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Research Article

The *Pseudomonas aeruginosa* lectin LecB modulates intracellular reactive oxygen species production in human neutrophils

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Pseudomonas aeruginosa is a Gram-negative bacterium and an opportunistic pathogen ubiquitously present throughout nature. LecB, a fucose-, and mannose-binding lectin, is a prominent virulence factor of *P. aeruginosa*, which can be expressed on the bacterial surface but also be secreted. However, the LecB interaction with human immune cells remains to be characterized. Neutrophils comprise the first line of defense against infections and their production of reactive oxygen species (ROS) and release of extracellular traps (NETs) are critical antimicrobial mechanisms. When profiling the neutrophil glycome we found several glycoconjugates on granule and plasma membranes that could potentially act as LecB receptors. In line with this, we here show that soluble LecB can activate primed neutrophils to produce high levels of intracellular ROS (icROS), an effect that was inhibited by methyl fucoside. On the other hand, soluble LecB inhibits *P. aeruginosa*-induced icROS production. In support of that, during phagocytosis of wild-type and LecB-deficient *P. aeruginosa*, bacteria with LecB induced less icROS production as compared with bacteria lacking the lectin. Hence, LecB can either induce or inhibit icROS production in neutrophils depending on the circumstances, demonstrating a novel and potential role for LecB as an immunomodulator of neutrophil functional responses.

Keywords: glycan-binding proteins · host-pathogen interaction · immunomodulation · NETs · phagocytes



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Neutrophils are the most abundant leukocytes in human blood, acting as the first line of defense by providing a ruthless killing machinery continuously combating microbial infections [1]. Neutrophils deploy a plethora of defense mechanisms upon

encountering a microbe, most often starting with the internalization of the prey, that is, the process of phagocytosis, accompanied by the release of proteases and other antimicrobial molecules into the phagolysosome, contributing to the efficient intracellular killing [2]. Alternatively, the release of neutrophil extracellular traps (NETs), cobweb-like structures made up predominantly of released DNA and proteases, can lead to the entrapment of the pathogens extracellularly [3, 4]. A major set of antimicrobial molecules is the toxic reactive oxygen species (ROS) produced

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by activation of the membrane-associated electron-transporting enzyme NADPH-oxidase [5]. The ROS are produced at distinct subcellular sites in neutrophils depending on at which membrane the NADPH-oxidase is assembled; (1) extracellularly (ecROS) at the plasma membrane, or (2) retained intracellularly (icROS) either in phagosome (during phagocytosis) or in nonphagosomal organelles (in the absence of phagocytosis) [6]. Different agonists induce signals leading to specific localization of NADPH-oxidase activation and, consequently, ROS production. Also, the excitation state of the neutrophils, ranging from resting to highly responsive (primed) influences ROS production. To our knowledge, no soluble stimulus that specifically induces icROS, without simultaneously triggering ecROS release, has been described so far.

Among the known modulators of immune cell function, lectins binding to carbohydrate-containing cell surface receptors play important roles [7, 8]. Lectins are expressed in the majority of cells, including mammalian, microbial, and plant cells [9]. Galectins, a widely studied family of mammalian lectins, are known modulators of neutrophil function, as is the plant lectin wheat germ agglutinin (WGA). Although having very different origins and different carbohydrate specificities, galectins (e.g. galectin-1 and 3) and WGA similarly activate both ecROS and icROS production in primed but not resting or unprimed neutrophils [10–13]. Bacterial lectins are known to be involved in phagocytosis by binding to carbohydrate surface receptors, a process dubbed “lectinophagocytosis”. For example, type-1 fimbriae on *E. coli* and type-2 fimbriae on *Actinomyces* spp., interacting with mannose and lactose, respectively, are shown to be involved in bacterial attachment, ingestion, and production of ROS by neutrophils [14–16].

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium ubiquitously present throughout nature. *P. aeruginosa* infection is one of the major problems in healthcare settings as the bacteria are inherently resistant to many antimicrobials in addition to forming biofilms [17]. Healthy individuals are usually not susceptible to *P. aeruginosa* infections but in immunocompromised individuals, such as cystic fibrosis (CF) patients, the bacterium can cause severe acute and chronic respiratory infections [18]. The importance of neutrophils for the protection against *P. aeruginosa* has been proven both in neutropenic patients [19], who are susceptible to frequent infections caused by this bacterium, and in neutropenic mouse and rabbit models [20, 21]. Patients with rare neutrophil disorders, including specific granule deficiency and leukocyte adhesion deficiency [22, 23] are also prone to develop *P. aeruginosa* infections.

The relatively large genome (6.3 Mb) of *P. aeruginosa* holds a vast number of virulence factors that enable the bacteria to adapt to diverse growth conditions and facilitate bacterial invasion and evasion of host defense [24]. Among those virulence factors are the two soluble lectins LecA and LecB, encoded by *lecA* and *lecB*, respectively, which are unique to the *Pseudomonas* genus [25]. Initially isolated from *P. aeruginosa* clinical isolate (PAO1), LecA and LecB occur as homotetramers comprised by subunits of 121 and 114 amino acids, respectively. Both lectins are Ca²⁺-dependent, sugar-binding proteins with strong binding affinity

toward galactose- and fucose/mannose-containing glycoproteins and glycolipids, respectively [25].

LecB is localized on the bacterial surface, noncovalently linked to carbohydrate ligands on the outer membrane of the bacterium. This can be through its tetrameric organization it can function as an adhesion molecule, mediating carbohydrate-dependent binding between the bacterium and host cells. It can also exist in a soluble form as it can be released from the bacterium surface by mere interference with soluble carbohydrates [26, 27]. The importance of LecB for bacterial virulence has been shown in vitro as LecB-deficient *P. aeruginosa* strain is less pathogenic and appears to have reduced biofilm formation compared with a LecB-positive, WT strain [27, 28]. A recent and intriguing study has shown soluble LecB to interact with endothelial cells and thereby disrupting the transendothelial migration of leukocytes in vitro [29]. Moreover, Alhede et al. [30] found significant upregulation of the *lecB* gene in *P. aeruginosa* biofilms after 2 h exposure of human neutrophils, and a similar upregulation was not seen in unexposed biofilms.

With regards to neutrophil interaction with LecB, we have in our map of the neutrophil glycome found several fucosylated and mannosylated glycans on mobilizable intracellular granules and plasma membrane [31] which could potentially act as receptors. Yet, to our knowledge, the interaction of these *P. aeruginosa* lectins with neutrophils and the functional neutrophil response to lectin binding has not been previously investigated. Therefore, the aim of our study was to investigate the role of LecB in modulating neutrophil functions in vitro. For this purpose, we determined the ability of LecB to bind to neutrophils and to induce ROS production, calcium signaling, and formation of NETs. Using soluble LecB and LecB-deficient *P. aeruginosa* strains, we present unequivocal evidence that LecB is a significant modulator of neutrophil functional responses. This is the first report demonstrating the potential role of LecB as a virulence factor of *P. aeruginosa* that mediates modulation of neutrophil function in vitro.

