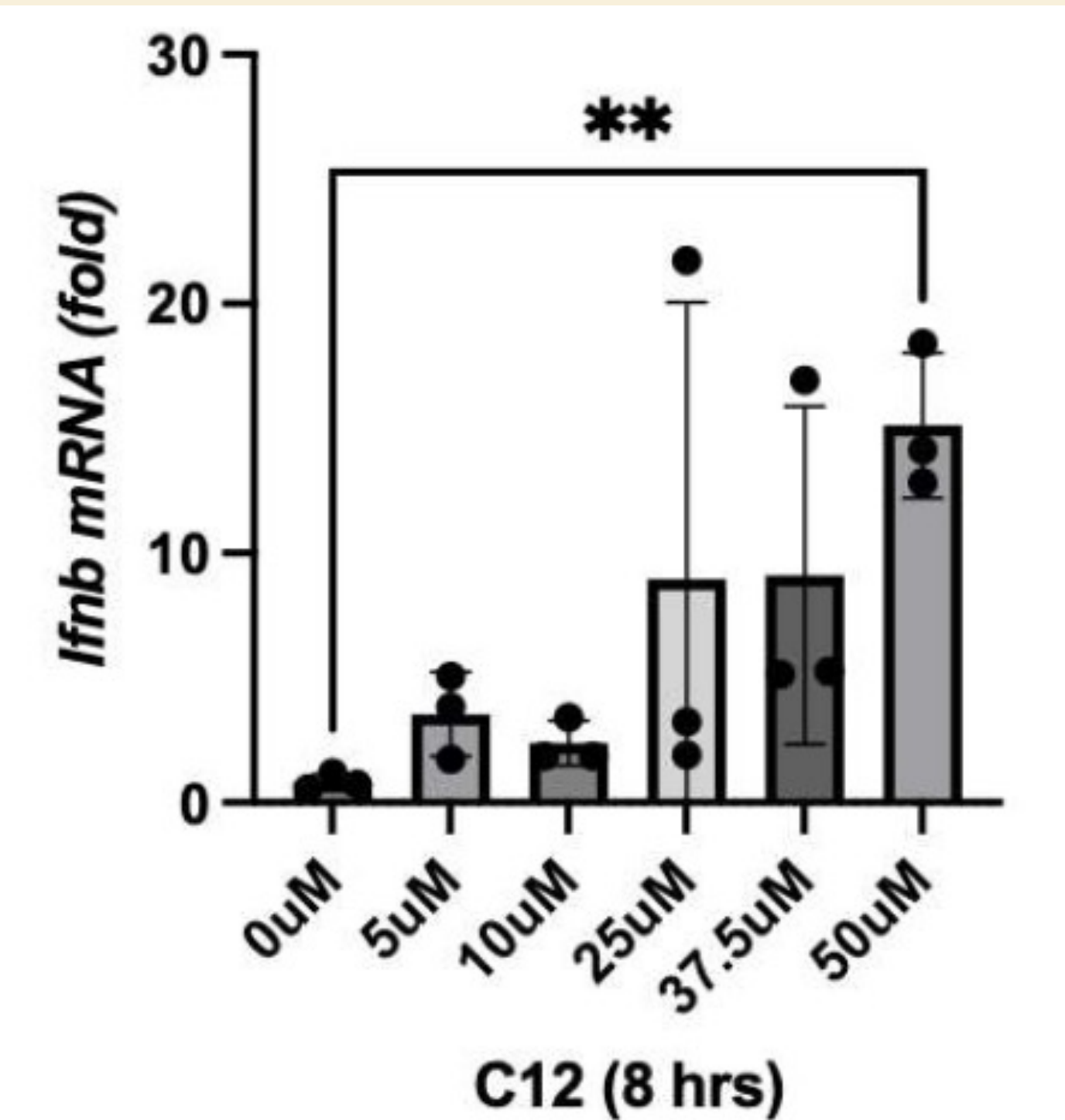


Figure 3. C12 titration

- Initial experiments aimed to confirm that C12 induces a type I interferon (IFN-I) response in mammalian immune cells.
- Reverse Transcription q-PCR (RTqPCR) analysis of RAW 264.7 murine macrophages showed an increase in IFNβ transcript levels in a C12 dose-dependent manner, indicating transcriptional activation of IFN-I.
- Asterisk indicates statistical significance (p < 0.05) as determined by a two-tailed unpaired t test.

