

Released Bacterial ATP Shapes Local and Systemic Inflammation during Abdominal Sepsis

Reviewed Preprint

v2 • July 15, 2024

Revised by authors

Reviewed Preprint

v1 • April 19, 2024

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Abstract

Sepsis causes millions of deaths per year worldwide and is a current global health priority declared by the WHO. Sepsis-related deaths are a result of dysregulated inflammatory immune responses indicating the need to develop strategies to target inflammation. An important mediator of inflammation is extracellular adenosine triphosphate (ATP) that is secreted by inflamed host cells and tissues, and also by bacteria in a strain-specific and growth phase-dependent manner. Here, we investigated the mechanisms by which bacteria release ATP. Using genetic mutant strains of *Escherichia coli* (*E. coli*), we demonstrate that ATP release is dependent on ATP synthase within the inner bacterial membrane. In addition, impaired integrity of the outer bacterial membrane and bacterial death notably contribute to ATP release. In a mouse model of abdominal sepsis, local effects of bacterial ATP were analysed using a transformed *E. coli* bearing an arabinose-inducible periplasmic apyrase hydrolyzing ATP to be released. Abrogating bacterial ATP release shows that bacterial ATP suppresses local immune responses, resulting in reduced neutrophil counts and impaired survival. In addition, bacterial ATP has systemic effects via its transport in outer membrane vesicles (OMV). ATP-loaded OMV are quickly distributed throughout the body and upregulated expression of genes activating degranulation in neutrophils, potentially contributing to the exacerbation of sepsis severity. This study reveals mechanisms of bacterial ATP release and its local and systemic roles in sepsis pathogenesis.

eLife assessment

This **fundamental** study advances our understanding of the role of bacterial-derived extracellular ATP in the pathogenesis of sepsis. The evidence supporting the conclusions is **compelling**, although not all concerns from a previous round of reviews were adequately addressed. The work will be of broad interest to researchers on microbiology and infectious diseases.

<https://doi.org/10.7554/eLife.96678.2.sa3>

Introduction

Worldwide, 11 million sepsis-related deaths were reported in 2017, which accounted for an estimated 19.7% of all global deaths (Rudd et al., 2020 [DOI](#)). Given its high incidence and immense socio-economic burden, the World Health Organization (WHO) has declared sepsis as a global health priority (Reinhart et al., 2017 [DOI](#)).

Sepsis is defined as life-threatening organ dysfunction caused by an imbalanced host immune response to infection. Antibiotic treatment remains the main approach to treat sepsis; however, despite the use of broad-spectrum antibiotics, lethality remains high. Immune-modulating strategies are an additional approach to antibiotics to curb excessive inflammation and to support an effective defense against the infectious agents. Until recently however, most trials inhibiting cytokine responses or toll-like receptors failed, indicating that alternative approaches for immunomodulation are required (Cao et al., 2023 [DOI](#)).

It has been shown that adenosine triphosphate (ATP), as soon as it is secreted into the extracellular space, critically modulates inflammatory and immune responses (Di Virgilio et al., 2020 [DOI](#); Eltzschig et al., 2012 [DOI](#)) by activating ionotropic P2X and metabotropic P2Y receptors (Burnstock, 2020 [DOI](#); Junger, 2011 [DOI](#)). Also, such purinergic signaling critically alters immune responses during sepsis (Dosch et al., 2019 [DOI](#); Ledderose et al., 2016 [DOI](#); Spari & Beldi, 2020 [DOI](#)). In particular, we have described that the connexin-dependent secretion of ATP by macrophages initiates an autocrine loop of over-activation, resulting in altered local and systemic cytokine secretion, which exacerbates abdominal sepsis (Dosch et al., 2019 [DOI](#)). Such sepsis-promoting effects of host-derived extracellular ATP are secondary to inflammation initiated by bacterial pathogens.

Recently, it has been discovered that also bacteria release ATP into the extracellular space (Mempin et al., 2013 [DOI](#)). Such ATP release might be a conserved mechanism of protection from host defense and precede host responses. ATP release has been shown for a variety of bacteria including the sepsis-associated *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) from the Proteobacteria phylum or *Enterococcus faecalis* (*E. faecalis*) and *Staphylococcus aureus* (*S. aureus*) from the Firmicutes phylum (Diekema et al., 2019 [DOI](#); Hironaka et al., 2013 [DOI](#); Iwase et al., 2010 [DOI](#); Mempin et al., 2013 [DOI](#); Murejan et al., 2018).

The mechanisms by which inflammatory and immune responses in the host are modulated by such released bacterial ATP have just begun to be elucidated (Spari & Beldi, 2020 [DOI](#)). In colonized compartments such as the intestine, it has been shown that ATP released by mutualistic bacteria modulates local cellular and secretory immune responses (Atarashi et al., 2008 [DOI](#); Perruzza et al., 2017 [DOI](#); Proietti et al., 2019a [DOI](#)) and in the mouth, bacterial ATP release leads to biofilm dispersal and periodontitis (Ding et al., 2016 [DOI](#); Ding & Tan, 2016 [DOI](#)). However, the role of ATP released by bacteria invading non-colonized compartments, such as the abdominal cavity or the blood in the context of local and systemic infections, remains to be determined.

In this study, we investigated if ATP released from bacteria influences the outcome of abdominal sepsis. We first isolated sepsis-associated bacteria and measured the amount of ATP they release. Second, we analysed the importance of ATP generation at the inner bacterial membrane on ATP release over time in *E. coli*, using *E. coli* with mutations in integral respiratory chain proteins (Mempin et al., 2013 [DOI](#)). Third, the function of the outer bacterial membrane on ATP release during exponential growth was assessed using porin mutants (Alvarez et al., 2017 [DOI](#); Choi & Lee, 2019 [DOI](#)). Fourth, we investigated local effects of ATP in the abdominal cavity. Lastly, based on the finding that bacteria secrete outer membrane vesicles (OMV) (Schwechheimer & Kuehn, 2015 [DOI](#)), which contain ATP (Alvarez et al., 2017 [DOI](#); Pérez-Cruz et al., 2015 [DOI](#)), we investigated systemic consequences of OMV-derived bacterial ATP.

Results

***E. coli*, one of the major pathogens in sepsis, releases ATP in a growth phase-dependent and strain-specific manner**

To assess ATP release of sepsis-associated bacteria, abdominal fluid of patients with abdominal sepsis was sampled and an-/aerobically incubated on LB agar plates (**Figure 1A**). Twenty-five different colonies were randomly picked and analysed by whole 16S-rRNA sanger sequencing, which resulted in twelve different bacterial species (**Figure 1B**). From these, the four most clinically important sepsis-associated bacteria *E. coli*, *K. pneumoniae* (both gram^{neg}), *E. faecalis* and *S. aureus* (both gram^{pos}) (Diekema et al., 2019; Murejan et al., 2018) were further cultivated for experimental studies. We quantified released ATP over time (**Figure 1-figure supplement 1**) using a luciferin-luciferase-based assay. A growth phase-dependent release of ATP was observed in all species, peaking during exponential growth (**Figure 1C**). Cumulative amount of released ATP was quantified using the area under the curve (AUC) of released ATP over time (AUC ATP). ATP release was detected across all assessed species (**Figure 1D**). To model these findings in mice, abdominal sepsis was induced using a standardized cecal ligation and puncture model (Dosch et al., 2019) (**Figure 1E**). Similar to human samples, Proteobacteria and Firmicutes phyla were predominating (**Figure 1F**, see **Figure 1B**). In the mouse model, *E. coli* released notably more cumulative ATP compared to *E. faecalis* and *S. aureus* (**Figure 1G-H**). The bacterial species assessed in humans and mice (*E. coli*, *E. faecalis*, *S. aureus*) differed on the strain level (sequences deposited, see data availability statement) confirming that ATP release is strain-specific (Mempin et al., 2013). In summary, sepsis-associated bacteria release ATP in a growth phase-dependent and strain-specific manner. *E. coli*, which is one of the most frequent facultative pathogens in patients with abdominal sepsis, released the highest amount of ATP isolated from a standardized mouse model of abdominal sepsis.

Bacterial ATP release is dependent on ATP synthesis at the inner bacterial membrane and correlates with bacterial growth

After having demonstrated that sepsis-associated bacteria release ATP, we next questioned whether and how ATP release is dependent on ATP generation. ATP synthase and cytochrome oxidases, which are located in the inner bacterial membrane, are key components of ATP generation under aerobic conditions. Therefore, ATP release of the *E. coli* parental strain (PS) was compared to all available mutants of ATP synthase subunits ($\Delta atpA$, $\Delta atpB$, $\Delta atpC$, $\Delta atpD$, $\Delta atpE$, $\Delta atpF$, $\Delta atpH$) and cytochrome *bo3* oxidase subunits ($\Delta cyoA$, $\Delta cyoB$, $\Delta cyoC$, $\Delta cyoD$) from the Keio collection (Baba et al., 2006; Yamamoto et al., 2009). The use of different mutants allows to identify the most relevant subunits influencing ATP generation and release. Also it allows to identify a possible correlation of bacterial ATP release with growth, which is a function of ATP generation (**Figure 2A**) (Galber et al., 2021; Ihssen et al., 2021; Ivančić et al., 2008; Lundin, 2000).

Bacterial growth (OD₆₀₀) and ATP release were measured over time and cumulative ATP release (AUC ATP) was assessed (**Figure 2B**). We first noticed that mutations in subunits of ATP synthase, which is the key enzyme of ATP generation, were generally associated with significantly lower cumulative ATP release compared to mutations in cytochrome *bo3* oxidase subunits (**Figure 2C**). Therefore, we suspected an interrelation between ATP generation and released ATP (see growth and ATP release curves in **Figure 2B**). We determined cumulative growth (AUC growth) in addition to cumulative ATP release and indeed, cumulative ATP release and cumulative growth were positively correlated (**Figure 2D**). Since OD₆₀₀ measurements are most accurate during exponential growth, we additionally assessed released ATP and growth at the individual ATP peak,

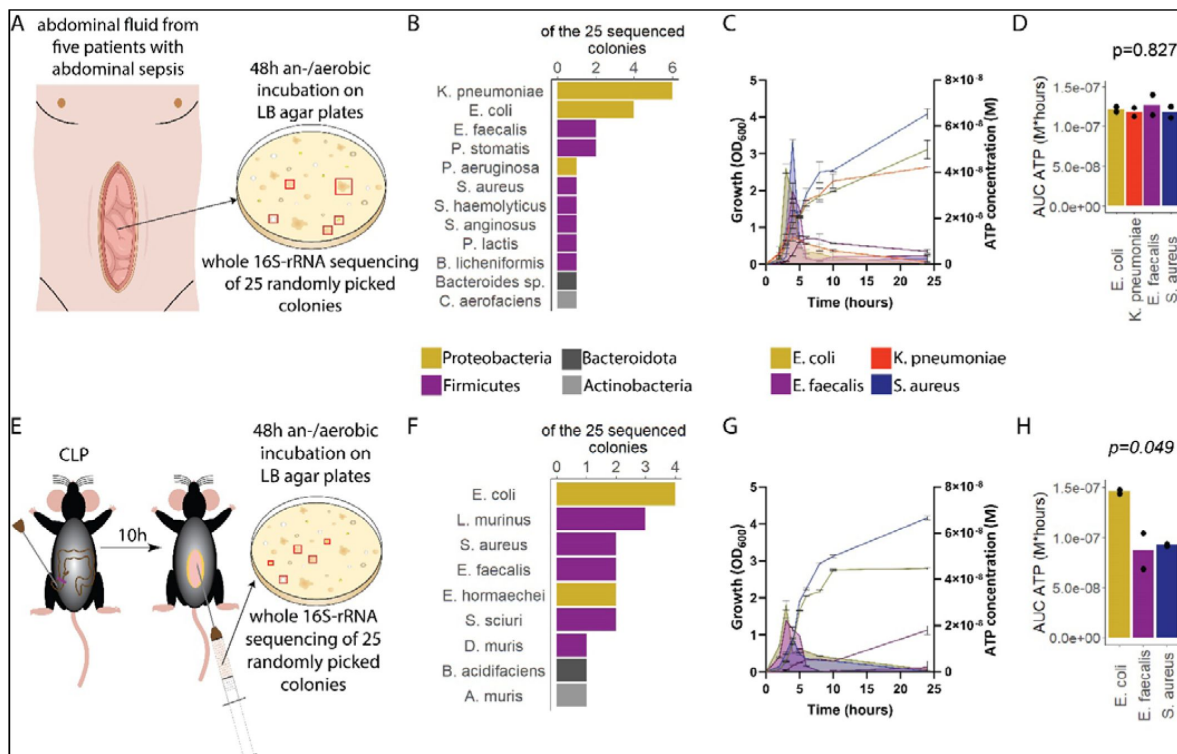


Figure 1

Sepsis-associated bacteria release ATP in a growth phase-dependent manner.