Results

LecB activates icROS production in primed neutrophils in a carbohydrate-dependent manner

The ability of LecB to induce ROS production in human neutrophils was investigated. LecB at a concentration of 1.5 μM was added to (1) unprimed, (2) 37°C-primed (weak priming), (3) LPS-primed and (4) TNFα-primed neutrophils and icROS and ecROS production was measured separately using luminol- or isoluminol-amplified CL, respectively. LecB induced substantial icROS production in neutrophils that were primed with either LPS or TNFα but not in unprimed or 37°C-primed neutrophils (Fig. 1A). Hence, priming is a prerequisite for LecB-induced activation of icROS production. The magnitude and the kinetics of the LecB-induced icROS response in TNFα-primed neutrophils were equivalent to the well-characterized stimulus PMA (Fig. 1B). LecB did not induce any ecROS production, neither in primed nor in

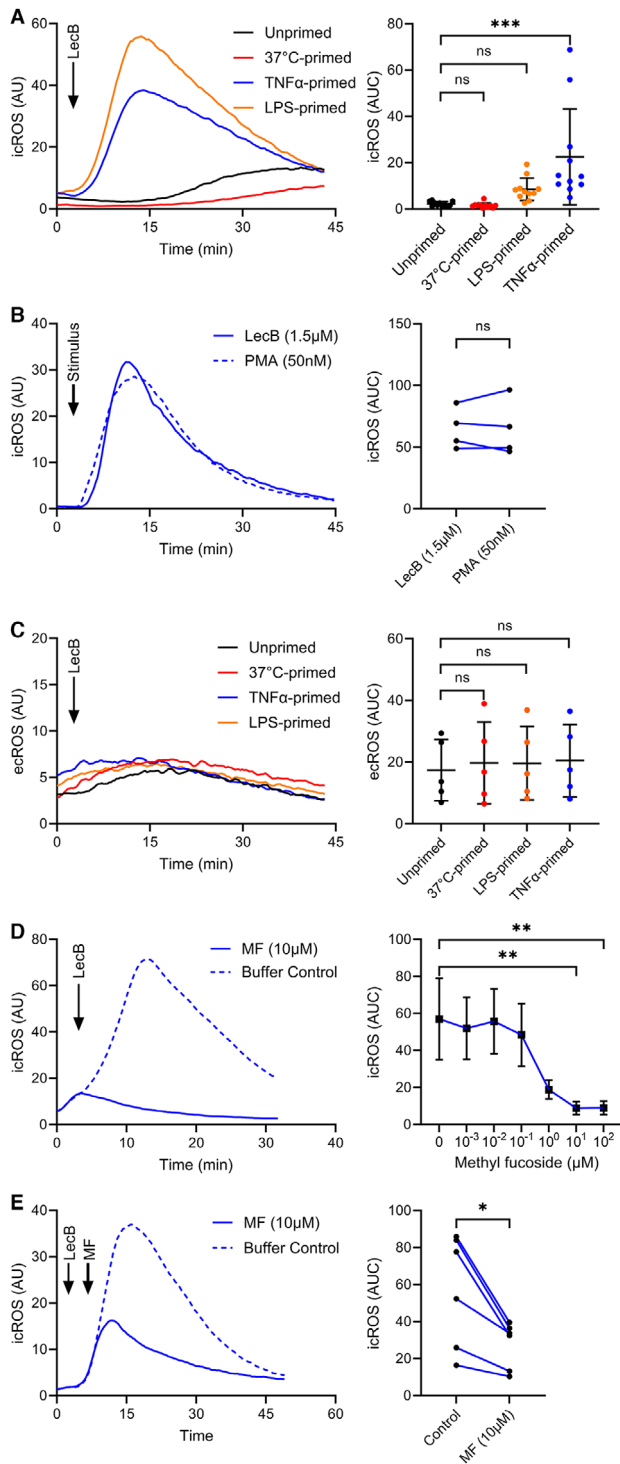


Figure 1. LecB directly induces icROS production in TNF α - and LPS-primed neutrophils. (A) LecB (1.5 μ M) directly induces icROS production in TNF α - or LPS-primed neutrophils but not in unprimed or 37°C-primed neutrophils, measured using luminol-amplified CL over time. (B) The magnitude and kinetics of LecB-induced icROS in primed neutrophils are similar to PMA (50 nM) induced icROS production, measured using luminol-amplified CL over time. (C) LecB (1.5 μ M) does not induce ecROS production in TNF α -, LPS-, 37°C-primed neutrophils, or unprimed neutrophils. This was measured using isoluminol-amplified CL over time. (D) Preincubating TNF α -primed neutrophils with methyl fucoside (10 μ M) inhibited LecB induced icROS production analyzed using luminol-

unprimed neutrophils (Fig. 1C), making it radically different to the other so far established lectin agonists [11, 12]. Unlike LecB, the *P. aeruginosa* lectin LecA did not induce any ROS production, neither in unprimed nor in primed neutrophils (Fig. S1).

Since LecB is a carbohydrate-binding protein (binding to fucose- and mannose-containing epitopes), we investigated the role of carbohydrates in mediating the icROS-activating mechanism. When preincubating TNF α -primed neutrophils with methyl fucoside (LecB inhibitor), the LecB-induced activation of icROS production was inhibited in a concentration-dependent manner (Fig. 1D). Also when methyl fucoside was added during LecB-induced icROS production, added 5 min after addition of the lectin, similar inhibition was detected (Fig. 1E). Taken together, this demonstrates a critical role for carbohydrate-containing neutrophil epitopes in LecB-induced icROS production.

Equal amounts of LecB bind to primed and unprimed neutrophils

Neutrophil granule membranes and the plasma membrane are decorated with an array of fucosylated glycoconjugates [31] that could act as binding receptors for LecB. Using confocal microscopy, LecB binding to the unprimed neutrophil surface was qualitatively visualized using fluorescein-labeled LecB (Fig. 2A). Priming of neutrophils with LPS or TNF α is accompanied by an increase of receptors on the neutrophil surface due to fusion of intracellular receptor-containing granules with the plasma membrane [32]. We, therefore, quantified the LecB binding to the surface of unprimed and primed (37°C, LPS and TNF α) neutrophils using flow cytometry. Irrespective of the priming status, the binding levels of fluorescein-labeled LecB to the neutrophil cell surface appeared to be quantitatively similar and the binding of LecB was in all cases completely inhibited by the addition of methyl fucoside (Fig. 2B and C).

Next, we measured the binding of fluorescein-labeled LecB at different concentrations (0.015–3 μ M) to unprimed and TNF α -primed neutrophils. The binding increased with increasing concentrations, both in unprimed and TNF α -primed neutrophils (Fig. 2D). Correspondingly, icROS production was measured in TNF α -primed neutrophils using the same LecB concentrations as stimulus. LecB induced icROS production at a concentration of 1.5 μ M but the response was more pronounced at a concentration

amplified CL over time and the panel on the right confirms the inhibition is concentration dependent as shown using the quantitative area under the curve analysis (mean \pm SD, $n = 6$). (E). The addition of methyl fucoside (10 μ M), 5 min after the stimulating TNF α -primed neutrophil with LecB inhibited the LecB-induced icROS production as measured using luminol-amplified CL over time. For each figure, the left panel displays the time traces of one representative experiment, while the right panel presents the corresponding quantitative area under the curve analysis (mean \pm SD). The statistical analysis for (A), (C), and (D) was performed using Friedman test and nonparametric paired Wilcoxon test was used for (B) and (E). *, **, and *** indicate P value less than or equal to 0.05, 0.01, and 0.001, respectively.