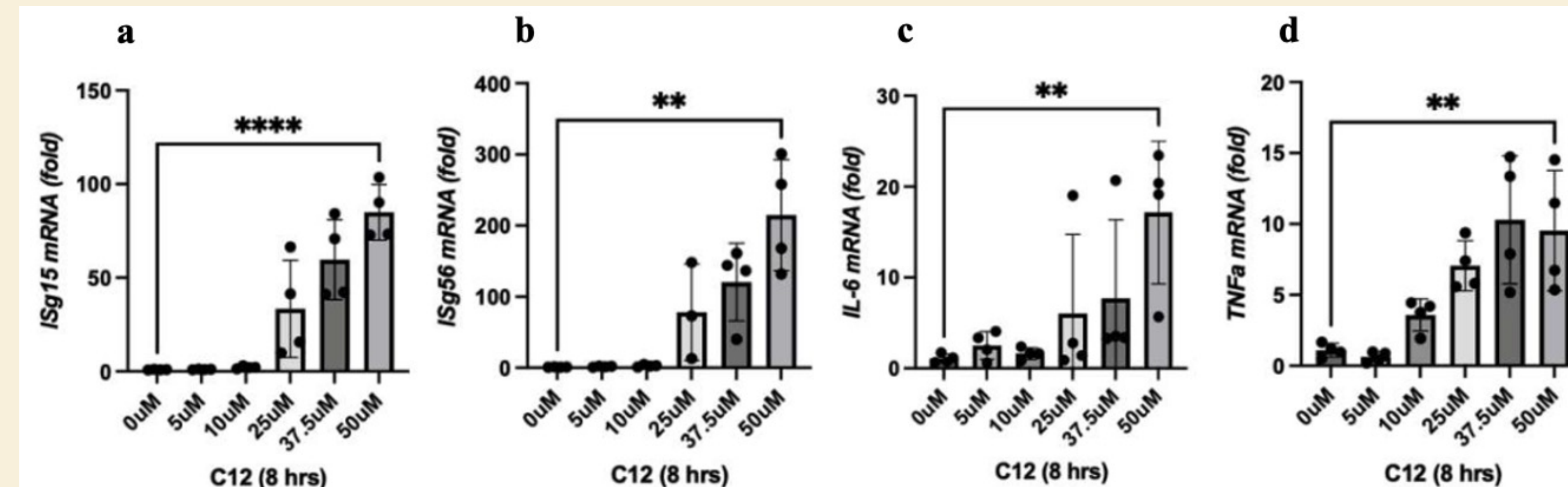


Figure 5. Physiological relevance

- To assess whether C12 induction of IFN-I is physiologically relevant, we evaluated the expression of three interferon signature genes (ISGs) in RAW 264.7 cells at both the protein and transcriptional level.
- RSAD2 is an antiviral protein induced by IFNβ and has been shown to suppress viral replication by interfering with RNA synthesis. ISG15 and ISG56 are proteins with roles in limiting viral replication and translation.
- Immunoblot analysis of RSAD2 showed increased RSAD2 levels at the 6 h C12 timepoint.
- RTqPCR analysis of ISG15 and ISG56 showed that their mRNA transcripts increased in a C12 dose-dependent manner (Fig. 5a,b).
- We also assessed the expression of two pro-inflammatory cytokines, IL-6 and TNFα.
- RTqPCR analysis indicated that mRNA transcripts of both IL-6 and TNFα were upregulated in a dose-dependent manner following C12 treatment (Fig. 5c,d).
- Asterisk indicates statistical significance (p < 0.05) as determined by a two-tailed unpaired t test.

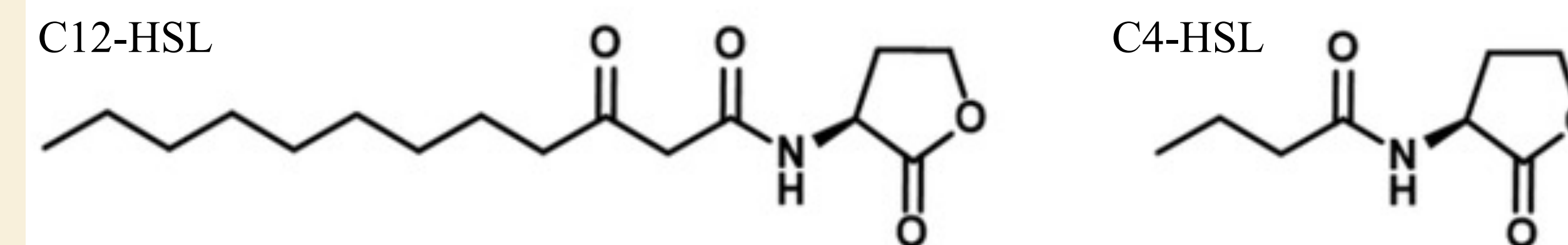


Figure 2. The two major HSLs of *P. aeruginosa*, C12 and C4.