- (A)** Experimental approach to isolate and cultivate sepsis-associated bacteria from abdominal fluid of patients with abdominal sepsis.
- (B)** Bacterial species identified by whole 16S-rRNA sanger sequencing from abdominal fluid of patients with abdominal sepsis. Three colonies out of 25 could not be identified.
- (C)** Measurement of released ATP (M) and growth (OD₆₀₀) over time (hours) from the four sepsis-associated bacteria *E. coli*, *K. pneumoniae*, *E. faecalis* and *S. aureus* isolated from patients. N = 2 independent bacteria cultures. Means and standard deviations are shown.
- (D)** Area under the curve (AUC) of released ATP over time (M*hours) of the previously assessed bacteria (cumulative ATP). One-way ANOVA, N = 2 independent bacteria cultures. Means and individual values are shown.
- (E)** Experimental approach to isolate and cultivate sepsis-associated bacteria from abdominal fluid of mice with abdominal sepsis.
- (F)** Bacterial species identified by whole 16S-rRNA sanger sequencing from abdominal fluid of mice with abdominal sepsis. Seven colonies out of 25 could not be identified.
- (G)** Measurement of released ATP (M) and growth (OD₆₀₀) over time (hours) from the three sepsis-associated bacteria *E. coli*, *E. faecalis* and *S. aureus* isolated from mice. N = 2 independent bacteria cultures. Means and standard deviations are shown.
- (H)** AUC of released ATP over time (M*hours) of the previously assessed bacteria (cumulative ATP). One-way ANOVA, N = 2 independent bacteria cultures. Means and individual values are shown.

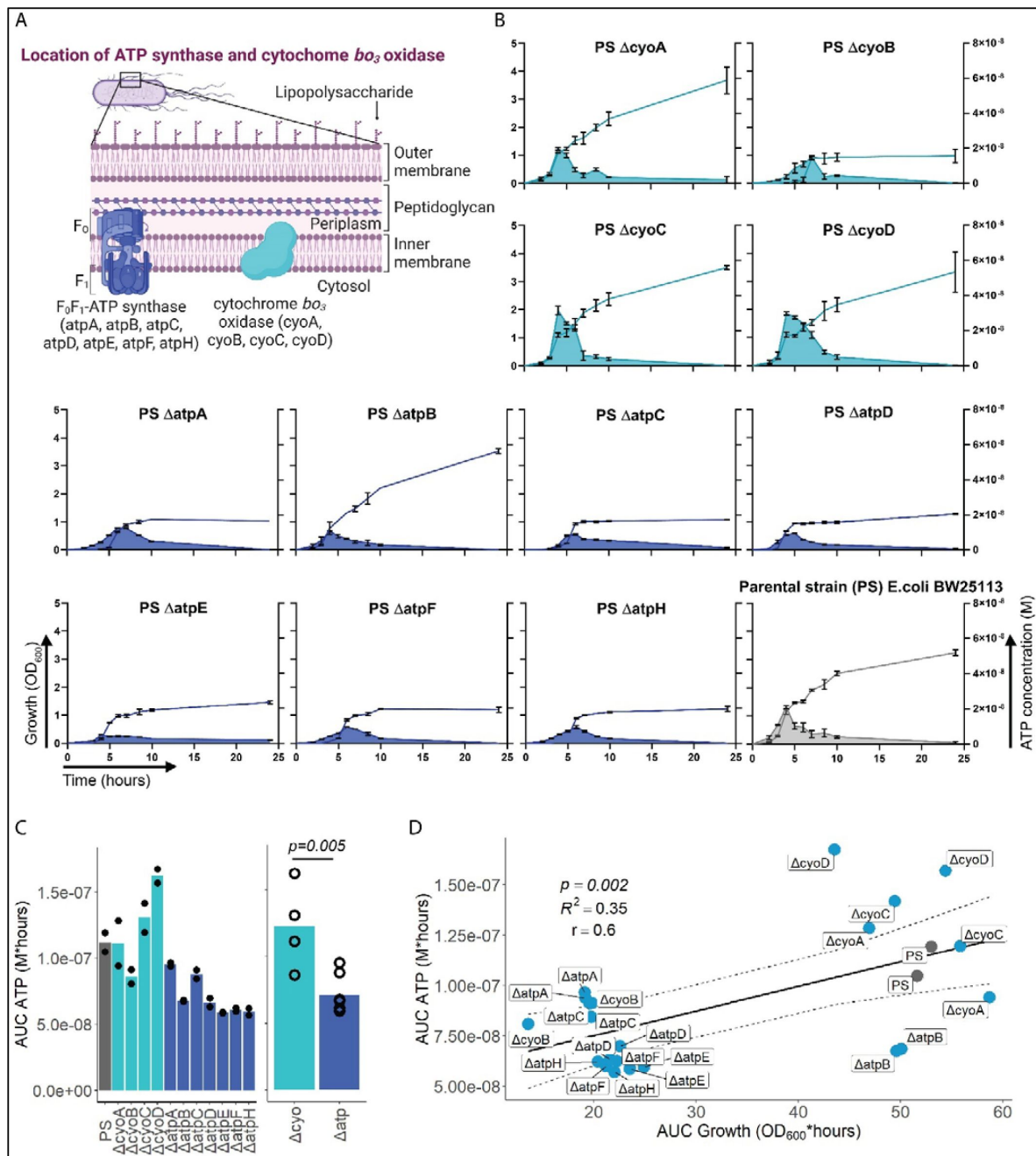


Figure 2

ATP release is dependent on ATP synthesis.

(A) Illustration depicting the location of ATP synthase and cytochrome bo_3 oxidase in gram^{neg} bacteria.

(B) Measurement of released ATP (M) and growth (OD₆₀₀) over time (hours) from cytochrome bo_3 oxidase (cyo) and ATP synthase (atp) mutants. The PS was added as a control. N = 2 independent bacteria cultures. Means and standard deviations are shown.

(C) Area under the curve (AUC) of released ATP over time (M*hours) of the previously assessed bacteria (cumulative ATP) is shown individually in the left panel. N = 2 independent bacteria cultures. Means and individual values are shown. Means of grouped cyo and atp mutants are compared in the right panel. T-test. Means and individual values are shown.

(D) Cumulative ATP (M*hours) and cumulative growth (OD₆₀₀*hours) of all assessed cyo and atp mutants and the PS were plotted against each other. Pearson's correlation (r) and coefficient of determination (R^2) of the applied linear model are depicted. 95% confidence level is shown by the black dashed lines.

which was even better positively correlated, strengthening our hypothesis (**Figure 2-figure supplement 1**). In summary, ATP release is directly dependent on ATP generation at the inner bacterial membrane. Mutations in subunits of bacterial ATP synthase have a higher impact on ATP generation, growth, and ATP release than mutations in subunits of cytochrome *bo3* oxidase (**Figure 2C**).

Outer bacterial membrane integrity and bacterial death determine bacterial ATP release during exponential growth

We next focused on the outer membrane by challenging its integrity while leaving ATP generation and the inner membrane intact. For that purpose, we used the *E. coli* porin mutants $\Delta ompC$, $\Delta ompF$, $\Delta lamB$ and $\Delta phoE$ (**Figure 3A**) (Baba et al., 2006; Yamamoto et al., 2009), which have been shown to suffer from impaired outer membrane integrity in varying degrees (Choi & Lee, 2019). ATP release and growth were measured over time including *E. coli* PS as baseline and membrane destabilizing EDTA and stabilizing Ca^{2+} as additional controls (Leive, 1968). Cumulative ATP release (AUC ATP) from the porin mutants were notably different compared to the PS, being lowest in $\Delta ompC$ and highest in $\Delta ompF$ (**Figure 3B-C**).

Interestingly, the $\Delta ompF$ mutant had also a very high peak of released ATP (**Figure 3B**). We hypothesized that this is because of impaired membrane integrity, resulting in ATP release and potentially bacterial death. Thus, we focused on the individual ATP peaks during exponential growth, which were observed after 4h of culturing. There was a strong negative correlation between the individual peak of released ATP (marked by the red line in **Figure 3B**) and growth at the same time point (**Figure 3D**).

We did not interfere with ATP generation at the inner membrane but deliberately challenged the outer membrane and tested therewith if destabilization of the outer membrane integrity is associated with bacterial death. Indeed, outer membrane integrity and bacterial death are significantly increased in $\Delta ompF$ compared to $\Delta ompC$ and the PS after 4h (ATP peak) of culturing (**Figure 3E, F**), akin to the amount of released ATP (**Figure 3G**). We conclude from these data that destabilization of the outer bacterial membrane (as observed with the $\Delta ompF$ mutant), results in bacterial death that is associated with ATP release.

In summary, outer membrane integrity and finally bacterial death notably contribute to the amount of bacterial ATP release during exponential growth.

Released bacterial ATP reduces neutrophil counts and impairs survival during abdominal sepsis in mice

Next, we wanted to investigate the function of bacterial ATP release *in vivo*. To study this, we transformed the *E. coli* PS with an arabinose-inducible apyrase (PS+pAPY) and compared it to the PS transformed with the empty vector (PS+pEMPTY) (Proietti et al., 2019b). In this model, ATP released by bacteria is hydrolyzed and consequently depleted by a periplasmic apyrase (Santapaola et al., 2006; Scribano et al., 2014).