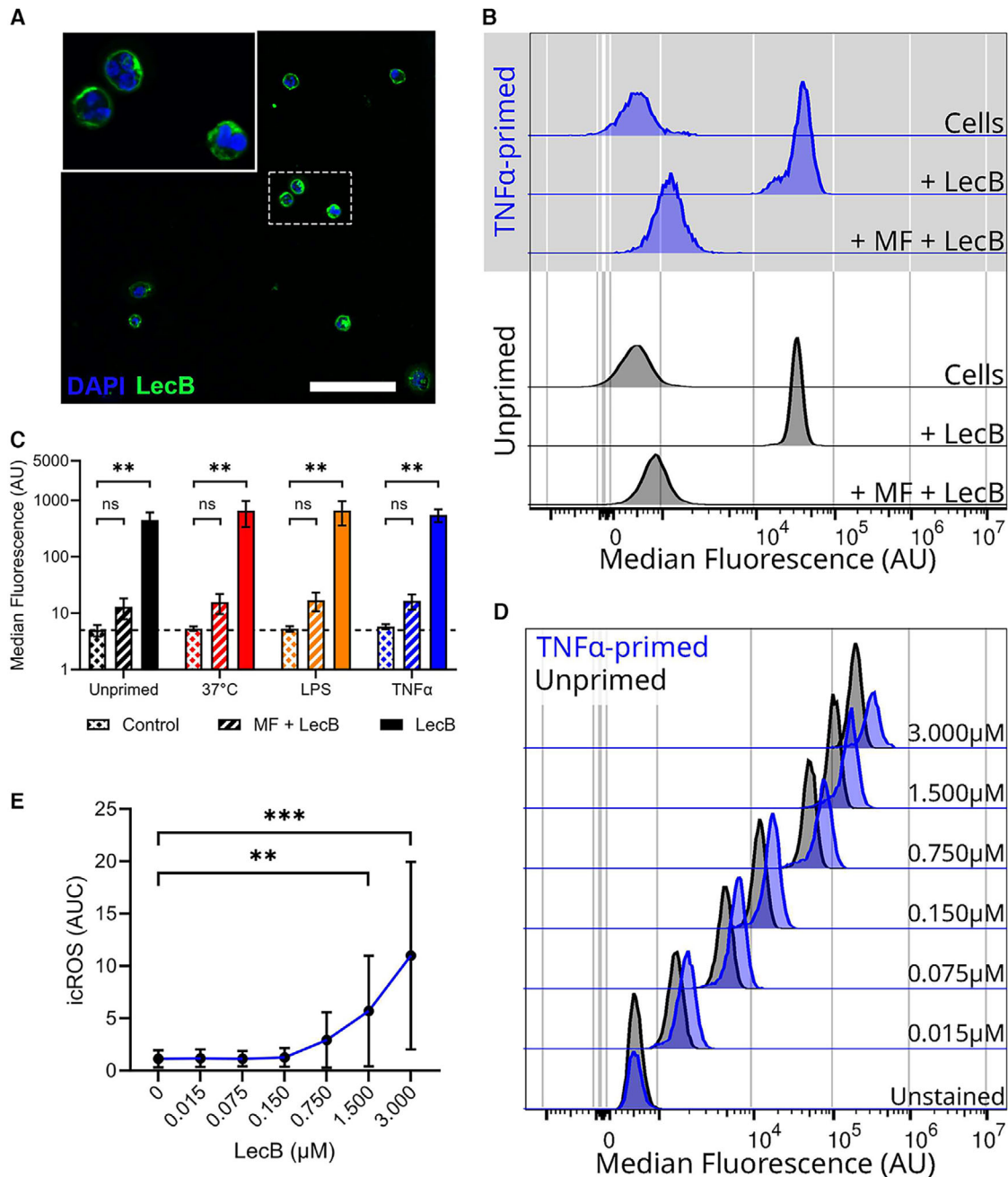


Figure 2. LecB binding to unprimed and primed neutrophils is similar. (A) Neutrophils were incubated with fluorescein-labeled LecB for 30 min (at RT) and placed on frosted microscopy slides for 15 min at 37°C. After fixation with 4% PFA, the slides were stained with DAPI, mounted and imaged by confocal microscopy. Window shows a close-up of the highlighted group of neutrophils. Scale bar corresponds to 50 μ m. (B) Unprimed and TNF α -primed neutrophils were incubated with fluorescein-labeled LecB, in the presence and absence of methyl fucoside (10 μ M) and binding was analyzed using flow cytometry. (C) Unprimed and primed (37°C, LPS, or TNF α) neutrophils were either incubated with buffer control, fluorescein-labeled LecB or methyl fucoside (10 μ M) with fluorescein-labeled LecB and the binding was measured as median fluorescence using flow cytometry. (D) TNF α -primed neutrophils were incubated with different concentrations of fluorescein-labeled LecB and measured using flow cytometry. (E) LecB-induced icROS production in TNF α -primed neutrophils is concentration-dependent as measured using luminol-amplified CL over time. The quantitative area under the curve showing statistically significant icROS production induced by LecB at concentrations above 1.5 μ M is represented as mean \pm SD, $n = 7$. The statistical analysis in (C) and (E) was performed using the Friedman test. ** and *** indicate P -value less than or equal to 0.01, and 0.001, respectively.

of 3 μM (Fig. 2E). Hence, both LecB binding and activation of icROS production were clearly dose-dependent. The concentrations of LecB used were chosen based on the optimal levels required to elicit a detectable response within our in vitro assays and do not necessarily represent the in vivo LecB concentrations during *P. aeruginosa* infection.

LecB does not induce intracellular Ca^{2+} signaling or NET formation but can bind to NETs

To better understand the functional effects that LecB induces in neutrophils we tested the ability of LecB to trigger Ca^{2+} mobilization from intracellular stores, as ROS production is often preceded by such a Ca^{2+} release. LecB did not induce any intracellular Ca^{2+} signals in unprimed (Fig. 3A) or $\text{TNF}\alpha$ -primed neutrophils (Fig. 3B), in contrast to the rapid and transient Ca^{2+} signals induced by the potent neutrophil chemoattractant N-formyl-methionine-leucyl-phenylalanine (fMLF; Fig. 3A, B).

As icROS has previously been shown to trigger NET release, we also tested whether a similar response could be induced by LecB stimulation and quantified extracellular DNA using a sytox green fluorescence assay [33]. Neutrophils typically formed NETs from 2 h after addition of PMA (positive control; Fig. 3C). However, LecB did not, during 4 h of measurement, induce NETs in unprimed (Fig. 3C) or $\text{TNF}\alpha$ -primed neutrophils (Fig. 3D). The absence of NET structures in LecB stimulated neutrophils was also confirmed by microscopic examination (data not shown).

