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A Bacterial Small Molecule Undermining Immune Response Signaling

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The innate immune system defends our body from infection by other organisms such as fungi, viruses and bacteria. Innate immunity does not lead to immunological memory and is comprised of a network of soluble factors and cells that protect the host in the first hours and days against invading pathogens. The immune cells recognize and uncover the intruding microorganisms with the help of specific receptors that bind to characteristic and unique pathogenic structures. They are thus detected as "foreign." For example, gram-negative bacteria contain lipopolysaccharide (LPS), a compound that is specifically recognized by Toll-like receptor (TLR) 4 on the host cell, as an important constituent of their outer membrane.[1] TLR binding stimulates multiple signaling cascades within the immune cell, which in turn responds with an increased expression of genes that alert the innate immune system. Among these inducible produced gene products are inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF), which are secreted by LPS-stimulated cells.[2] These two cytokines are themselves activators of specific receptors in the cell membrane and thus rapidly amplify the signaling events that occur in the LPS-stimulated cells. In addition, IL-1 and TNF alert neighboring cells that have not yet encountered TLR4 activation.[3]

LPS activates diverse signaling pathways including the inducible transcription factor NF-κB and mitogen-activated protein (MAP) kinases such as p38.[4] Work from more than two decades has mounted compelling evidence that the NF-κB transcription factor is a key component of the innate immune system. In most cells, NF-κB is kept away from its site of action in the nucleus by association with an inhibitory IκB protein that traps it in the cytosol.[5] Adverse conditions such as LPS-induced TLR4 stimulation trigger a signaling cascade that leads to the activation of the so-called IKK (IκB kinase) complex, which is composed of the enzymatically active subunits IKKα and IKKß and the regulatory subunit IKKγ/NEMO. The activated IKK complex phosphorylates the inhibitory IκB protein and thus marks it for subsequent Lys48-linked polyubiquitination and proteasomal destruction.[6,7] The DNA-binding dimer is then free to move to the nucleus, where it induces the expression of a plethora of genes including chemokines, cell adhesion proteins, and further mediators of the inflammatory process. In addition, the function of the DNA-binding subunits can also be regulated by posttranslational modifications including phosphorylation, which is relatively well characterized for the strongly transactivating p65 subunit.[8] One of the earliest NF-κB target genes is IκBα, which is resynthesized quickly after its proteolytic destruction and thus ensures termination of the early NF-κB response by an autoregulatory feed-back mechanism. The LPS-induced NF-κB activation pathway and the molecular events discussed here are schematically displayed in Figure 1. Given the central relevance of NF-κB for innate immunity, pathogens have evolved multiple strategies to interfere with the activation of this transcription factor. For example, viruses can either activate intracellular NF-κB signaling (when this is needed for their effi-

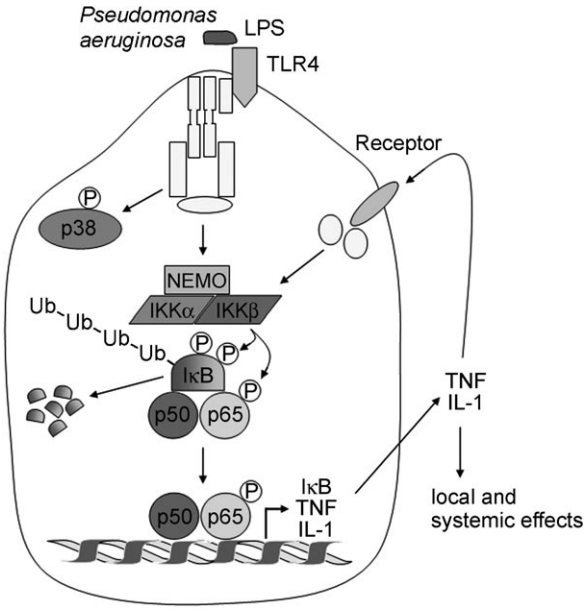

Figure 1. Schematic summary of the signaling mechanisms regulated by *Pseudomonas aeruginosa*. Lipopolysaccharide (LPS) at the surface of the bacterium stimulates Toll-like receptor 4 (TLR4), thus leading to the activation of NF-κB and p38. While p38 signaling is activated, NF-κB signaling is inhibited by C12. The molecules with relevance to this text are shown; Ub: ubiquitin.