Indeed, apyrase induction resulted in a significant reduction of ATP release in PS+pAPY, compared to PS+pEMPTY (**Figure 4-figure supplement 1A-B**). To test the consequences *in vivo*, apyrase was induced by arabinose three hours before intraabdominal (i.a.) injection into wild type C57Bl/6 mice (**Figure 4A**). Thereby ATP release was abrogated in the bacteria cultures that were used for injection (**Figure 4B**). *In vivo*, no difference in ATP levels were detected when ATP was measured directly in the abdominal fluid after four (**Figure 4C**) and eight hours (**Figure 4-figure supplement 1C**). This is not surprising given that ATP is rapidly hydrolysed by ectonucleotidases *in vivo* (Eltzschig et al., 2012). After both four (**Figure 4D-E**) and eight hours (**Figure 4-figure supplement 1D-E**), no differences in local or systemic bacterial counts were

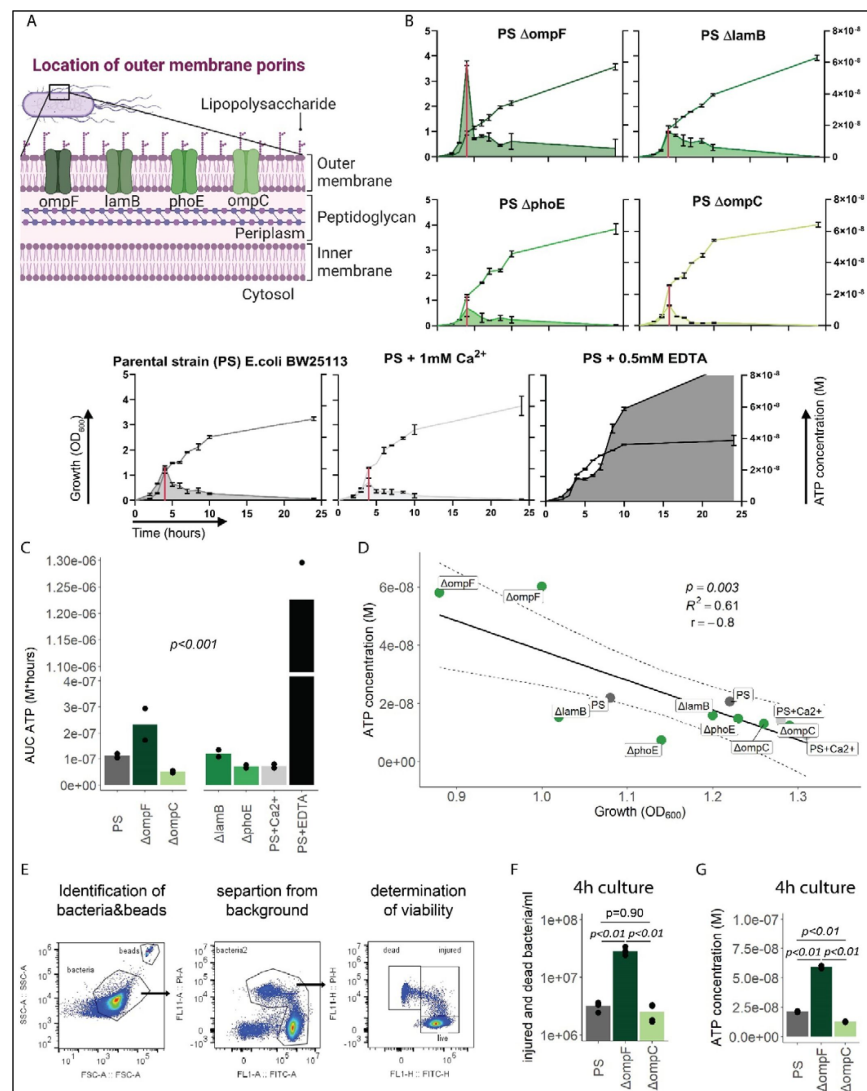


Figure 3

Outer membrane integrity and bacterial death determine bacterial ATP release during growth.

- (A) Illustration depicting the location of outer membrane porins in gram^{neg} bacteria.
- (B) Measurement of released ATP (M) and growth (OD₆₀₀) over time (hours) from outer membrane porin mutants. The PS and the PS supplemented with either 1mM Ca²⁺ or 0.5mM EDTA were added as controls. N = 2 independent bacteria cultures. Means and standard deviations are shown. The red line marks the individual peak of ATP release and growth (OD₆₀₀) at that time point.
- (C) Area under the curve (AUC) of released ATP over time (M*hours) of the previously assessed bacteria (cumulative ATP). One-way ANOVA, N = 2 independent bacteria cultures. Means and individual values are shown.
- (D) ATP concentration (M) and growth (OD₆₀₀) at the individual peak of ATP release of all assessed outer membrane porin mutants, the PS and the PS+Ca²⁺ (no peak for the EDTA control) were plotted against each other. Pearson's correlation (r) and coefficient of determination (R²) of the applied linear model are depicted. 95% confidence level is shown by the black dashed lines.
- (E) Gating strategy to identify added counting beads, live, injured and dead bacteria.
- (F) Quantitative assessment of injured and dead bacteria, as identified by flow cytometry after 4h of culturing (ATP peak) of the PS, $\Delta ompF$ and $\Delta ompC$. One-way ANOVA followed by Tukey post-hoc test, N = 4 independent bacteria cultures. Means and individual values are shown.
- (G) ATP concentration (M) after 4h of culturing (ATP peak) of the PS, $\Delta ompF$ and $\Delta ompC$. One-way ANOVA followed by Tukey post-hoc test, N = 2 independent bacteria cultures. Means and individual values are shown.

observed. Yet, despite similar bacterial counts, the survival was significantly higher in the absence of bacterial ATP (PS+pAPY) compared to ATP-generating controls (PS+pEMPTY) after i.a. injection (Figure 4F).

Next, we asked how this difference in bacterial ATP release affects the immune system. Therefore, inflammatory cells in the abdominal cavity were characterized using flow cytometry (Figure 4G). As expected, a disappearance reaction of large peritoneal macrophages (LPM) was observed after both, *E. coli* PS+pEMPTY and *E. coli* PS+pAPY i.a. injection compared to sham controls after four (Figure 4H) and eight hours (Figure 4-figure supplement 1F) (Ghosh et al., 2010). Such LPM disappearance following abdominal *E. coli* infection is a result of free-floating clots composed of LPMs and neutrophils and important for effective pathogen clearance (Salm et al., 2023; Vega-Pérez et al., 2021; Zindel et al., 2021) but not dependent on ATP release according to our data. Interestingly, however, the number of small peritoneal macrophages (SPM) and CX3CR1^{pos} monocytes was significantly reduced, whereas neutrophils were significantly increased almost up to 8 hours (Figure 4I, Figure 4-figure supplement 1G) in bacterial ATP-depleted abdominal sepsis (PS+pAPY). This effect is potentially dependent on released bacterial ATP given that no differences in bacterial counts were observed (see Fig. 4D and 4E, Figure 4-figure supplement 1D-E).

In summary, ATP released by bacteria suppresses abdominal inflammatory responses and impaired survival of mice in a model of abdominal sepsis.

Establishing ATP-loaded OMV as a model system to assess the systemic relevance of bacterial ATP

ATP is rapidly metabolized in the extracellular space and therefore, the mode of action of released bacterial ATP is limited to the immediate cellular vicinity (Junger, 2011). However, the outcome of sepsis is not only dependent on local but also on systemic responses to microorganisms. Therefore, we hypothesized that bacterial ATP has systemic effects as protected cargo in OMV (Alvarez et al., 2017). OMV are small (20-300nm) spherical particles that are released by both gram^{neg} and gram^{pos} bacteria (Schwechheimer & Kuehn, 2015). In gram^{neg} bacteria, they bulge off the outer membrane and disseminate throughout the body (Jang et al., 2015). They are equipped with typical bacterial surface features lacking the machinery for self-reproduction, and contain DNA, proteins, and metabolites (Baeza & Mercade, 2021; Bitto et al., 2017; Kulp & Kuehn, 2010; E.-Y. Lee et al., 2007). Therefore, OMV are suited as a systemic delivery system for bacterial ATP. Indeed, recently, ATP has been detected in OMV derived from pathogenic *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* PAO1 and *Acinetobacter baumannii* AB41 (Pérez-Cruz et al., 2015).

To assess the potential of OMV as ATP carriers, we compared the OMV production from several hypervesiculation *E. coli* mutants ($\Delta mlaE$, $\Delta mlaA$, $\Delta rfaD$, $\Delta degP$, $\Delta rodZ$, $\Delta nlpI$, $\Delta tolB$) (Figure 5A) (McBroom et al., 2006). The $\Delta nlpI$ and $\Delta tolB$ strains showed a 20- and 30-fold increase of OMVs when compared with the PS (Figure 5B). We then assessed ATP release and growth over time from $\Delta nlpI$, $\Delta tolB$ and the PS, to identify their individual peak of ATP release and isolated OMV at their individual peak of ATP release and after 24h (Figure 5-figure supplement 1A). ATP was detected in OMV from all assessed strains at the individual peak of ATP release but only in minimal detectable levels after 24h (Figure 5C). The $\Delta tolB$ OMV isolated after 24h were then used as ATP-depleted vehicles. After density gradient ultracentrifugation, most $\Delta tolB$ OMV were either in fraction 3a or 3b and therefore of similar density (Figure 5D, Figure 5-figure supplement 1B). They were equipped with outer membrane ompF but not cytoplasmic ftsZ (Figure 5D), indicating that they are outer membrane derived. In order to generate OMV with known and constant ATP concentrations, we used electroporation to load OMV with ATP while empty mock-electroporated OMV ($\Delta tolB$ OMV harvested from 24h culture) served as ATP-depleted controls (Fu et al., 2020; Lennaárd et al., 2021). Before and after electroporation, the OMV size

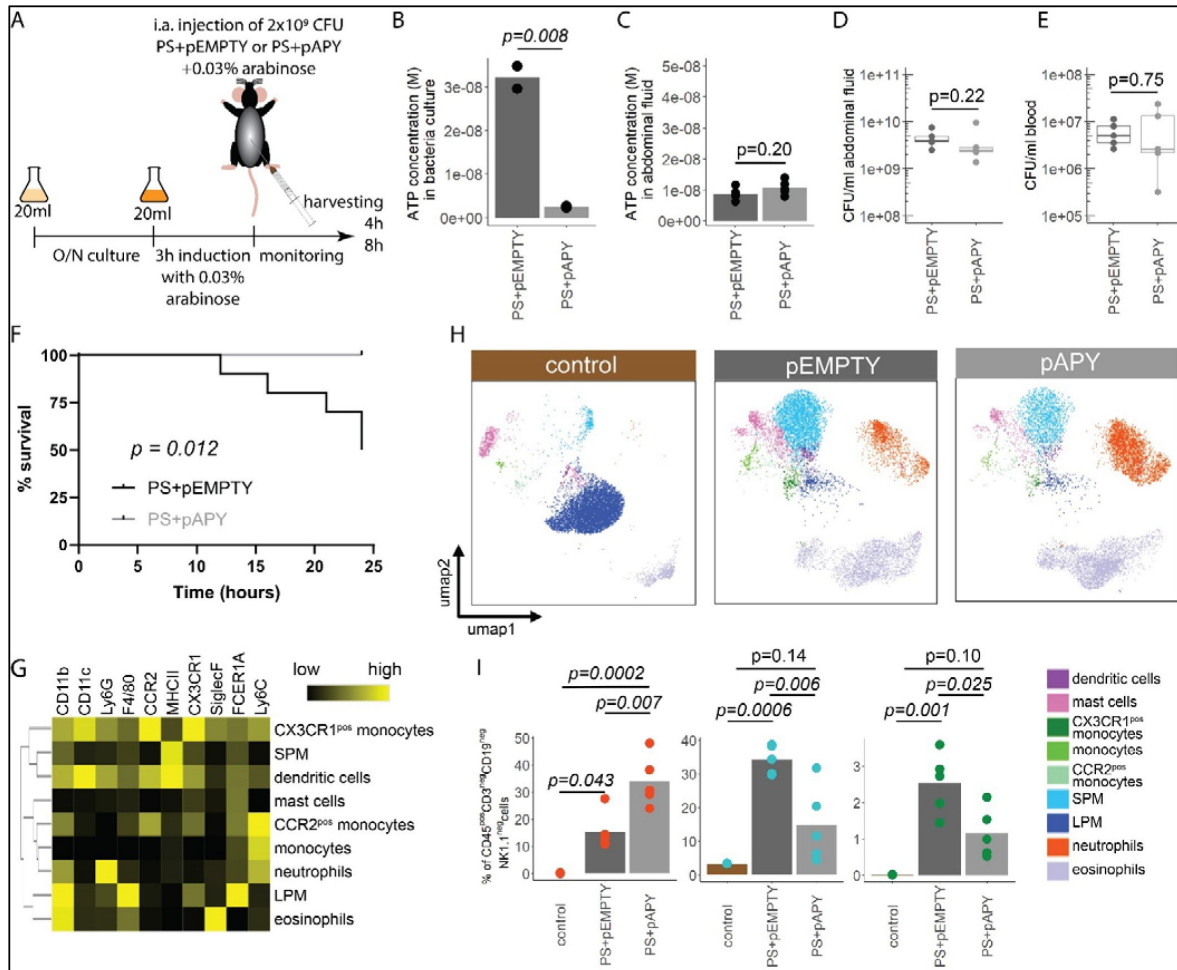


Figure 4

Bacterial ATP reduces neutrophil counts and reduces survival in a mouse model of abdominal sepsis.