Since LecB is a bacterial surface lectin we investigated whether LecB can bind to already released NETs, known to trap bacteria as an antimicrobial feature. To test this, we induced NETs by incubating neutrophils with PMA for 3 h on a microscopy slide and added fluorescein-labeled LecB to the slides in the presence and absence of the inhibitor methyl fucoside. The slides were then analyzed by confocal microscopy after staining the NETs for DNA and myeloperoxidase (MPO), an established NETs constituent. As seen in Fig. 3E, fluorescein-labeled LecB colocalized with MPO and DNA, indicating that LecB binds to NETs. The addition of methyl fucoside completely blocked the binding (Fig. 3E), demonstrating that the binding is carbohydrate-dependent.

LecB inhibits *P. aeruginosa* induced icROS

Phagocytosis is one of the most critical neutrophil functions to eliminate pathogenic microorganisms. During phagocytosis, icROS is generated in the phagolysosome, as one of the many neutrophil antimicrobial mechanisms [6]. We measured icROS production in unprimed neutrophils exposed to human-serum-opsinized *P. aeruginosa* (mPAO1) and found that phagocytosis of the bacteria-induced clear icROS production. The level of response was dependent on the MOI, that is, the number of bacteria per neutrophil. MOI of 30 showed a stable and high icROS response (Fig. 4A) and was therefore used in further experiments. Serum opsonization was necessary for the activation to occur as

unopsonized *P. aeruginosa* induced negligible amounts of icROS production even at MOI 30 (Fig. S2).

To investigate whether LecB influences the phagocytosis-induced icROS production, the lectin was added to the cells 5 min prior to the addition of mPAO1. Interestingly, the phagocytosis-induced icROS production was much lower in the presence of LecB, that is, LecB inhibited the phagocytosis-induced icROS production (Fig. 4B and C). Interestingly, this inhibiting effect was opposite to the LecB-induced direct activation of icROS production (Fig. 1A), that is, soluble LecB can directly activate icROS production but also inhibit phagocytosis-induced icROS.

To gain insights into the inhibitory mechanism mediated by LecB, we introduced the lectin to neutrophils during an ongoing phagocytosis-induced icROS response, choosing 20 min after the addition of bacterial prey in order to coincide with a stage where the phagocytic process must have advanced significantly, and the induced response is midway toward reaching its peak. Upon addition of LecB, the ongoing response was rapidly inhibited (Fig. 4D, E), suggesting that the soluble LecB interacts with the neutrophils per se and not with the bacteria that are being phagocytosed, inducing an inhibitory signal that blocks further icROS production related to the phagocytic process. The LecB-mediated inhibition of phagocytosis-induced icROS is in fact not specific to *P. aeruginosa* as the same effect was observed for *Staphylococcus aureus*-induced icROS production (Fig. 4F). While LecB inhibited phagocytosis-induced icROS, it did not inhibit PMA-induced icROS or fMLF-induced icROS production in unprimed neutrophils (Fig. S3) suggesting that the inhibition is specific to phagocytosis-induced NADPH-oxidase activation.

LecB-deficient *P. aeruginosa* induces higher icROS production compared with the WT strain

Since exogenously added LecB inhibited the mPAO1-induced icROS production (Fig. 4B–E), we hypothesized that endogenous surface expressed LecB, could potentially influence the phagocytosis-induced response. When investigating the LecB-deficient mutant strains PW6664 and PW6665, both derived from the WT mPAO1 strain, it was evident that both the mutated strains induced higher icROS production than the LecB-expressing WT mPAO1 strain (Fig. 5A–C). This further strengthens the involvement of LecB in inhibiting phagocytosis-induced icROS production.

Discussion

Lectins are found in almost all cell types and are important to mediate cell interactions and thereby play critical roles in health and diseases. The significance of the *P. aeruginosa* lectin LecB frequently revolves around its involvement in host-bacterium interactions, where it plays a crucial role in adhesion and colonization through its binding to carbohydrate structures on host cells. We have recently published a comprehensive analysis of neutrophil