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cient replication) or prevent the activation of this signaling pathway (when they want to escape from the immune response).[9] Bacteria have also learned to exploit key cellular responses to allow their efficient survival in the infected host.[10] For example, *Mycobacterium tuberculosis* secretes the ESAT-6 protein, which binds to TLR2 and thus prevents downstream signaling in macrophages.[11] Many more examples of bacterial strategies to undermine the host's immune system could be discussed here, and a new clue into the mechanisms used by bacteria was recently published by Kravchenko and colleagues in the July issue of *Science*.[12]

This paper reports on a small molecule that is released by *Pseudomonas aeruginosa* and inhibits NF-κB-dependent gene expression, thus ensuring the attenuation of the innate immune system to establish a local persistent infection with this bacterium.[12] This bacterium can cause persistent infections in susceptible humans, including those who are immune suppressed or suffer from cystic fibrosis. The starting point for the study was the question how opportunistic bacteria such as *P. aeruginosa* establish persistent infections, while highly virulent pathogens such as *Staphylococcus aureus* and *Salmonella typhimurium* trigger an acute and severe disease. The authors compared the ability of these three different bacteria to trigger NF-κB activation, which is the main usual suspect when it comes to innate immunity signaling. The results showed efficient NF-κB activation by the two virulent pathogens, as determined by monitoring the elimination and subsequent NF-κB-dependent resynthesis of IκB. In contrast, *P. aeruginosa* allowed the degradation of IκB but prevented its resynthesis, providing the first hint of its ability to manipulate NF-κB signaling. *P. aeruginosa* is known to produce *N*-(3-oxo-dodecanoyl) homoserine lactone (C12), a small molecule involved in several bacterial functions such as sensing bacterial crowding in biofilms.[13] When the pure C12 compound was tested for its effects on LPS-induced NF-κB activation, Kravchenko and co-workers found impaired phosphorylation of IκBα and a total block in IκBα resynthesis, thus demonstrating its efficacy for the inhibition of NF-κB signaling. However, C12 is not a general inhibitor of LPS-induced signaling cascades, as it potently activates the p38 MAPK signaling pathway, a result that is in accordance with a previously published paper of the same group.[14] The key finding of C12-mediated NF-κB inhibition was corroborated by a further experiment that compared the effects on NF-κB between wildtype *P. aeruginosa* and a mutant bacterial strain lacking the *lasI* gene, which is required for the efficient synthesis of C12. These experiments confirmed the inhibitory activity of C12 on IκBα resynthesis, as the *lasI* mutant failed to repress IκB resynthesis. Further experiments substantiated the result of C12-dependent inhibition of LPS-induced transcription for many NF-κB target genes. These blocking effects were already visible with C12 concentrations (10 μM) only slightly above the C12 concentrations found to be in *P. aeruginosa* samples (4.7 μM). The inhibitory effect on target gene expression occurred in different cell lines and also in response to NF-κB activation by TNF, but was not detected for interferon-triggered transcription programs. Kravchenko and co-workers extended these assays and also found the inhibitory effects of C12 on NF-κB-dependent target genes in intact animals, as revealed by the analysis of transgenic mice harboring a luciferase reporter gene driven by an NF-κB-dependent promoter. While all these different experimental approaches uncovered a specific and clear inhibitory activity of the C12 compound on NF-κB-dependent gene expression, the effects on the upstream steps of NF-κB signaling did not reveal the candidate protein targeted by this small molecule.

In all experiments, either *P. aeruginosa* or C12 did not prevent IκBα degradation, which is the classical key event in the canonical NF-κB activation cascade and a standard indicator for a functional NF-κB activation pathway. C12 concentrations of 50 μM, which is more than ten-times the C12 concentration contained in the *P. aeruginosa* cultures, allowed the detection of slightly impaired phosphorylation of the IKK substrates IκBα and p65. In contrast, the same concentration virtually wiped out transcription of NF-κB target genes. These results make any direct effects of C12 on the IKK complex rather unlikely. Accordingly, in vitro kinase assays failed to reveal any significant inhibitory effect of this compound on IKK activity. Thus, the molecular target mediating the inhibitory activity of C12 on NF-κB signaling remains to be identified in future studies. Intriguingly, C12 is a potent inducer of the p38 signaling pathway and causes phosphorylation of the p38 target cAMP response element-binding protein (CREB). Also the receptor structure that allows for C12-induced p38 activation is still unknown, and it is now an exciting goal to find the cellular receptor(s) that mediate these two opposite effects (NF-κB inhibition and p38 activation). This dual effect of C12 can be also taken as an indication that C12 inhibits NF-κB at a rather late stage in the signaling cascade after the branching point on the road leading from the TLR4 to p38 activation. This hypothesis can be easily tested, as the compound should interfere with target gene expression triggered by expression of the IKKs or other downstream components of the NF-κB pathway. Also dose dependence experiments to measure the effects of C12 on NF-κB signaling will help to clarify these issues in the future.