(A) Experimental approach to determine the local role of bacterial ATP *in vivo*, i.a. injecting PS+pEMPTY or PS+pAPY.

(B) Measurement of released ATP (M) in bacteria culture supernatant immediately before bacteria were i.a. injected. T-test, $N = 2$ independent bacteria cultures. Means and individual values are shown.

(C) Measurement of ATP (M) in abdominal fluid from mice four hours after i.a. injection of bacteria. T-test, $n = 5$ animals per group of $N = 2$ independent experiments. Means and individual values are shown.

(D) Quantitative assessment of colony forming units in abdominal fluid and (E) blood from mice four hours after i.a. injection of bacteria. Wilcoxon rank sum test, $n = 5$ animals per group of $N = 2$ independent experiments. Means and individual values are shown. No growth for controls was detected.

(F) Kaplan-Meier curves of mice after i.a. injection of bacteria. Log-rank test, $n = 10$ animals per group.

(G) Heatmap showing surface marker expression (x-Axis), which was used to characterize the different immune cell populations (y-Axis).

(H) Concatenated ($n = 5$ animals for each treatment group, $n = 3$ animals for control group of $N = 2$ independent experiments) and down-sampled images of immune cell populations characterized in the abdominal cavity four hours after sham treatment or i.a. injection of bacteria.

(I) Abundance of neutrophils, small peritoneal macrophages (SPM) and CX3CR1^{pos} monocytes in abdominal fluid from mice four hours after sham treatment or i.a. injection of bacteria. One-way ANOVA followed by Tukey post-hoc test, $n = 5$ animals for each treatment group, $n = 3$ animals for control group of $N = 2$ independent experiments. Means and individual values are shown.

distribution was assessed by nanoparticle tracking analysis and morphology by electron microscopy (**Figure 5E** [↗](#), **Figure 5-figure supplement 1C** [↗](#)). OMV were loaded with ATP (**Figure 5F** [↗](#)) and over time, the amount of ATP in OMV decreased, especially at physiologic (37°C) temperature (**Figure 5G** [↗](#)) as opposed to 4°C (**Figure 5-figure supplement 1D** [↗](#)).

In summary, OMV contain ATP and release ATP at physiological temperatures. To use OMV as an ATP delivery system, empty OMV were loaded using electroporation.

OMV-derived bacterial ATP induces degranulation processes in neutrophils after lysosomal uptake

OMV are potent inducers of inflammation and sepsis (Park et al., 2010 [↗](#), 2013 [↗](#)), which travel throughout the body and are taken up by a variety of cells (Bittel et al., 2021 [↗](#); Kim et al., 2013 [↗](#); J. Lee et al., 2018 [↗](#)). To test the hypothesis that ATP within OMV mediates systemic effects of invasive bacteria, we injected ATP-loaded and empty OMV i.a. and investigated the resulting inflammation one hour after injection (**Figure 6A** [↗](#)).

In the abdominal fluid, uptake of DiI-stained OMV by leucocytes was independent of ATP cargo (**Figure 6B** [↗](#), **Figure 6-figure supplement 1** [↗](#)) and all major cell populations with phagocytic ability (LPM, SPM, neutrophils) were highly positive for OMV (**Figure 6-figure supplement 2A-B** [↗](#)). LPM dramatically decreased, whereas neutrophils increased in response to OMV when compared to sham controls (**Figure 6-figure supplement 2C** [↗](#)).

Within one hour, the DiI-stained OMV were distributed throughout the body and mainly taken up by neutrophils, with remarkable differences between organs (**Figure 6-figure supplement 3A-C** [↗](#)). The highest ratio of OMV^{pos} neutrophils compared to all other OMV^{pos} cells was observed in the lung in an ATP-independent manner (**Figure 6C** [↗](#)). Intracellular fate of engulfed OMV is still a matter of discussion but one possibility is the degradation in the endolysosomal compartment (Bielaszewska et al., 2017 [↗](#); Juodeikis & Carding, 2022 [↗](#)). We therefore injected DiI-stained OMV i.a. to assess to which membrane compartment OMV co-localize. A strong co-localization with the endolysosomal compartment of pulmonary neutrophils was identified (**Figure 6D** [↗](#)).

Next, we aimed to determine what pathways OMV-derived bacterial ATP initiates in neutrophils after uptake into the endolysosomal compartment. During infection and sepsis, neutrophils are highly abundant in the lungs and most OMV were engulfed by neutrophils (see **Figure 6C** [↗](#) and **Figure 6-figure supplement 3C** [↗](#)). Because of its anatomically central position in the blood circulation and extensive vasculature, the lung is often affected in sepsis and a significant cause of mortality (Wang et al., 2024 [↗](#)). We therefore isolated again pulmonary neutrophils (**Figure 6-figure supplement 4** [↗](#)) for RNA sequencing. Uptake of ATP-loaded OMV resulted in a significantly different transcriptional activity compared to empty OMV (**Figure 6E** [↗](#)): The strongest differences were found in lysosomal (LYSO) and neutrophil degranulation pathways (NDG) (**Figure 6F** [↗](#), **Figure 6-figure supplement 5** [↗](#)). In the lysosomal pathway, transcripts for proteins involved in autophagosome-lysosome fusion (Gabarap), endosome to lysosome transport (Hgsnat, Laptm5), or enzymatic cleavage (Capn1, Fuca1, Ctsb, Ctsd, Gba) were significantly higher expressed in response to ATP-loaded OMV when compared to empty OMV (**Figure 6F-G** [↗](#)). Furthermore, neutrophil degranulation was significantly increased in response to ATP-loaded OMV as indicated by increased expression of gene transcripts resulting in antibacterial activity (Lyz2, S100a8, Camp) or NETosis (Tlr2, Itgb2) (**Figure 6F** [↗](#) and **H** [↗](#)).

In summary, OMV are phagocytosed locally by major cell populations with phagocytic ability. Remotely, OMV were mainly taken up by neutrophils, where they co-localize with the endolysosomal compartment. Delivery of ATP by OMV resulted in an upregulation of lysosomal activation and neutrophil degranulation.

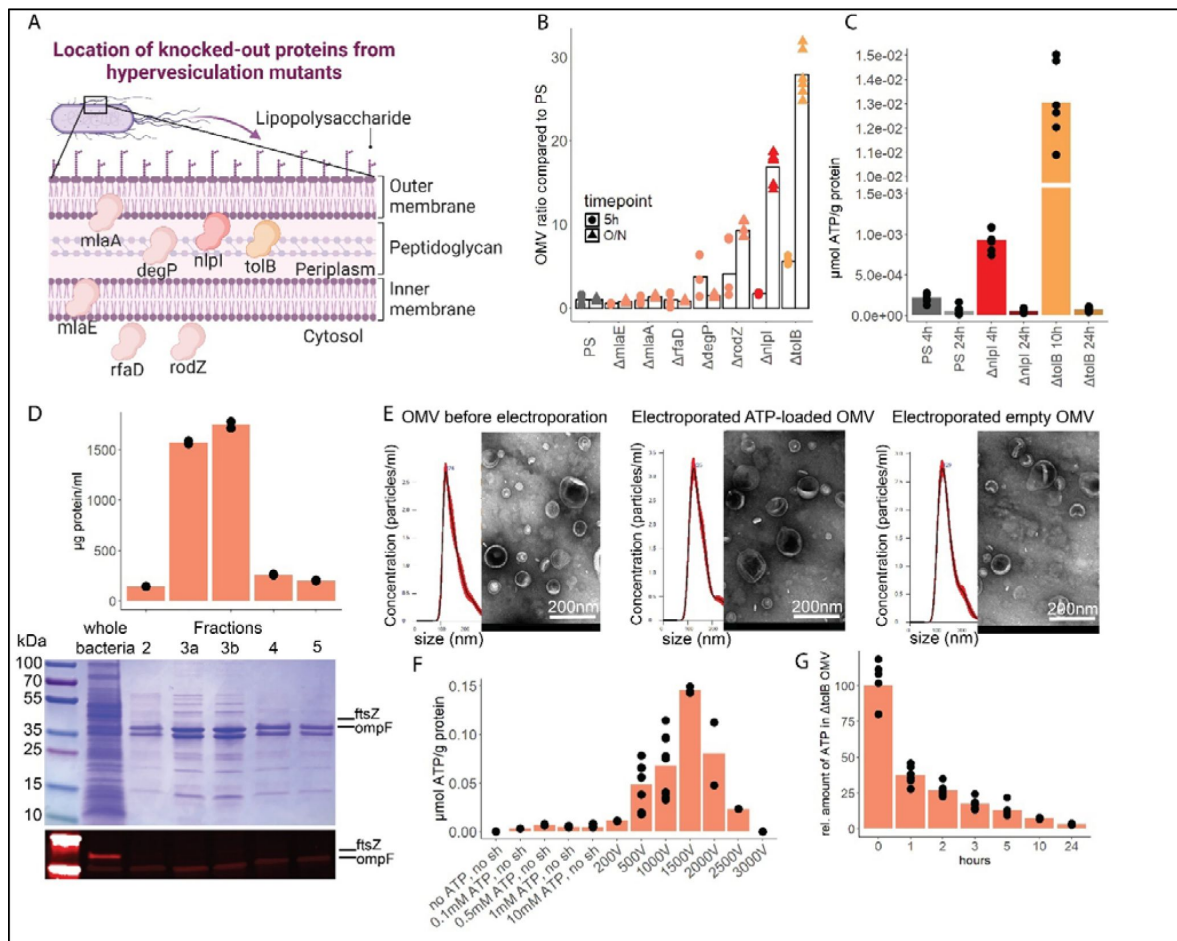


Figure 5

OMV contain ATP and can be exploited as a model to assess the systemic relevance of bacterial ATP.

(A) Illustration depicting the location of assessed proteins that lead to a hypervesiculation phenotype if knocked-out in the gram^{neg} bacterium *E. coli*.

(B) Relative amount of OMV ($\mu\text{g protein/ml}$) compared to the PS isolated from growth cultures of the assessed hypervesiculation mutants after five hours (exponential growth) and O/N (stationary phase). $n = 2$ measurements of $N = 3$ independent bacteria cultures. Means and individual values are shown.

(C) Absolute quantification of ATP in OMV isolated from growth cultures of the PS, $\Delta nlpI$ and $\Delta tolB$ at their individual peak of ATP release and after 24 hours. $n = 2$ measurements of $N = 3$ independent bacteria cultures. Means and individual values are shown.

(D) Amount of protein (BCA assay) detected in different fractions after density gradient ultracentrifugation. $n = 2$ measurements of the different fractions. $20\mu\text{l}$ of *E. coli* growth culture and $20\mu\text{l}$ of each fraction were then characterized by Coomassie blue staining and specific detection of outer membrane ompF and cytoplasmic ftsZ.

(E) Characterization of OMV by nanoparticle tracking analysis ($n = 5$ measurements per sample) and electron microscopy (representative image) before and after electroporation.

(F) Absolute quantification of ATP in OMV, which were loaded using different strategies. Column 2-5: different concentrations of ATP incubated for one hour at 37°C (passive filling). Column 6-12: different voltages with fixed settings for Resistance (100Ω) and Capacitance ($50\mu\text{F}$). $N = 2-9$ independent experiments. Means and standard deviations are shown.

(G) Relative quantification of ATP in OMV over 24 hours at 37°C after electroporation (0h = 100%). $N = 2$ measurements of $N = 3$ independent experiments. Means and individual values are shown.