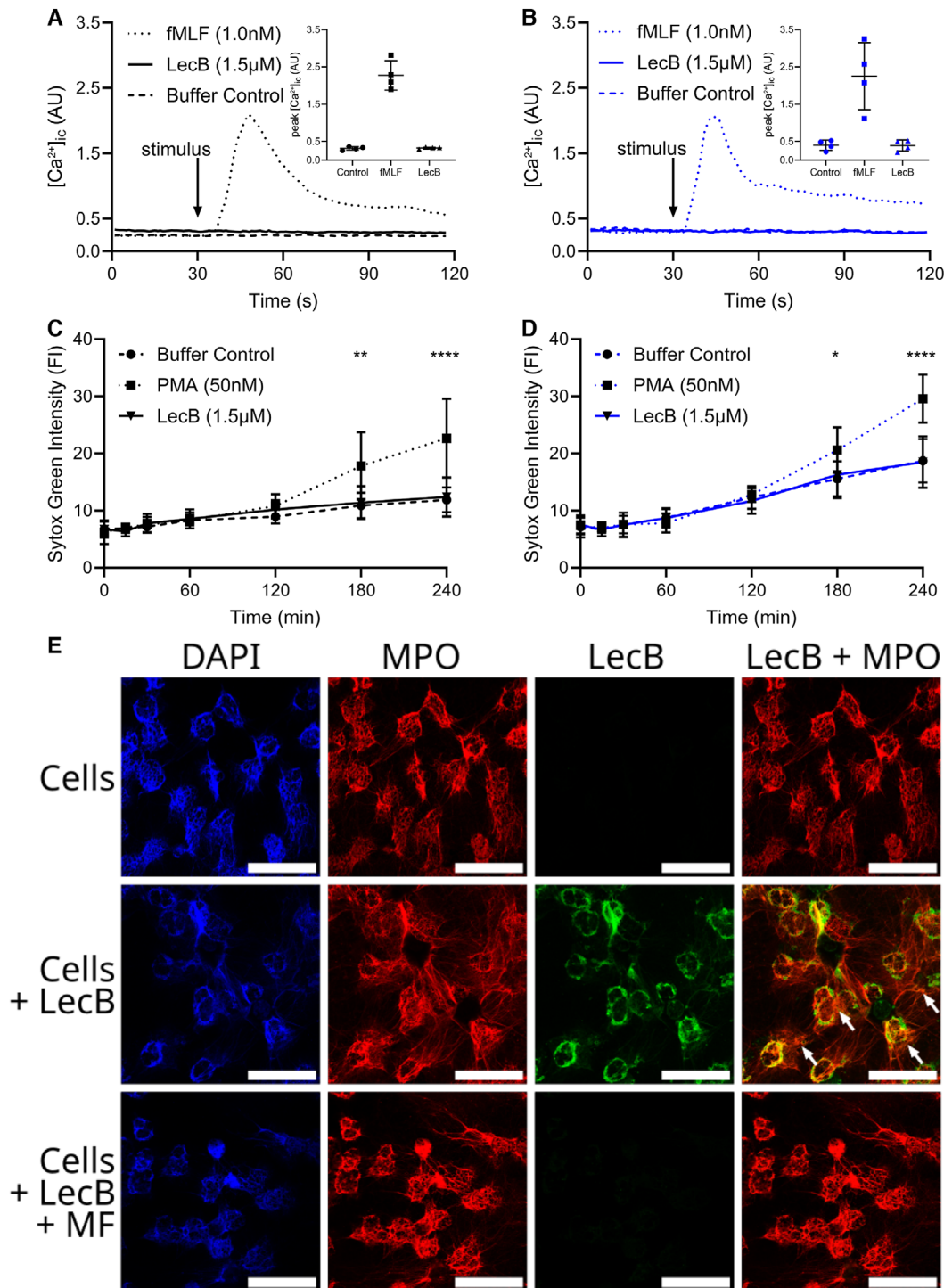


Figure 3. LecB does not induce intracellular Ca²⁺ response or NETosis in neutrophils but binds to PMA-stimulated NET structures. Representative Ca²⁺ responses of neutrophils to fMLF (1.0 nM), LecB (1.5 μ M), and buffer control as stimuli based on four independent experiments with (A) unprimed neutrophils and (B) TNF α -primed neutrophils, shown here. For investigation of NET formation in response to LecB, neutrophils were incubated with LecB (1.5 μ M), PMA (50 nM; positive control), or buffer (spontaneous NET formation) as a control for 4 h. Corresponding quantitative data is shown in the figure insets. The formation of NETs in unprimed (C) and TNF α -primed (D) neutrophils was measured with the Sytox green (DNA-labeling probe) and at regular intervals using a plate reader. Data represented as mean \pm SD from four biological replicates each with three technical repeats. (E) NETosis was induced by incubating unprimed neutrophils with PMA (50 nM) for 3 h on microscopy slides. After incubation, the samples were incubated for 30 min at 4°C with fluorescein-labeled LecB in the absence (cells + LecB) and presence of methyl fucoside (10 μ M; Cells + LecB + MF). After fixation with 4% PFA, the samples were stained for DNA (DAPI), and MPO (AF647) and imaged on a confocal microscope. Representative micrographs of three independent experiments. Scale bar corresponds to 50 μ m. The statistical analysis in (C and D) was performed using two-way ANOVA. *, **, and **** indicate P-value less than or equal to 0.05, 0.01, and 0.0001 respectively.

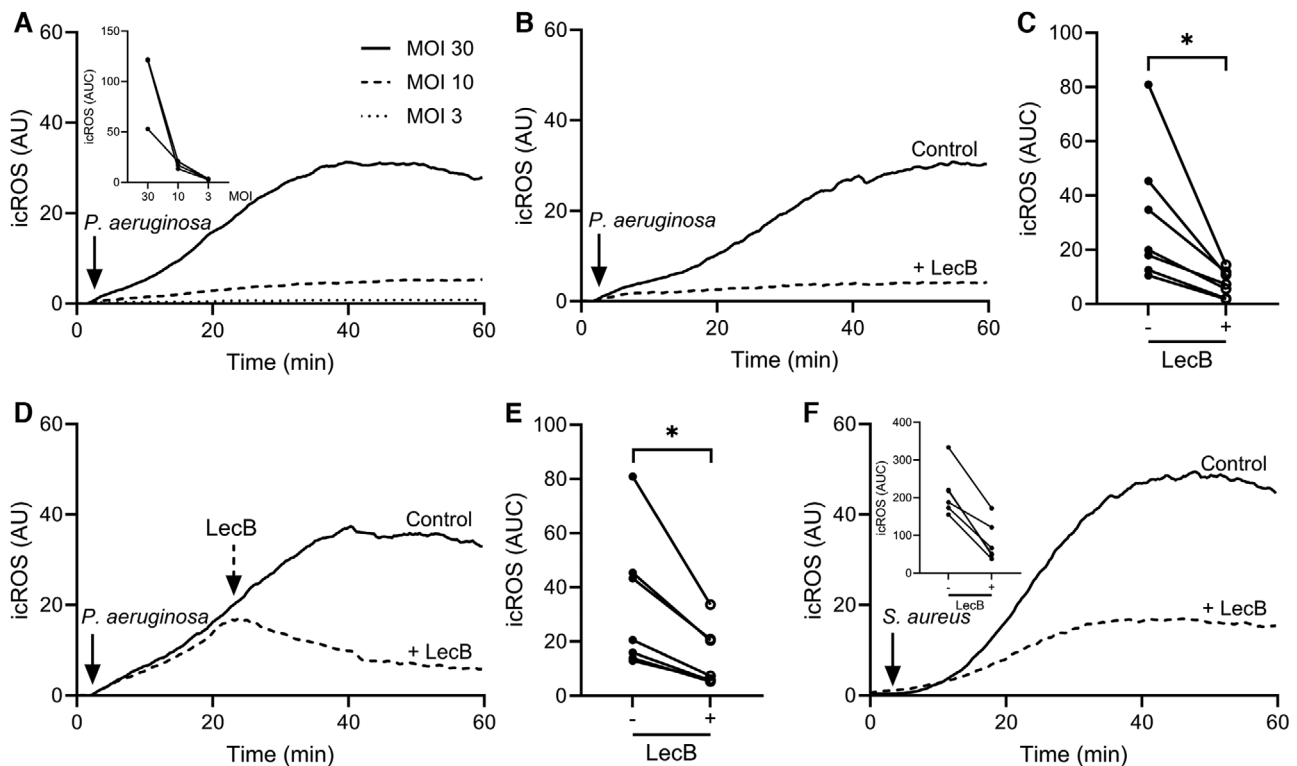


Figure 4. LecB inhibits phagocytosis-induced icROS production in neutrophils stimulated by *P. aeruginosa*. (A) Suspensions of 10% serum opsonized *P. aeruginosa* at MOI 3 (dotted line), 10 (dashed line), and 30 (solid line) were added to unprimed neutrophils (as shown using arrow) and luminol-amplified CL was measured over time. Representative phagocytosis-induced icROS production kinetics is shown ($n = 4$) and the corresponding quantitative area under the curve is shown in the inset. (B) Suspensions of 10% serum opsonized *P. aeruginosa* at MOI 30 was added to neutrophils preincubated without (solid line) and with (dashed line) LecB ($1.5 \mu\text{M}$) for 5 min at 37°C and luminol-amplified CL was measured over time. Representative phagocytosis-induced icROS production kinetics is shown ($n = 7$) and the corresponding quantitative area under the curve values is shown in (C). (D) Suspensions of 10% serum-opsonized *P. aeruginosa* at MOI 30 were added to neutrophils as shown using arrow and LecB was added after 20 min during the ongoing kinetic response (as shown using dashed arrow). Representative phagocytosis-induced icROS production kinetics is shown ($n = 7$) and the corresponding quantitative area under the curve values is shown in (E). (F) Suspensions of 10% serum opsonized *S. aureus* at MOI 10 was added to neutrophils preincubated without (solid line) and with (dashed line) LecB for 5 min at 37°C and luminol-amplified CL was measured over time. Representative phagocytosis-induced icROS production kinetics is shown ($n = 5$) and the corresponding quantitative area under curve is shown in the inset. The statistical analysis was performed using nonparametric paired Wilcoxon test and * indicate P-value less than or equal to 0.05.