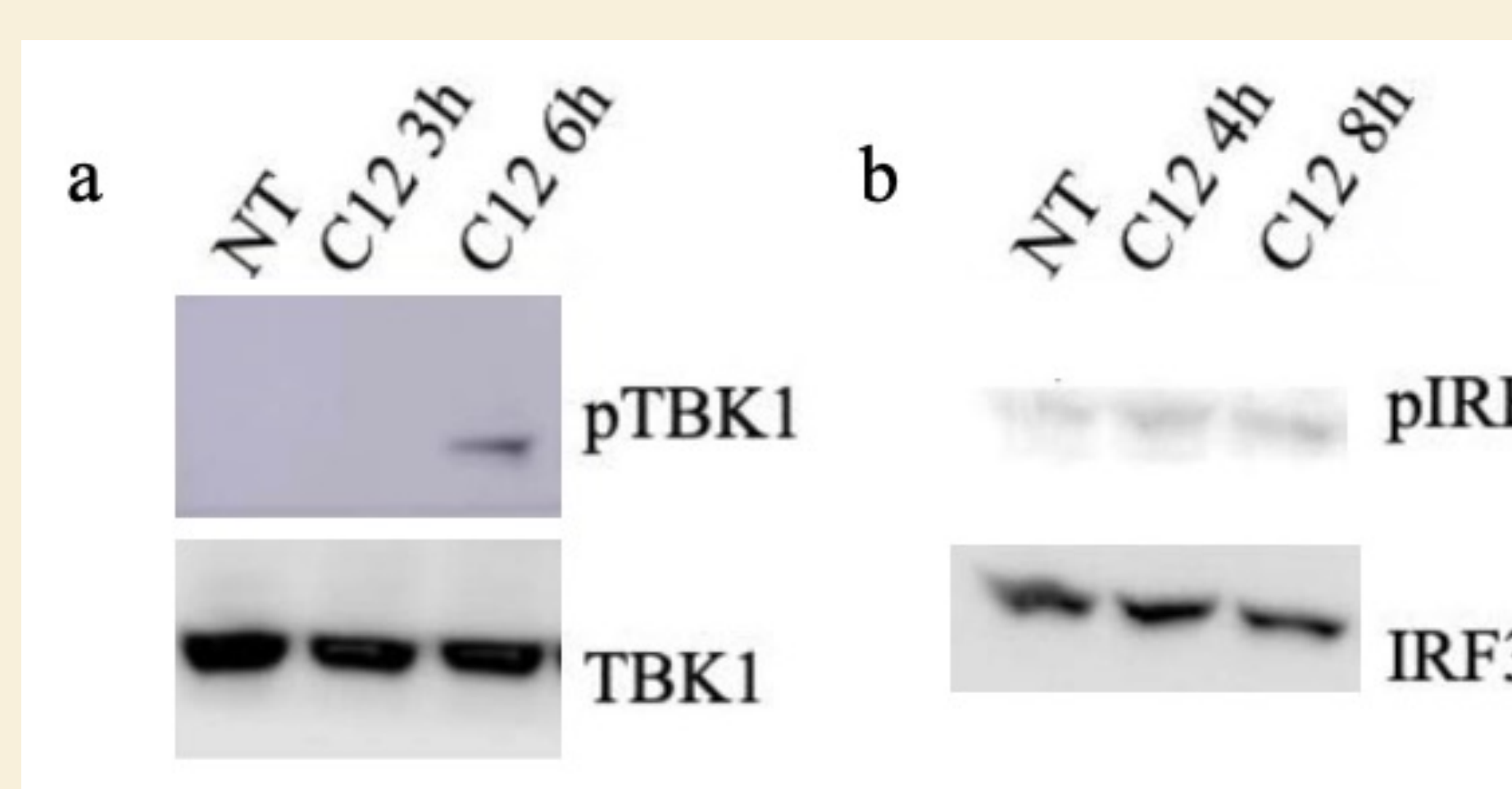
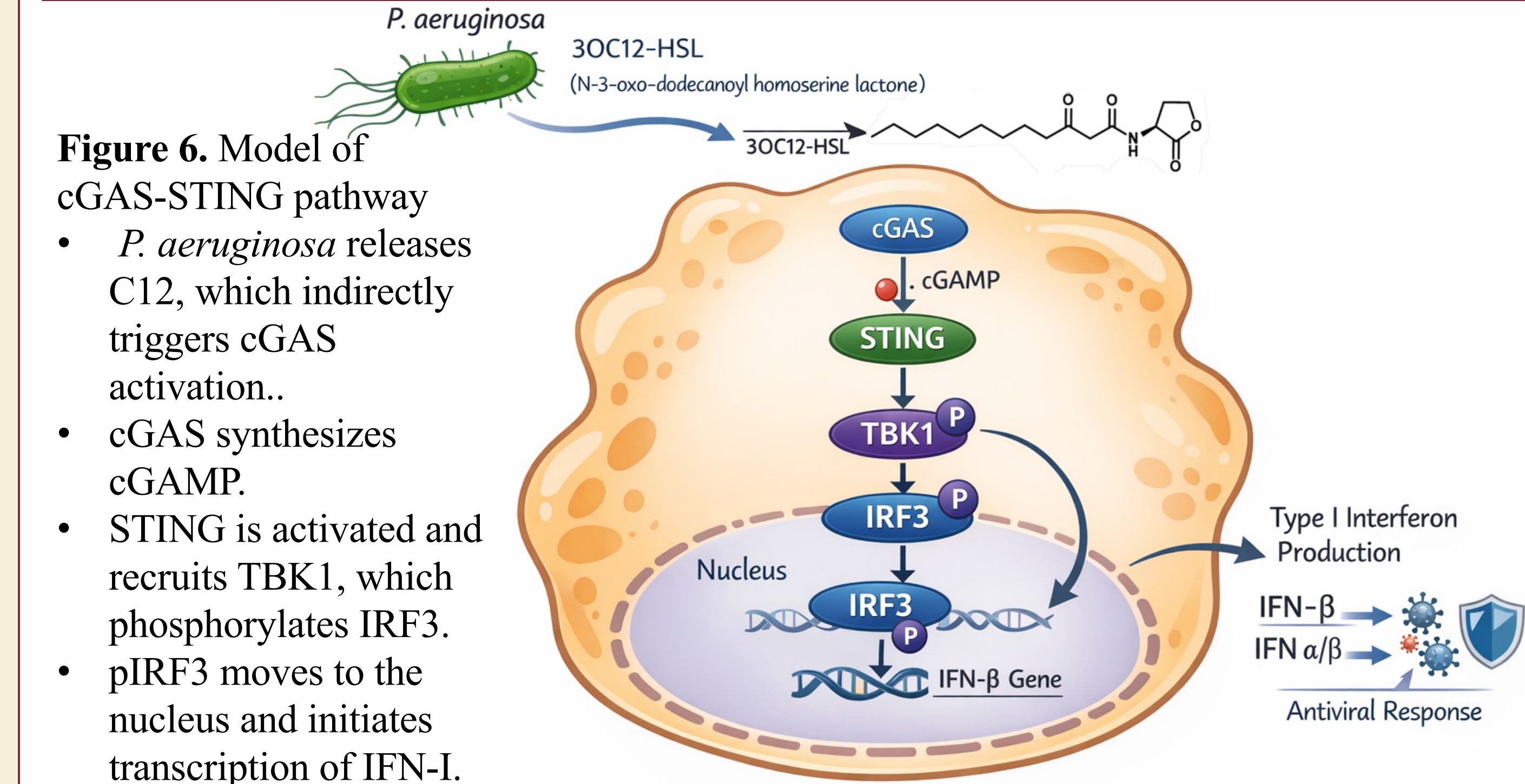