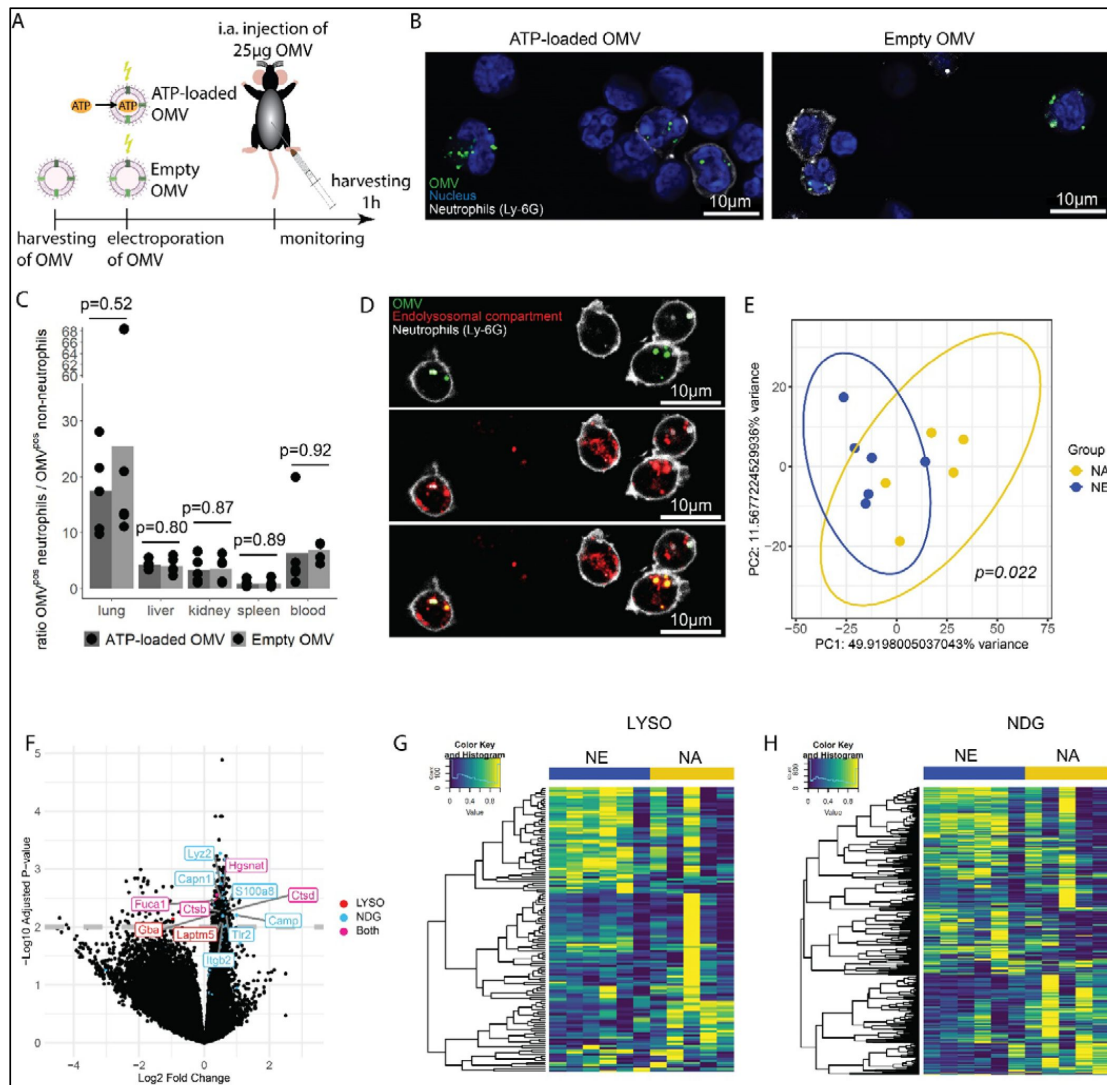


Figure 6

Bacterial ATP within OMV upregulates lysosome-related pathways and degranulation processes in neutrophils.

- (A)** Experimental approach to determine the systemic role of bacterial ATP *in vivo*, i.a. injecting ATP-loaded or empty OMV.
- (B)** Representative microscopic images of cells from the abdominal cavity one hour after i.a. injection of either ATP-loaded or empty OMV. OMV: DiI, Nucleus: DAPI, Neutrophils: Ly-6G-FITC.
- (C)** Cells from remote organs were isolated one hour after i.a. injection of either ATP-loaded or empty OMV. OMV were mainly taken up by neutrophils (except in the spleen, ratio ≈ 1). T-test with Benjamini-Hochberg correction, $n = 5$ animals per group of $N = 2$ independent experiments. Means and individual values are shown.
- (D)** Representative microscopic image of pulmonary neutrophils one hour after i.a. injection of either ATP-loaded or empty OMV. OMV co-localize with the endolysosomal compartment. OMV: DiI, Endolysosomal system: Deep Red LysoTracker, Neutrophils: Ly-6G-FITC.
- (E)** Pulmonary neutrophils were isolated one hour after i.a. injection of ATPys-loaded or empty OMV, bead-sorted and RNA sequencing was done. Principal component analysis shows significantly different clustering between neutrophils that took up ATPys-loaded (NA) or empty OMV (NE). PERMANOVA, $n = 6$ animals in the NE group, $n = 5$ animals in the NA group. Ellipses represent 95% confidence level.
- (F)** Volcano plot of RNA sequencing results shows an upregulation of genes mainly in the NA group. Genes classified in either lysosome (LYSO) or neutrophil degranulation pathways (NDG) or both, which were mentioned in the text, were highlighted.
- (G)** Heatmap of the lysosome pathway (LYSO) showing the gene expression per sample.
- (H)** Heatmap of the neutrophil degranulation pathway (NDG) showing the gene expression per sample.

Discussion

In this study, we have demonstrated that ATP release is dependent on the respiratory chain located at the inner bacterial membrane. ATP synthase is most likely dominant compared to cytochrome *bo*₃ oxidase because of its non-redundant function for ATP generation and bacterial growth. Mutations in one of the cytochrome *bo*₃ oxidase subunits *cyoA*, *cyoC* or *cyoD* could be compensated by another subunit. This is supported by lower ATP release in the *cyoB* mutant, which harbors all three prosthetic groups of the cytochrome *bo*₃ oxidase and is therefore indispensable for proper function (Tsubaki et al., 2000 [\[1\]](#)). Alternatively, deficiency in the *bo*₃-type oxidase could be partially compensated by *bd*-type oxidases (Borisov et al., 2011 [\[2\]](#); Grauel et al., 2021 [\[3\]](#)).

Instability of the outer bacterial membrane as we have seen for the $\Delta ompF$ mutant (Choi & Lee, 2019 [\[4\]](#)) is associated with bacterial death and ATP release. It remains unclear whether loss of ATP results directly in bacterial death or bacterial death is the direct result of impaired outer bacterial membrane stability and ATP release is secondary to bacterial death. However, since the inner membrane and ATP generation remains intact and the periplasmic space is considered generally devoid of ATP, the latter seems more likely (Kulp & Kuehn, 2010 [\[5\]](#)). Previous studies used Sytox/PI staining and microscopy to identify bacterial death and did not identify bacterial death as a relevant source of extracellular ATP during exponential growth (Hironaka et al., 2013 [\[6\]](#)). Conversely, we used flow cytometry, a quantitatively more sensitive method and our data demonstrated that impaired membrane integrity and bacterial death are critical for ATP release during growth. Despite low rates of bacterial death during exponential growth (Koch, 1959 [\[7\]](#)), and a low ratio of dead to live bacteria, the high gradient between intra- and extracellular ATP (approximately 100 - 1000x higher) might be sufficient to explain the amount of released ATP measured (Leduc & Van Heijenoort, 1980 [\[8\]](#); Mempo et al., 2013 [\[9\]](#)).

In this study, we used the above-mentioned outer membrane porin mutants to study impaired membrane integrity and we used hypervesiculation mutants to study the effects of OMV-derived bacterial ATP. It is important to note that these impaired membrane integrity and hypervesiculation phenotypes are not clearly distinct but rather overlapping. However, outer membrane porin mutants are not known as high OMV producers. Thus, measured ATP from outer membrane porin mutants was most likely directly released and not OMV-derived ATP. To assess ATP within OMV, we switched to typical hypervesiculation mutants.

ATP was detected within OMV and the concentration of ATP within OMV is sufficient to activate P2-type receptors in lysosomes (Araujo et al., 2020 [\[10\]](#); Junger, 2011 [\[11\]](#)). Furthermore, OMV are released in response to environmental stresses like infection and sepsis (Kulp & Kuehn, 2010 [\[5\]](#); Orench-Rivera & Kuehn, 2016 [\[12\]](#)). ATP is likely to activate P2 receptors, which have been shown to be expressed in lysosomes, such as P2X1, P2X3 or P2X4 (Qureshi et al., 2007 [\[13\]](#); Robinson & Murrell-Lagnado, 2013 [\[14\]](#)). OMV can therefore be considered as non-living bacteria-resembling highly inflammatory vesicles (Jang et al., 2015 [\[15\]](#); Kulp & Kuehn, 2010 [\[5\]](#)) that effectively distribute ATP throughout the body to activate cells, such as neutrophils, initiating systemic inflammatory responses. It has already been shown that OMV are engulfed by neutrophils (Sartorio et al., 2021 [\[16\]](#)), however, the finding that they actually have a functional effect is a novelty. Activation of neutrophils in response to ATP-loaded OMV resulted in upregulation of inflammatory, lysosomal, and neutrophil degranulation pathways as well as an upregulation of the apoptosis pathway and genes involved in NETosis by a hitherto unknown mechanism. These pathways explain in part the low neutrophil counts we observed in mice. Such remote degranulation in response to OMV is unlikely to control bacteria at the source of sepsis but rather harmful to host tissues increasing sepsis severity (Eichelberger & Goldman, 2020 [\[17\]](#)).

The study has several limitations: Bacterial ATP release is strain-specific (**Figure 1**) (Mempin et al., 2013). As we focused on the laboratory BW25113 *E. coli* strain, it remains to be elucidated if impaired outer membrane integrity and bacterial death are also of such importance in other gram^{neg} or also gram^{pos} bacteria. The approach to load OMV with ATP is rather artificial; it is, however, the only way to assure that ATP-loaded and empty OMV only differ in their ATP cargo. OMV have surface antigens and contain DNA, proteins and other metabolites (Kulp & Kuehn, 2010), which are known to elicit inflammation as well. This was controlled using the same OMV as baseline vehicles and including electroporation also for the controls.

Given the magnitude of the difference in concentrations we detected between directly released and OMV-derived ATP, the role of OMV-derived ATP was not assessed locally, and we focused on its remote effects. However, the signaling mechanisms between directly released ATP (extracellular) and OMV-delivered ATP (endolysosomal compartment) are different. Therefore, the local role of OMV-derived ATP should be explored in future studies, including potential differences in transcription or cytokine profiles.

Other open questions need to be addressed in future projects: It remains to be determined how OMV are physiologically loaded with ATP. Despite the lack of a transporter, ATP may leak across the inner membrane into the periplasmic space. Such leakage may be similar to baseline leakage in eukaryotic cells (Yegutkin et al., 2006). Different types of OMV have been described in recent years, for example outer-inner membrane vesicles (O-IMV) or explosive OMV (Juodeikis & Carding, 2022; Takaki et al., 2020; Toyofuku et al., 2019; Turnbull et al., 2016; Turner et al., 2015), which are composed of outer membrane, inner membrane and cytoplasmic content. The mechanisms, how these types of OMV are generated, could explain that ATP is found within them. However, the *ΔtolB* mutant produces, unlike other *tol* mutants, only very few O-IMV (0.1%-2% of all OMV) (Pérez-Cruz et al., 2013; Reimer et al., 2021; Takaki et al., 2020) and our Western blot analysis suggests that our OMV are primarily outer membrane derived. Future studies may address the ATP cargo of the different OMV subgroups. Another open question is generally the physiological role of OMV-delivered ATP. We detected concentrations around 1-10 nmol/g protein, which is in accordance with Pérez-Cruz et al. (Pérez-Cruz et al., 2015). Interestingly, Alvarez et al. reported 1000x higher concentration (~13nmol/μg protein). This difference might be explained by the techniques used. Considering our chosen voltage of 1100V and the ATP decrease in OMV over time, we reach similar concentration as measured in our isolated OMV. It is therefore reasonable to assume that our shown effects are physiological, which has, however, to be confirmed by other studies.