glycan composition, revealing the presence of fucosylated epitopes that are potential binding sites for LecB on both the plasma membrane and the intracellular granule membranes. [31]. It can be assumed that only plasma membrane-localized glycans are important for LecB interaction with neutrophils, but the granule-localized glycans can be mobilized by priming. Neutrophils contain at least four different intracellular organelles, such as hard-to-mobilize azurophilic granules, not-so-hard-to-mobilize specific and gelatinase granules, and easy-to-mobilize secretory vesicles. While priming by 37°C -incubation can mobilize secretory vesicles, only $\text{TNF}\alpha$ and LPS priming can mobilize specific and gelatinase granules as well [32, 34]. In vitro priming by $\text{TNF}\alpha$ and LPS can mimic the in vivo priming that takes place during extravasation [35]. LecB binds predominantly to fucosylated epitopes and to some extent mannose as well, carbohydrates which are quite abundant on both plasma membrane and specific/gelatinase granule membranes [31]. The presence of abundant LecB binding carbohydrates on the plasma membrane and on the surface of mobilizable granule membranes, explains our findings of similar

fluorescent LecB binding intensity to both unprimed and primed neutrophils (Fig. 2C). This could likely be due to the saturation of lectin binding capacity. Apart from neutrophils, LecB also binds to other white blood cells, including monocytes and lymphocytes (Fig. S4), which also possess fucose-containing glycocalyx [36].

Priming with $\text{TNF}\alpha$ or LPS appears not to be a prerequisite for the binding of LecB to neutrophils, but it is indeed essential for LecB-induced icROS production. Since LecB can only induce icROS in $\text{TNF}\alpha$ - or LPS-primed neutrophils, it is reasonable to suggest that the yet unidentified functional receptor(s) responsible for LecB-induced icROS activation are localized in the specific/gelatinase granule membrane of resting cells and can be mobilized to the cell surface upon priming. Therefore, it is of paramount importance to identify the receptors involved in LecB binding to the neutrophil plasma membrane and granule membranes, respectively, to allow for elucidation of the downstream functional events following LecB binding to the cells. The LecB-induced activation of icROS is very similar to the effect seen using the human lectins galectin-1 and 3 [10, 12] as well as the plant

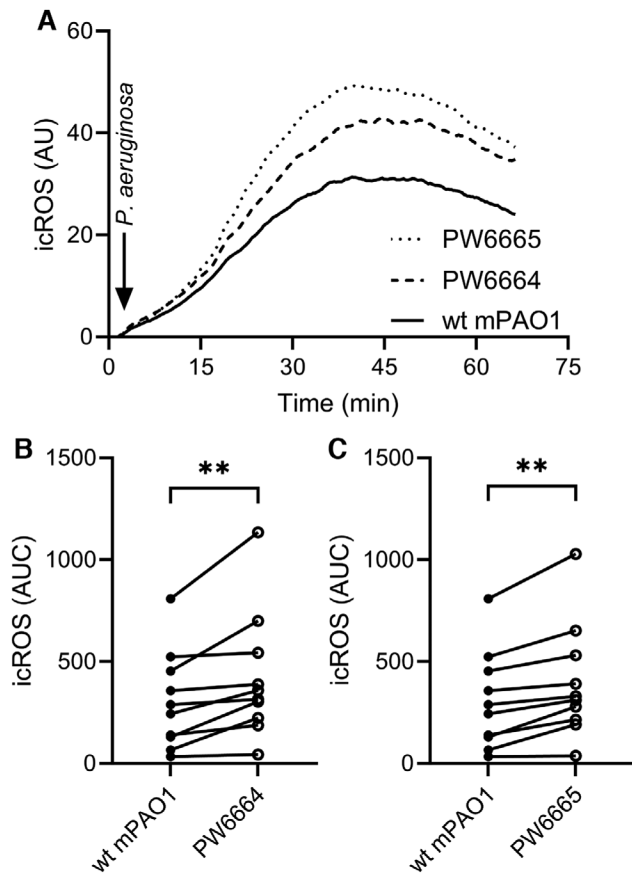


Figure 5. LecB-deficient *P. aeruginosa* strains induce higher phagocytosis-induced icROS production than WT mPAO1 strain. (A) Suspensions of 10% serum opsonized WT *P. aeruginosa* strain (mPAO1) and corresponding LecB-deficient strains (PW6664 and PW6665) were added to neutrophils (as shown by arrow) and luminol-amplified CL was measured over time. Representative phagocytosis-induced icROS production kinetics is shown ($n = 10$) and the corresponding quantitative area under the curve values were calculated (mean \pm SD, $n = 10$; B and C). The statistical analysis was performed using nonparametric paired Wilcoxon test and ** indicate a P -value less than or equal to 0.01.

lectin WGA [11]. Unlike galectins and WGA, LecB does not induce ecROS release from neutrophils, regardless of priming status; this specific induction of icROS appears to be a unique feature of LecB. This implies that there are distinct receptor(s) involved in each lectin-neutrophil interaction, probably due to different receptor carbohydrate composition.

The precise localization of icROS has been a debatable topic in neutrophil cell biology. The prevailing view is however that icROS can either be localized in heterotypically fused organelles (consisting of azurophil and specific granules) or in granule-fused phagolysosomes during phagocytosis [6]. We have shown that soluble LecB directly activates icROS production (Fig. 1A) but inhibits the phagocytosis-induced icROS production (Fig. 4B, D), which was intriguing. The inhibition of phagocytosis-induced icROS is further evidenced by the experiments using LecB-deficient *P. aeruginosa* strains, which induced increased icROS as compared with the WT strain (Fig. 5A–C). We have recently

published a similar finding of activation and inhibition effect induced by the addition of galectin-3 [37].

When adding soluble LecB during bacterial phagocytosis, the localization of LecB is likely influenced by the action of specific endocytic/phagocytic receptors. Prior research by Thunauer et al. [38] has demonstrated that LecB strongly interacts with glycans on $\beta 1$ -integrins located on the plasma membrane of epithelial cells, leading to rapid integrin endocytosis. Since integrins also serve as phagocytic receptors on neutrophils, it is reasonable to hypothesize that the presence of soluble LecB during phagocytosis might result in LecB uptake into phagosomes. This hypothesis has prompted our investigation into whether LecB if taken up into phagolysosomes during phagocytosis, can inhibit intraphagosomal MPO by binding to its fucose/mannose moieties [39, 40]. However, the enzymatic activity of MPO was not altered after incubating native human MPO with soluble LecB (Fig. S5), which suggests that LecB inhibition of MPO is not the cause of inhibition of the phagocytosis-induced icROS. Therefore, while it is plausible that LecB could be found within phagosomes, the specific signaling events governing the modulation of phagocytosis-induced icROS production remain a subject for further inquiry.

The efficient LecB-mediated inhibition of phagocytosis-induced icROS production in neutrophils could potentially result in improved survival of *P. aeruginosa* in the neutrophil phagolysosome. *P. aeruginosa* also utilizes extracellular Psl polysaccharides to evade neutrophil phagocytosis and subsequent ROS production [41]. *P. aeruginosa* has been shown to reduce ROS production by utilizing its effector proteins ExoS and ExoT and the inhibition depends on the ADP-ribosyltransferase activities of the proteins [42]. In this study, using LecB-deficient strains we have shown how *P. aeruginosa* can utilize LecB to reduce phagocytosis-induced icROS production. Taken together, we can speculate that *P. aeruginosa* appears to use multiple virulence factors to evade from neutrophil-triggered immune defense.