While the target molecule(s) for C12 in the host cell await their identification, the main advancement made by the Kravchenko study is the view on the mechanisms that allow chronic bacterial infections. The study implies that the opportunistic bacterium *P. aeruginosa* ensures its chronic residence in the host by its ability to produce a small molecule that dampens NF-κB immune signaling. This exciting concept can be experimentally tested in the future by comparing the infectiousness and persistence of wildtype *P. aeruginosa* and the *lasI* mutant, which shows impaired C12 synthesis.[13] The relative concentration of C12 that actually occurs in the microenvironment at the site of infection in the host also remains to be determined. But even suboptimal amounts of the compound should at least partially inhibit NF-κB target gene transcription and therefore target an early amplification step of the innate immune response. In

addition, the inhibition of NF-κB prevents the anti-apoptotic activity of this transcription factor, and the authors accordingly show that C12 confers pro-apoptotic activity to TNF. Thereby, C12 would not only down-modulate NF-κB signaling, but also eliminate immune cells by apoptosis. The concept that bacterial virulence correlates with the strength of TLR4 signaling is corroborated by a study that compared two *Yersinia pestis* strains. While the wildtype strain, which caused only a mild TLR4 response, was highly virulent, a modified strain that causes a potent TLR4 signal showed a largely decreased virulence.[15] The everlasting battle between our body and pathogens employs numerous mechanisms and both combatants use a large array of deceptions and tricks. The Kravchenko paper reveals a new clue by demonstrating that small molecules can also be used by bacteria.

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[1] N. J. Gay, M. Gangloff, *Annu. Rev. Biochem.* **2007**, *76*, 141–165.
[2] M. W. Covert, T. H. Leung, J. E. Gaston, D. Baltimore, *Science* **2005**, *309*, 1854–1857.
[3] T. Kawai, S. Akira, *Trends Mol. Med.* **2007**, *13*, 460–469.
[4] J. Schulze-Luehrmann, S. Ghosh, *Immunity* **2006**, *25*, 701–715.
[5] M. S. Hayden, S. Ghosh, *Cell* **2008**, *132*, 344–362.
[6] T. Henkel, T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, P. A. Baeuerle, *Nature* **1993**, *365*, 182–185.
[7] I. Alkalay, A. Yaron, A. Hatzubai, A. Orian, A. Ciechanover, Y. Ben-Neriah, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10599–10603.
[8] M. L. Schmitz, I. Mattioli, H. Buss, M. Kracht, *ChemBioChem* **2004**, *5*, 1348–1358.
[9] S. Ludwig, S. Pleschka, O. Planz, T. Wolff, *Cell. Microbiol.* **2006**, *8*, 375–386.
[10] L. A. O'Neill, *Nat. Med.* **2008**, *14*, 370–372.
[11] S. K. Pathak, S. Basu, K. K. Basu, A. Banerjee, S. Pathak, A. Bhattacharyya, T. Kaisho, M. Kundu, J. Basu, *Nat. Immunol.* **2007**, *8*, 610–618.
[12] V. V. Kravchenko, G. F. Kaufmann, J. C. Mathison, D. A. Scott, A. Z. Katz, D. C. Grauer, M. Lehmann, M. M. Meijler, K. D. Janda, R. J. Ulevitch, *Science* **2008**, *321*, 259–263.
[13] T. T. Hoang, H. P. Schweizer, *J. Bacteriol.* **1999**, *181*, 5489–5497.
[14] V. V. Kravchenko, G. F. Kaufmann, J. C. Mathison, D. A. Scott, A. Z. Katz, M. R. Wood, A. P. Brogan, M. Lehmann, J. M. Mee, K. Iwata, Q. Pan, C. Fearns, U. G. Knaus, M. M. Meijler, K. D. Janda, R. J. Ulevitch, *J. Biol. Chem.* **2006**, *281*, 28822–28830.
[15] S. W. Montminy, N. Khan, S. McGrath, M. J. Walkowicz, F. Sharp, J. E. Conlon, K. Fukase, S. Kusumoto, C. Sweet, K. Miyake, S. Akira, R. J. Cotter, J. D. Goguen, E. Lien, *Nat. Immunol.* **2006**, *7*, 1066–1073.

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