OMV are promising diagnostic molecular biomarkers in gram^{neg} sepsis (Michel & Gaborski, 2022) and it would be of relevance to compare OMV isolated from septic and control patients to assess possible differences in ATP cargo. Furthermore, it remains to be elucidated, which mechanisms lead to low local neutrophil counts. Since ATP is involved in cell death (Proietti et al., 2019b) as well as in chemotaxis (Junger, 2011), increased cell death, impaired infiltration or a combination of both is possible.

This study reveals that ATP is released by bacteria during growth because of impaired membrane integrity and bacterial death. ATP is also being released via OMV and therefore acts locally (direct release) and systemically (via OMV). Bacterial ATP reduces neutrophil counts, activates the endolysosomal system and upregulates neutrophil degranulation, which together increase the severity of abdominal infection and early sepsis. These findings have the potential to lead to the development of novel treatments for abdominal sepsis, e.g. by *in vitro* generated OMV that modulate neutrophil function via delivery of inhibitors to intracellular purinergic receptors during sepsis.

Materials and Methods

Human data

From five patients that underwent revision laparotomy because of abdominal sepsis, swabs were taken from abdominal fluid, streaked on LB agar plates (15g Agar, 5g Bacto Yeast Extract, 10g Bacto Tryptone, 5g NaCl in 1L ddH₂O; key resources table) and cultivated an-/aerobically for 48h. The human experimental protocol was approved by the Cantonal Ethics Commission Bern, Switzerland (ethical approval 2017-00573, NCT03554148). Written informed consent was obtained from all patients and the study has been performed in accordance with the Declaration of Helsinki as well as the CONSORT statement.

Mouse handling

Specific-pathogen-free C57Bl/6J RccHsd mice (key resources table) were purchased at the age of 8 weeks from Inotiv (earlier Envigo, the Netherlands) and were housed in ventilated cages in the Central Animal Facility, University of Bern, Switzerland. All experiments were performed in the morning, mice were supplied with a 12-hour light/dark cycle at 22°C and fed ad libitum with chow and water. To minimize cage effects, we mixed mice over several cages and therefore only used female mice. All animal procedures were carried out in accordance with the Swiss guidelines for the care and use of laboratory animals as well as in accordance with the ARRIVE guidelines and were approved by the Animal Care Committee of the Canton of Bern (Switzerland) under the following number: BE41/2022.

Cecal ligation and puncture (CLP) sepsis model

To isolate bacteria from mice with abdominal sepsis, cecal ligation and puncture was performed as described elsewhere with some minor modifications (Rittirsch et al., 2009 [↗](#)). In brief, mice were anesthetized s.c. injecting (3μl/g body weight) a mixture of fentanyl (0.05mg/ml), dormicum (5mg/ml) and medetor (1mg/ml) and were then shaved and disinfected with Betadine. Mid-line laparotomy was performed (approx. 1 cm) and the cecum was exposed. The proximal 1/3 of the cecum was ligated with Vicryl 4-0 (Ethicon, cat# V1224H) and perforated with a 23 G needle. The cecum was returned to the abdominal cavity and the laparotomy was sutured continuously in two layers with prolene 6-0 (Ethicon, cat# MPP8697H). At the end, the antidote (naloxone (0.1mg/ml), reverter (5mg/ml), temgesic (0.3mg/ml)) was s.c. injected (9μl/g body weight). A semi-quantitative score sheet was used to predict animal postoperative well-being. Mice were evaluated every four hours according to the following criteria: appearance, level of consciousness, activity, response to stimulus, eye shape, respiratory rate and respiratory quality and analgesia was applied if necessary. After 10 hours, abdominal fluid was collected, spread on LB agar plates, and cultivated an-/aerobically for 48h.

Whole 16S-rRNA sanger sequencing

Twenty-five colonies cultivated from abdominal fluid of patients and mice each described above were randomly picked and collected in separate sterile Eppendorf tubes and resuspended in 1ml of sterile PBS. Each sample was centrifuged for 5 min. at 20'000g and washed once with 1ml sterile PBS. The pellet was resuspended in 20μl of sterile PBS and samples were incubated 5min. at 100°C. 1μl was used for PCR using GoTaq G2 Green Master Mix (key resources table) and the following primers (key resources table) at a final concentration of 0.2μM:

-fD1: 5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3'

-fD2: 5'-AGA-GTT-TGA-TCA-TGG-CTC-AG-3'

-rP1: 5'-ACG-GTT-ACC-TTG-TTA-CGA-CTT-3'

PCR conditions were as follows: Initial 5min. at 94°C for denaturation, followed by 35 cycles of 1min. denaturation at 94°C, 1min. annealing at 43°C, and 2min. extension at 72°C, with a final extension for 10min at 72°C. This resulted in a PCR product of optimally ~1400 - 1500bp. 20µl PCR product were run on 2% agarose gel for 90min., cut out and purified using Qiaquick Gel Extraction Kit (key resources table). The amplicon concentration was measured using nanodrop (Thermo Fisher Scientific) and sanger sequencing was done by microsynth.

Quantification of released bacterial ATP

Bacteria were aerobically grown in 20ml LB medium (5g Bacto Yeast Extract, 10g Bacto Tryptone, 5g NaCl in 1L ddH₂O; key resources table) overnight (O/N) (16h / 37°C / 200rpm) and 0.25ml of O/N culture were diluted in 100ml fresh LB medium. Up to 24 hours, 0.25ml bacteria culture were taken at several time points for growth assessment (OD₆₀₀) and 1ml was taken for ATP quantification. OD₆₀₀ was measured using a Tecan Spark spectrophotometer. The 1ml sample for ATP quantification was centrifuged for 5min. at 16'000g, the supernatant was filtered through a 0.2µm syringe filter and stored at -80°C. ATP was quantified using a luciferin-luciferase-based assay according to the manufacturer's protocol (ATP Kit SL, key resources table) and bioluminescence was measured using a Tecan Spark spectrophotometer. In [Figure 3](#), LB medium was supplemented with 1mM calcium or 0.5mM EDTA for the controls.

Absolute quantification of bacteria and assessment of viability

Growth culture was set up as described above and after 4 hours (ATP peak), samples were taken to quantify bacteria and assess viability. Bacteria were diluted in PBS and stained using the Cell Viability Kit with BD Liquid Counting Beads (key resources table) according to the manufacturer's protocol. In brief, 100µl of diluted bacterial growth culture was stained with 1µl TO dye, 1µl PI dye and 10µl of counting beads were added. The sample was acquired on a CytoFlex S (Beckman Coulter), setting the thresholds for the TO- and the PI-channel to 1000 and analysis was done with FlowJo software (key resources table). Bacterial biomass was calculated to bacteria/ml culture according to the manufacturer's protocol.

Transformation of *E. coli* PS

The Keio collection (key resources table) *E. coli* PS was aerobically grown in LB medium O/N (16h / 37°C / 200rpm). 0.5ml O/N culture was diluted in 35ml fresh LB medium and incubated until OD₆₀₀ was 0.35-0.45. Cultures were chilled in ice-water and washed two times with ice-cold 20ml ultra-pure water (Thermo Fisher Scientific, cat# 10977035) (20min. / 4°C / 3200g). The supernatant was carefully decanted, and the bacterial pellet was gently resuspended by pipetting (no vortex). After the final wash, the pellet was resuspended in 240µl ice-cold ultra-pure water and kept on ice until electroporation. 80µl of ice-cold bacterial cells were mixed with 1µl plasmid (pBAD28 == pEMPTY or pHND10 == pAPY, 100-200ng, key resources table) in chilled 0.1cm cuvettes (Bio-Rad, cat# 1652089) and immediately electroporated (Voltage = 1.8kV, Capacitance = 25µF, Resistance = 200Ω). Directly after electroporation, 1ml of warm LB medium was added to the cuvette, bacteria were transferred to a tube containing 10ml pre-warmed LB medium and cultivated for 1h at 37°C. Then, bacteria were dispersed on LB agar plates supplemented with ampicillin (100 µg/ml) and incubated at 37°C. The next day, three colonies were picked, streaked on a new ampicillin supplemented LB agar plate, and incubated for 24h. The following day, two cryostocks were made from single colonies in LB medium supplemented with 20% glycerol.

Bacteria injection sepsis model

The Keio collection (key resources table) *E. coli* PS transformed with either pEMPTY or pAPY was aerobically grown O/N (16h / 37°C / 200rpm) in 20ml LB medium supplemented with ampicillin (100 µg/ml). PS+pEMPTY and PS+pAPY were washed with PBS (20min. / 22°C / 3200g) and

resuspended in 20ml fresh LB medium supplemented with ampicillin (100 µg/ml). To induce the apyrase, L-arabinose (0.03%, Sigma-Aldrich, cat# A3256-25G) was added. After three hours, bacteria were washed, resuspended in PBS supplemented with L-arabinose (0.03%) and 2×10^9 colony forming units were injected i.a. ATP in bacteria cultures and in the abdominal fluid was assessed using the same assay as described above (ATP Kit SL, key resources table). Mice were evaluated for post-operative well-being every four hours as described above. After four and eight hours and when the score reached specific criteria (for the survival experiment), animals were sacrificed using pentobarbital (150mg/kg bodyweight) followed by organ collection.

Collection of abdominal fluid, blood and organs

After pentobarbital injection, the mice were placed on a surgical tray, fixed, the abdomen was shaved, the abdominal skin was disinfected with betadine and the skin (but not peritoneum) was cut. Abdominal cells were isolated as described elsewhere with some modifications (Ray & Dittel, 2010). In brief, a 22 Gauge peripheral IV catheter was inserted into the abdominal cavity through the peritoneum. The abdominal cavity was flushed two times with 5ml MACS buffer (PBS supplemented with 3% FBS (Gibco, cat# 10500-064), 2% HEPES (Sigma-Aldrich, cat# H0887-100ml) and 2mM EDTA (Sigma-Aldrich, cat# E5134-500G)). The first 5ml were used to flush the upper abdomen under pressure, and the second 5ml, to flush the lower abdomen under pressure. Part of the aspirated fluid was used for aerobic plating, if needed, and the rest was centrifuged for 5min at 700g to pellet the abdominal cells for various downstream applications. To collect blood, the peritoneum was opened. 300µl blood was collected from the inferior vena cava using a 22 Gauge peripheral IV catheter and a 1ml syringe, which was prefilled with 30µl 2mM EDTA. Before organ collection (lungs, liver, kidney, and spleen), the mouse was flushed with 5ml PBS. Organs were excised and used for downstream processing.

Digestion of organs and preparation of single cell suspension

Harvested organs were digested (key resources table; kidney&lungs: 1mg/ml Col I + 1mg/ml Col IV + 1mg/ml Col D + 0.1mg/ml DNase I in DMEM (Gibco, cat# 31966-047) + 3% FBS for 30 min.; liver: 1mg/ml Col IV + 0.1mg/ml DNase I in DMEM + 3% FBS for 20 min.) at 37°C with a spinning magnet or gently pushed through a 100µm mesh (spleen). After washing with MACS buffer (5min. / 4°C / 700g), erythrocytes were lysed using self-made RBC buffer (90g NH₄Cl, 10g KHCO₃, 370mg EDTA in 1L ddH₂O for 10x stock). Cells were washed again and stained as described below.