While it has been demonstrated that soluble LecB can elicit partial calcium release in B cells [43], our present study did not reveal any transient increase in intracellular calcium levels upon the addition of soluble LecB, in both unprimed and TNF α -primed neutrophils. To the best of our knowledge, neither the influence of LecB attached to the bacterial surface on induction of intracellular calcium signaling in neutrophils has been previously investigated. In contrast to LecB, other soluble lectins such as galectin-3 [44] and WGA (unpublished data) induce a transient calcium response. With regards to NET formation, our findings indicate that soluble LecB does not induce NET formation in vitro, but there is no information in the literature on the effect of LecB-expressing *P. aeruginosa* on NET formation. Our study is the first to provide evidence that soluble LecB does not induce calcium signaling and NET formation in vitro. However, the potential role of surface-bound LecB on living *P. aeruginosa* bacteria in mediating these responses remains an area that warrants further investigation. We acknowledge that this aspect is yet to be explored, and we hope that our data stimulate future research in this direction.

The presence of NET structures is a common feature in *P. aeruginosa*-infected CF lungs [45]. *P. aeruginosa* is a potent

inducer of NETosis [46, 47], but the specific mechanisms triggering NET release are still unknown. Using liquid chromatography and mass spectrometry, we and others have identified an abundance of azurophil granule proteins, including MPO, neutrophil elastase, cathepsin G, and azurocidin, in sputum from *P. aeruginosa* infected CF patients [39, 48]. We profiled the glycosylation pattern in CF sputum, particularly the glycans on azurophil granule proteins. One common feature of those azurophil granule proteins was the presence of abundant paucimannosidic glycans [39, 40]. Paucimannosidic glycans carry both mannose and fucose that could potentially act as binding partners to LecB. As seen in Fig. 3D, the binding of LecB to PMA-induced NETs co-localized with MPO, and this interaction was inhibited using methyl fucoside, suggesting that LecB may bind to the paucimannosylated glycoproteins. To be remembered though is that even if LecB binds to MPO it does not alter its enzymatic activity (Fig. S5). Therefore, we can speculate that *P. aeruginosa* carrying LecB utilizes the lectin as an adhesion molecule by binding to the NET-associated proteins and thereby may assist the bacteria to further colonize and form biofilms.

The ability of the surface-bound LecB on *P. aeruginosa* to bind to host glycoproteins [49, 50] suggests that these lectins are involved in bacterial adhesion to host tissues. In support of that, studies have shown that the binding of the LecB-negative *P. aeruginosa* mutant strain to human lung epithelial cell line A549 [28] is significantly decreased compared with the WT PAO1 strain, the latter being inhibitable by methyl fucoside. CF sputum is characterized by decreased sialylation and increased fucosylation of mucins (as reviewed in [51]), offering preferential binding sites for LecB which can promote bacterial adhesion in the lung. Supporting that, CF epithelial cells with differential glycosylation patterns than the normal epithelium were shown to provide better binding sites for *P. aeruginosa*, facilitated by LecB [52], thus establishing the fact that LecB is critical for bacterial adherence and colonization. To our knowledge, there is no previous study on LecB-mediated neutrophil-*P. aeruginosa* interaction. Our study has shown the importance of LecB in this context and set a necessary platform to further explore the involvement of this key lectin for *P. aeruginosa* evasion of neutrophil defense.

The physiological implications of soluble LecB-induced direct activation of iCROS and its inhibitory effect on phagocytosis-induced iCROS production are both intriguing and multifaceted. One pivotal question arises: who benefits most from these effects of soluble LecB the host or the bacteria? The production of ROS is indeed a double-edged sword, serving as a critical anti-microbial host defense mechanism. However, when produced excessively at the site of infection, ROS can inflict collateral damage on surrounding tissues. Neutrophils, upon deployment to infection sites, undergo priming and activation, rendering them highly responsive to external stimuli. In this scenario, the presence of soluble LecB may cause a local, continuous stimulation of neutrophils, driving the induction of ROS production and consequently tissue damage and inflammation. Conversely, during the phagocytosis of *P. aeruginosa*, soluble LecB inhibits this intracellular ROS production. This modulation gains particular significance during *P.*

aeruginosa infections, considering the probable presence of soluble LecB in close proximity to the bacteria. Consequently, *P. aeruginosa* might employ the inhibitory effect of LecB to safeguard itself from bactericidal ROS within the phagolysosome. Such a strategy could be of paramount importance during chronic *P. aeruginosa* infections in immunocompromised patients. Taking these two effects together, an increased, ROS-dependent tissue destruction and inflammation and a protection of intraphagosomal bacteria, suggests that LecB is a potent virulence factor primarily on the side of the pathogen.

In summary, we report for the first time that *P. aeruginosa* lectin LecB is a potent activating and inhibiting modulator of neutrophil ROS production. While soluble LecB binds strongly to unprimed and primed neutrophils, it activates iCROS only in primed neutrophils and inhibits phagocytosis-induced iCROS during *P. aeruginosa* phagocytosis, which was also supported by LecB-deficient mutant strains. The exact molecular mechanisms involved in the activating and inhibiting signaling pathways remain to be investigated. Our findings suggest that LecB could be a potential target to improve host immune defense against *P. aeruginosa* infection.

Data limitations and perspectives

Our study primarily relies on in vitro experiments with isolated human neutrophils. While this approach offers valuable insights into direct interactions of LecB with neutrophils, the complexities of in vivo host-pathogen interactions that have not been addressed are acknowledged. To address these limitations, future studies should extend their scope by investigating LecB's impact on neutrophil function within infection models. Despite our progress in understanding LecB's effects on neutrophils, the underlying signaling mechanisms remain uncharted territory. We need to elucidate processes following LecB binding to cells — whether they are receptor-induced signals or cellular uptake mechanisms driving ROS production. Such deepened understanding is pivotal for decoding not only lectin-induced neutrophil responses but also the role played by LecB in *Pseudomonas* infection. Therefore, we underscore the importance of identifying LecB-binding receptors involved in neutrophil activation. Utilizing mass spectrometry to identify LecB binding molecules, focusing on granule-localized mobilizable membrane receptors, would be the first step after which exploring the full spectrum of receptors and delineating their specific roles in LecB signaling is a much-coveted goal for future research.

Materials and methods

Isolation of neutrophils

Neutrophils from buffy coats (obtained from the blood bank at Sahlgrenska University Hospital) were isolated by dextran sedimentation and Ficoll-paque (Cytiva, GE-Healthcare Bioscience)

separation [53]. Remaining erythrocytes were removed by hypotonic lysis, and isolated granulocytes, with a minimum purity of 95%, predominantly consisting of neutrophils, were resuspended in Krebs Ringer phosphate buffer supplemented with 1 mM Ca^{2+} . For some experiments, isolated blood neutrophils were primed with either $\text{TNF}\alpha$ (10 ng/mL) or LPS (1 $\mu\text{g}/\text{mL}$) at 37°C for 20 min and stored on ice until use. Also, neutrophils were also incubated at 37°C for 20 min without any priming agent as a control for temperature-induced priming.