Figure 4. Upstream regulators

- To determine whether C12 activates canonical upstream regulators of IFN-I, we assessed the amount of total and phosphorylated TBK1 (pTBK1) and IRF3 (pIRF3) in RAW 264.7 murine macrophages.
- Immunoblot analysis in RAW 264.7 cells treated with the maximum nonlethal dose of C12 (50uM) indicated that C12 induced phosphorylation of TBK1 at the 6 h timepoint (Fig. 4a).
- Immunoblot analysis in RAW 264.7 cells treated with 50uM C12 indicated that C12 induced phosphorylation of IRF3 at the 4 and 8 h timepoints (Fig. 4b).

## Discussion

- This investigation sought to determine whether C12 induces an IFN-I response in mammalian immune cells and whether this response is cell type specific.
- Our data show that C12 induces a significant response at the transcriptional level in murine immune cell lines in a dose-dependent manner, but C4 does not.
- Western blot analysis confirmed that canonical upstream regulators TBK 1 and IRF3 were phosphorylated in response to C12, consistent with established IFN-I signaling cascades.
- Induction of ISGs including RSAD2, ISG15, and ISG56 supports the notion that this IFN-I response is not only transcriptionally initiated, but functionally relevant in specific immune cells.
- Increased IL-6 and TNFα expression suggests that C12 mediated IFN-I induction is potent enough to engage other inflammatory pathways like NF-KB, which is induced by these cytokines and contributes to a physiologically relevant immune response.
- These findings suggest that C12 may be a novel immunostimulatory molecule, acting as a noncanonical PAMP or inducer of damage-associated molecular patterns. This expands our understanding of how bacterial QS systems influence host immune surveillance and activation. Future studies will attempt to understand how C12 triggers cGAS activation.
- While our results demonstrate a functional IFN-I response, mechanistic activation upstream of TBK1 remains unknown. Future investigations will attempt to determine what upstream sensors mediate this activation.



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