Staining of cells and flow cytometry

First, viability dye (key resources table) and Fc-block (key resources table) were diluted in PBS and cells were incubated for 20min. at 4°C in the dark. Cells were washed with MACS buffer and surface staining was done with the listed antibody cocktail: (key resources table: Ly-6G FITC, Ly-6C PerCP-Cy5.5 or Ly-6C PE-Cy7, CD11b APC, CD206 AF700, CD11c APC-eFluor780, CD45 efluor450 or CD45 APC-Cy7, CD19 Super Bright 600, CD3 BV605, NK1.1 BV605, CCR2 BV650, I-A/I-E BV711, CX3CR1 BV785, Siglec F PE, FcεR1α PE/Dazzle 594, CD115 PE-Cy7, F4/80 BUV395) for 20min. at 4°C in the dark. Cells were washed again and resuspended in MACS buffer for acquisition on an LSR-Fortessa (BD Biosciences). Analysis was done with FlowJo software (key resources table) and OMIQ web-based analysis platform (<https://www.omiq.ai/>). To preserve the global structure of abdominal cell populations, uniform manifold approximation and projection (UMAP) was used as dimensionality reduction technique and cell populations were defined using FlowSOM clustering algorithm (Van Gassen et al., 2015).

Absolute quantification of bacteria by plating

To count bacteria in isolated abdominal fluid, blood or growth cultures, serial dilutions were done (1 to 1:100'000) and 50µl of each dilution was streaked on LB agar plates and aerobically incubated. Plates, which had between 20-200 colony forming units were used for quantification.

Collection of OMV and ATP measurement of OMV

- *E. coli* PS or hypervesiculation mutants from the Keio collection were grown in LB medium for 5h, O/N (16h) or 24h at 37°C and 200rpm. Bacteria cultures were then centrifuged (20min. / 4°C / 3200g) to pellet bacteria. The supernatant was filtered through a 0.45µm PES filter (key resources table) and ultracentrifuged (1.5h / 4°C / 150'000g) to pellet OMV. When ATP within OMV was measured, OMV were washed in PBS and directly stored at -80°C. ATP was quantified using a luciferin-luciferase-based assay according to the manufacturer's protocol (Microbial ATP Kit HS, key resources table) and bioluminescence was measured using Tecan Spark spectrophotometer.

If OMV were used for characterization or i.a. injection, first ultracentrifugation was followed by a density gradient ultracentrifugation using OptiPrep (Iodixanol, Stemcell technologies, cat# 07820). OMV were resuspended in 50% OptiPrep and OptiPrep gradient (10%, 20%, 30%, 40%, 45%, 50%) was made in underlay-technique starting with the 10% layer. Samples were ultracentrifuged (16h / 4°C / 150'000g) and 6 fractions (fractions 1, 2, 3a, 3b, 4, and 5) were defined. Fractions were washed separately with PBS (1.5h / 4°C / 150'000g) and the amount of OMV was determined measuring protein concentration using BCA assay according to the manufacturer's protocol (Thermo Fisher Scientific, cat# 23227). For experiments, only OMV from fraction 3 were used, which were washed with PBS (1.5h / 4°C / 150'000g), resuspended in PBS and stored at -80°C until further processing.

Electroporation (EP) and staining of OMV

If electroporation and staining of OMV was performed, OMV were thawed, pelleted (1.5h / 4°C / 150'000g), the pellet was resuspended in 720µl EP-buffer (500mM Sucrose and 1ml Glycerol in 10ml ultra-pure water) and kept on ice. This suspension was mixed with either 80µl EP-buffer (control) or 80µl 10mM ATP (or ATPys for RNA sequencing experiment) (key resources table), filled in chilled 0.4cm EP cuvettes (Bio-Rad, cat# 1652088) and immediately electroporated (Voltage = 1100V, Capacitance = 50µF, Resistance = 100Ω). After EP, the OMV were 1:1 diluted in warm PBS and kept at 37°C for 20min. DiI (key resources table) was added 1:100 to the sample during the incubation time. OMV suspension was then washed with PBS (1.5h / 4°C / 150'000g).

OMV injection sepsis model

After the final wash in PBS (see above), OMV were resuspended in NaCl 0.9% and filtered through a 0.45µm centrifuge filter tube (Sigma-Aldrich, cat# CLS8162-96EA). The final OMV suspension was quantified using a BCA assay. 25µg of either ATP-loaded OMV or empty OMV were i.a. injected and mice were evaluated as described above. After one hour, the animal was sacrificed using pentobarbital followed by organ collection. Tissue digestion and flow cytometry was done as described above.

Assessment of ATP release by OMV

OMV were electroporated as described above. After washing in PBS (1.5h / 4°C / 150'000g), the OMV pellet was resuspended in 6ml warm PBS and incubated at 37°C. At baseline, after 1, 3, 5, 10 and 24 hours, 1ml was taken and washed with PBS (1.5h / 4°C, 150'000g). The OMV pellet was then resuspended in 200µl PBS to assess protein concentration using a BCA assay, and ATP in OMV was quantified using a luciferin-luciferase-based assay according to the manufacturer's protocol (Intracellular ATP Kit HS, key resources table). Bioluminescence was measured using a Tecan Spark spectrophotometer.

SDS-PAGE, protein staining and Western blot

20µl of diluted *E. coli* culture and 20µl of the different OMV fractions (except fraction 1, since no protein could be detected) were diluted 1:1 with Laemmli/βME (Laemmli buffer solution containing 5% β-mercaptoethanol). The mixture was heated at 100°C for 5min., shortly centrifuged at 13'000rpm and loaded on Mini-PROTEAN TGX Gels (Bio-Rad, cat# 4561094). Bio-Rad marker (6µl) was added to one well and the gel was run at 100V for 1.5h. The gel was then either directly stained with Coomassie blue or transferred to a membrane.

Coomassie blue staining was done as follows: The gel was washed in ddH₂O and the staining solution (Coomassie blue 0.1%, 40% ethanol, 10% acetic acid) was heated for 15s in the microwave. Warm staining solution was added to the gel and gently shaken for 15min. Then, staining solution was removed, and the gel was washed with ddH₂O. Destaining solution (10% ethanol, 7.5% acetic acid) was added for 1h, exchanged and left overnight.

To transfer the proteins to a membrane, iBlot2 (Invitrogen) was used. Membranes were then blocked with 4% milk/PBS for 1h. FtsZ-antibody (1:200, key resources table) and ompF-antibody (1:500, key resources table) were added and incubated overnight at 4°C. The next day, membranes were washed with PBS tween (PBST, 0.05%) three times for 5min. Secondary fluorescent antibody was then added in milk (1:10'000, key resources table) and membrane was incubated for 1h. The membrane was washed again with PBST three times for 5min. and then scanned using a Licor Odyssey.

Nanoparticle tracking analysis (NTA)

Size distribution of OMV was analysed using the NanoSight NS300 Instrument (Malvern Panalytical, 405 nm laser) according to the manufacturer's protocols. OMV were resuspended in PBS and serial dilutions (1 to 1:100'000) were used to find suitable concentrations. Each experimental sample was analyzed 5 times. PBS was used to flush the system between the samples and to assess background. For each sample, the relative amount of OMV and the OMV size was recorded, which resulted in a size distribution curve. NTA 2.3 software was used to analyze the data and the following script was used for acquisition (Gheinani et al., 2018 [DOI](#)): SETTEMP 25; CAMERAON; CAMERAGAIN 12; CAMERALEVEL 11; REPEATSTART; SYRINGLOAD 100; DELAY 10; SYRINGSTOP; DELAY 15; CAPTURE 60; DELAY 1; **REPEAT 4**; SETTEMP OFF; PROCESSINGLESETTING; EXPORTRESULTS.

Electron microscopy negative staining

For imaging of negatively stained samples, 5µl of OMV suspension were adsorbed on glow discharged carbon coated 400 mesh copper grids (Plano) for 1-5min. After washing the grids 3 times by dipping in ultra-pure water, the grids were stained with 2% uranyl acetate solution (Electron Microscopy Science) in water for 45 s. The excess fluid was removed by gently pushing the grids sideways onto filter paper. The grids were then examined with a FEI Tecnai Spirit transmission electron microscope at 80kV, which was equipped with a Veleta TEM CCD camera (Olympus).

Immunofluorescent microscopy

One hour after i.a. injection of DiI-stained OMV, lungs were digested as described above. Single cell suspension was either fixed and imaged or live cells were imaged. For the fixed approach, single cell suspension was immunolabeled with FITC-tagged anti-Ly-6G (key resources table) at 1:100 dilution for 20 minutes at 4°C in the dark to distinguish neutrophils. Cells were washed once with PBS and applied onto glass slides using Cytospin (Thermo Shandon) for 5 minutes (1800 rpm). Slides were air dried for 2 minutes, washed with PBS and fixed using 4% paraformaldehyde solution for 10 min. Cell nuclei were stained with DAPI (key resources table) at 1:5000

concentration in IF buffer (0.25% BSA, 0.1% Triton X-100 in PBS) for 1.5 hours at room temperature in the dark. After washing 3×5 min. with IF buffer, the slides were covered and sealed with nail polish.

If live cells were imaged, single cell suspension was concurrently immunolabeled and stained with Hoechst 33342 (key resources table) at 1:1000 dilution for cell nuclei, FITC-tagged anti-Ly-6G (key resources table) at 1:100 dilution for neutrophils and LysoTracker Deep Red (key resources table) at 1:1000 dilution for lysosomes for 30 min. at 37°C in the dark. Cells were washed with MACS buffer and immediately imaged.

Fluorescence images were taken using either a Zeiss LSM710 or a Zeiss LSM980 inverted confocal laser scanning microscope equipped with a 63x(NA1.4) oil immersion objective. Detector wavelength cutoffs were set to minimize signal crosstalk between fluorophores.

RNA isolation from pulmonary neutrophils

One hour after OMV i.a. injection, lungs were digested as described above. One animal had to be excluded, since the surgical time point has been missed. Neutrophils were isolated from single cell suspension using Streptavidin MicroBeads (Miltenyi Biotec, cat# 130-048-101) according to the manufacturer's protocol. In brief, cells were counted and incubated with a biotinylated anti-mouse Ly-6G antibody (key resources table) for 20min. at 4°C. After washing, Streptavidin MicroBeads were added and incubated for 20min. at 4°C. After washing, a MidiMACS separator (Miltenyi Biotec, cat# 130-042-302) together with a LS column (Miltenyi Biotec, cat# 130-042-401) was used to isolate neutrophils and purity of positively selected neutrophils as well as unlabeled cells was assessed by flow cytometry (key resources table: Ly-6C PE-Cy7, CD11b APC). Neutrophils were pelleted, and supernatant was removed by pipetting. Neutrophils were snap frozen and stored at -80°C. RNA was isolated directly from frozen neutrophils pellets using Promega ReliaPrep RNA Cell Miniprep System (key resources table) according to the manufacturer's protocol and quality was assessed using Bioanalyzer and RNA 6000 Nano Kit (RQN 5.8-9.6, median 7.55). Samples were snap frozen and stored at -80°C until further processing by the Next Generation Sequencing Platform, University of Bern, www.ngs.unibe.ch [↗](#).

RNA sequencing

RNA sequencing libraries were prepared using Lexogen CORALL total RNA-seq library kit according to the manufacturer's protocol, which includes a rRNA depletion step. Sequencing was performed on an Illumina NovaSeq6000 SP flow cell, 2×50 cycles.

Alignment & quantification: The resulting fastq files were quality controlled using fastqc v0.11.9 (*Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data*, n.d.). Forward R1 reads were debarcoded by moving the first 12 nucleotides on the 5' end to the name of the read via fastp v0.19.5 ([Chen et al., 2018](#) [↗](#)). The alignment was performed with STAR v2.7.10a_alpha_220818 ([Dobin et al., 2013](#) [↗](#)). First, a Genome index was generated from the mouse reference genome GRCm39 ENSEMBL version 108. Second, the reads were then aligned to the reference using STAR with default parameters. Gene read counts were quantified using featureCounts from subread v2.0.1 using the mm108 GTF annotation with default parameters ([Liao et al., 2014](#) [↗](#)).