Preparation of bacterial strains

P. aeruginosa WT mPAO1 strain and its corresponding LecB-deficient mutant strains (PW6664 and PW6665) were grown aerobically on Luria-Bertani agar at 37°C, overnight and single colonies were added to Luria-Bertani broth, cultured overnight at 37°C on an orbital shaker. Bacterial growth curves were determined by measuring OD at 600 nm, and colony-forming units (CFU/mL) were counted. GFP-tagged *S. aureus* strain 8325-4 was inoculated in tryptic soy broth supplemented with 10 $\mu\text{g}/\text{mL}$ chloramphenicol, cultured aerobically overnight at 37°C on an orbital shaker and enumerated using an Accuri C6 flow cytometer (Becton Dickinson). *P. aeruginosa* strains and *S. aureus* were subsequently washed, diluted to a concentration of 10^9 cells/mL in Krebs–Ringer PBS (KRG; pH 7.3) with 1 mM Ca^{2+} , and stored in aliquots at -80°C for future use.

Isoluminol and luminol amplified chemiluminescence

ecROS and icROS generated by neutrophil NADPH-oxidase were determined by isoluminol and luminol-amplified chemiluminescence (CL), respectively, in a ClarioStar plate reader (BMG Labtech) [54]. The assays were performed in 96-well white microtiter plates with a total volume of 200 $\mu\text{L}/\text{well}$. For icROS measurements, luminol (50 μM , Sigma-Aldrich) was added together with the extracellular radical scavengers superoxide dismutase (50 U/mL, Worthington) and Catalase (2000 U/mL, Worthington) and mixed with neutrophils (2×10^5 cells/well). For ecROS measurements, isoluminol (50 μM , Sigma-Aldrich) and horseradish peroxidase (4 U/mL, Roche Diagnostics) was mixed with neutrophils (2×10^5 cells/well). The cells were equilibrated for 5 min at 37°C before the stimuli was added, and CL was recorded over 30–65 min. Stimuli used: soluble LecA and LecB lectins (concentration as stated in each figure legend, Elicityl), 10% normal human serum opsonized and unopsonized WT mPAO1 strain, opsonized LecB-deficient mutant strains PW6664 and PW6665 [55] and opsonized *S. aureus* (at a MOI, i.e., microbes per neutrophils as stated in each figure legend). During runs with bacteria as the stimuli, LecB was also added either before equilibration or added to the ongoing measurement, as stated in each figure legend. When the LecB inhibitor methyl fucoside (TCI Chemicals, Germany, cat no. M1051) was used, this was

added during measurements to a final concentration mentioned in the figure legend.

Lectin–neutrophil interaction analysis

Surface binding of LecB was assessed in unprimed and 37°C-, LPS-, and $\text{TNF}\alpha$ -primed neutrophils. Neutrophils were incubated with fluorescein-labeled LecB (1.5 μM) in the presence and absence of methyl fucoside (10 μM) for 30 min on ice. In addition, the cells were additionally-labeled with CD45-BV785 (BioLegend, cat#304048) and APC-conjugated Annexin-V (Invitrogen, cat#A35110). Finally, 7AAD (BD, Cat#51-68981E) was added directly before flow cytometry (CytoFlex, Beckman Coulter) analysis. Neutrophils were gated based on CD45 and forward- and side-scatter properties. Annexin-V+ (apoptotic cells) and 7AAD+ (necrotic cells) were excluded from the analysis (gating strategy in Fig. S6).

Unprimed neutrophils incubated with fluorescein-labeled LecB were allowed to adhere to superfrost microscopy slides, and placed in a 37°C incubator for 15 min. The cells were fixed with 4% paraformaldehyde (PFA) for 10 min in RT (dark). Slides were stained with DAPI (1 $\mu\text{g}/\text{mL}$) for 5 min in RT (dark) and mounted with ProLong Gold antifade medium (Invitrogen) followed by visualization with a 40 \times objective using a Leica SP8 confocal microscope controlled by the Leica Application Suite X software.

Measurement of cytosolic Ca^{2+} concentration

Unprimed and $\text{TNF}\alpha$ -primed neutrophils were labeled with Fluo-3 AM (ThermoFisher; 3.6 $\mu\text{g}/\text{mL}$) and Fura-Red (ThermoFisher; 10 $\mu\text{g}/\text{mL}$) in cell loading medium (KRG supplemented with Ca^{2+} with 1% heat-inactivated FCS) for 30 min, at 37°C. After washing and re-suspension in the cell loading medium, cells were incubated for 5 min at 37°C before flow-cytometric analysis. Baseline fluorescence was monitored for 30 seconds, followed by stimulation with LecB (1.5 or 7.5 μM), fMLF (1 nM), or buffer. Fluorescence emission of both Fluo-3 and Fura red were monitored simultaneously and a ratio (reflecting the relative cytosolic [Ca^{2+}]) between the dyes was calculated [56].

Measurement of neutrophil extracellular traps

Unprimed neutrophils in RPMI (without phenol red) culture medium (Gibco) supplemented with Sytox Green DNA stain (1.25 μM , Molecular Probes) was added to 96-well black microtiter plates (Thermo Scientific; 5×10^4 cells/well. Soluble LecB (1.5 μM) or PMA (50 nM) was added as stimuli and the plates were incubated for up to 4 h at 37°C and 5% CO_2 . Sytox Green fluorescence (485/535 nm) was measured at indicated time points in a ClarioStar plate reader (BMG Labtech).

Binding of LecB to NETs

Neutrophils were allowed to settle on sterilized and poly-lysine coated coverslips (placed within a 12-well plate) for 15 min at 37°C and 5% CO₂. NETosis was induced by addition of PMA (50nM). After 3 hours incubation with PMA, fluorescein-labeled LecB was added to the cell culture medium and the culture plate was incubated at 4°C for 30 min. Directly after, the culture medium was aspirated, and washed and the cells were fixed with 4% PFA at RT for 15 min. After washing and blocking with PBS (containing 10% normal donkey serum and 2% BSA) the cells were stained for MPO with rabbit-anti-human MPO (1:200, DAKO 0398) or isotype for 30 min at RT, and coupled with secondary antibody AF647 (IgG (H+L), Molecular Probes, A31573) for additional 30 min at RT. After final washing, the slides were mounted with ProLong Gold antifade medium containing DAPI (Invitrogen) onto microscopy slides. The samples were visualized with a 40× objective using a Leica SP8 confocal microscope controlled by the Leica Application Suite X software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.9.01 (GraphPad Software). Data represented as mean ± SD or mean ± SEM and either the Friedman test followed by Dunn's multiple comparison test or Wilcoxon matched-pairs signed rank test was used for comparison of datasets.

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Ethics approval statement: Buffy coats were obtained from the hospital blood bank after de-identification and according to the Swedish legislation section code 4§ 3p SFS 2003:460, no informed consent is needed.

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Abbreviations: **icROS:** intracellular ROS · **ecROS:** extracellular ROS
· **CL:** chemiluminescence · **NETs:** neutrophil extracellular traps

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