Data visualization: The data from the read count matrix was normalized to reads per million (RPM) and log-transformed, $x \rightarrow \log(1+x)$. The resulting data was used for Principal Component Analysis (PCA), which was performed with the R function *prcomp* and visualized with a custom script using ggplot2 ([Wickham, 2016](#) [↗](#)).

Differential Gene expression: Differentially expressed genes were computed using the R package DESeq2 ([Love et al., 2014](#) [↗](#)).

Pathway enrichment analysis: The differentially expressed genes obtained from DESeq2 with an adjusted p-value below 0.01 were uploaded to Metascape for pathway analysis on 5th December 2023 (Zhou et al., 2019 [↗](#)).

Heatmaps: The lists of genes of the pathways of interest were obtained from genome.jp for the KEGG pathways and using the R function *gconvert* from the R package gprofiler2 otherwise (Kolberg & Raudvere, 2023 [↗](#)). The log transformed data was used and the heatmaps were done with the heatmap.2 function of the R package gplots with the hierarchical cluster method “complete” using Pearson correlation distance (Warnes et al., 2022 [↗](#)).

Volcano plot: The volcano plots were performed with the R package ggplot2 and a custom R script (Wickham, 2016 [↗](#)).

Statistical analysis

Descriptive statistics and statistical tests were performed using Prism software (key resources table) or R and Rstudio (key resources table). For differences between two groups a t-test was applied when data was normally distributed (parametric). Otherwise, a Wilcoxon rank sum test was used (non-parametric). For differences between more than 2 groups, a one-way ANOVA followed by a Tukey post-hoc test (parametric), or a Kruskal-Wallis test followed by a pairwise Wilcoxon rank-sum test with Benjamini-Hochberg (BH) correction (non-parametric) was used. For survival analyses, a log-rank test was used. No one-tailed tests were used. No method was used to predetermine experimental sample sizes. A linear model and the correlation between ATP and OD₆₀₀ in **Figure 2** [↗](#) and **Figure 3** [↗](#) were computed using the R functions *lm* and *cor.test* with the following parameters:

```
stats::cor.test(data$variableATP, data$variablegrowth, alternative = “two.sided”, method =
“pearson”) summary(stats::lm(variableATP ~ variablegrowth, data = data))
```

$P < 0.05$ ($p == \text{padj}$, when correction for multiple testing was necessary) was considered significant in all statistical analyses unless stated otherwise in the figure legend. Significant differences are in *italic*.

Acknowledgements

We are grateful for the technical and financial support of the Inselspital, the DBMR and our laboratory. A special thank goes to Dana Leuenberger and Isabel Büchi for their support with the animal experiments and microscopy. We also want to thank the Microscopy Imaging Center (MIC) of the University of Bern for their assistance in sample preparation and imaging on the electron microscope. We want to thank Prof. Dr. Dr. Fabio Grassi and his group for providing us with the pBAD28 and pHND10 plasmids. We also want to thank Prof. Dr. Siegfried Hapfelmeier, Dr. Olivier Schären and Prof. Dr. Torsten Seuberlich for their extremely helpful scientific support. We want to thank Prof. Dr. Andrew Macpherson supporting us with his expertise and providing us with the Keio collection. Finally, we want to thank Prof. Dr. Dr. Joel Zindel, Dr. Simone Zwicky and Dr. Felix Baier for their critical contributions and ideas to complete this project. Figures were created using BioRender.com [↗](#).

Funding

This work was supported by the Swiss National Science Foundation (No: 166594) to G.B.

Data and code availability

RNA sequencing data will be made publicly available on the NCBI Sequence Read Archive (SRA) upon version of record (VOR) publication. In addition, we will then make publicly available all source data, metadata, and code to recreate the figures at BORIS portal of the University of Bern under a CC BY licence.

Author contributions

Conceptualization: D.S. and G.B.; Methodology: D.S., G.B. and D.St.; Software: D.S., D.S.-T. and K.K.; Formal analysis: D.S., A.S, D.S.-T. and N.E.; Investigation D.S., A.S., S.M., N.E. and L.S.; Resources: G.B. and D.St.; Data curation: D.S., D.S.-T. and K.K.; Writing – original draft preparation: D.S., G.B., L.S., D. St., D.S.-T., K.K.; Visualisation: D.S., D.S.-T.; Supervision: G.B.; Project administration: D.S. and G.B.; Funding acquisition: G.B. and D.St.

Competing interests

The authors declare no financial or other competing interests.

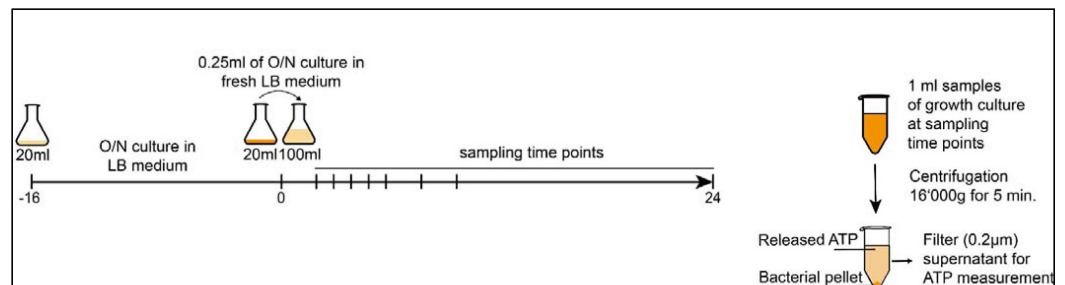


Figure 1-figure supplement 1

Experimental approach to measure released bacterial ATP and growth over time.

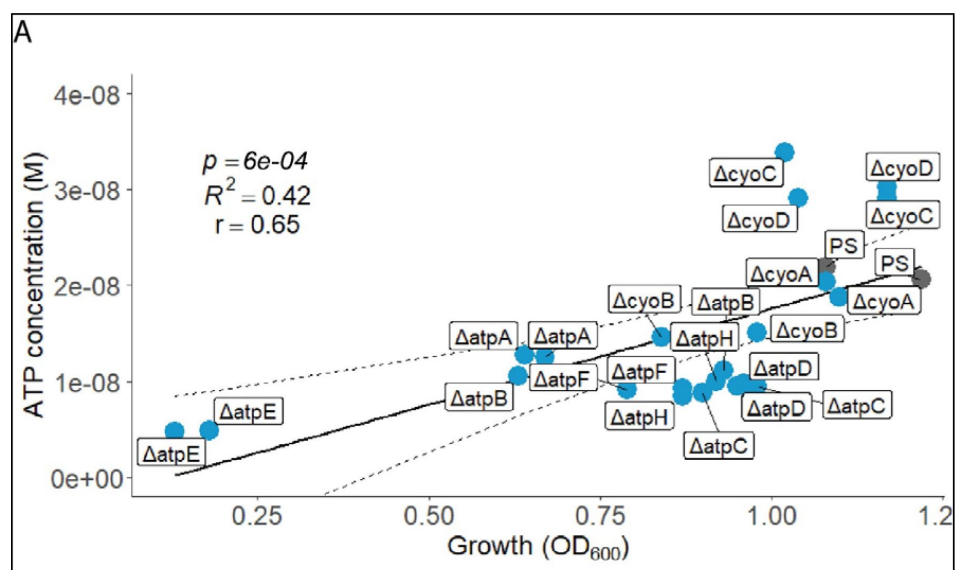


Figure 2-figure supplement 1

ATP concentration (M) and growth (OD_{600}) at the individual peak of ATP release of all assessed cyo and atp mutants and the PS were plotted against each other. Pearson's correlation (r) and coefficient of determination (R^2) of the applied linear model are depicted. 95% confidence level is shown by the black dashed lines.

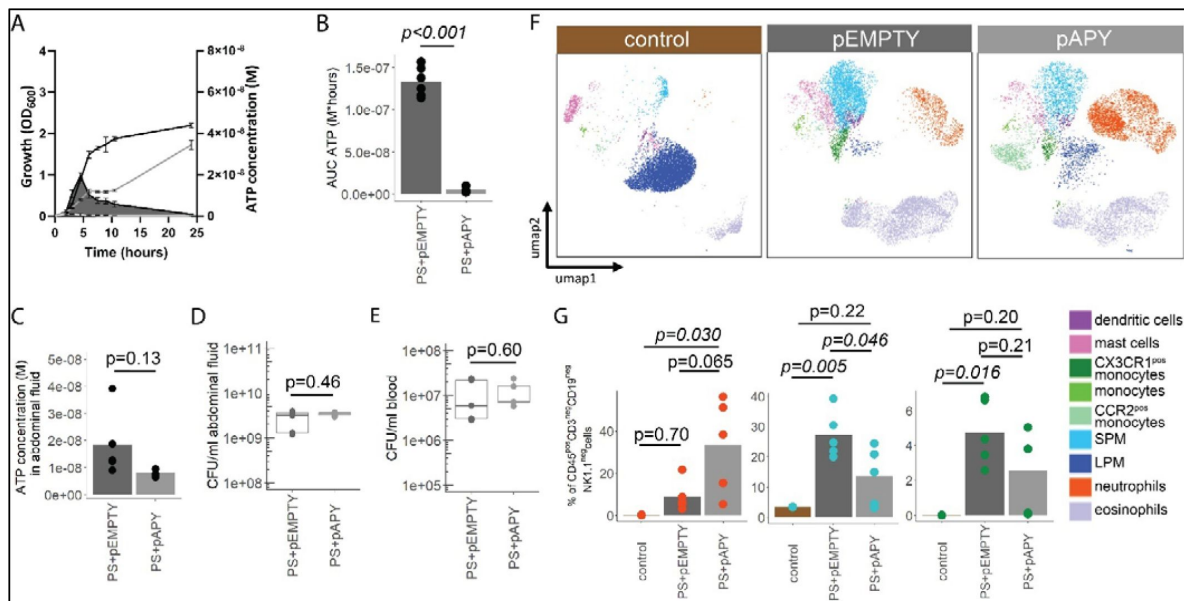


Figure 4-figure supplement 1

Immune cell characterization eight hours after i.a. injection of bacteria.

(A) Measurement of released ATP (M) and growth (OD_{600}) over time (hours) from PS+pEMPTY and PS+pAPY. $n = 2$ measurements of $N = 3$ independent bacteria cultures. Means and standard deviations are shown.

(B) Area under the curve (AUC) of released ATP over time (M*hours) of the previously assessed bacteria (cumulative ATP). T-test, $n = 2$ measurements of $N = 3$ independent bacteria cultures. Means and individual values are shown.

(C) Measurement of ATP (M) in abdominal fluid from mice eight hours after i.a. injection of bacteria. T-test, $n = 5$ animals per group of $N = 2$ independent experiments. Means and individual values are shown.

(D) Quantitative assessment of colony forming units in abdominal fluid and **(E)** blood from mice eight hours after i.a. injection of bacteria. Wilcoxon rank sum test, $n = 5$ animals per group of $N = 2$ independent experiments. Means and individual values are shown. No growth for controls was detected.

(F) Concatenated ($n = 5$ animals for each treatment group, $n = 3$ animals for control group of $N = 2$ independent experiments) and down-sampled images of immune cell populations characterized in the abdominal cavity eight hours after sham treatment or i.a. injection of bacteria.

(G) Abundance of neutrophils, small peritoneal macrophages (SPM) and CX3CR1^{pos} monocytes in abdominal fluid from mice eight hours after sham treatment or i.a. injection of bacteria. One-way ANOVA followed by Tukey post-hoc test, $n = 5$ animals for each treatment group, $n = 3$ animals for control group of $N = 2$ independent experiments. Means and individual values are shown.

Figure 5-figure supplement 1

ATP measurement of the PS, $\Delta nlpI$ as well as $\Delta tolB$ and OMV collection and characterization.

(A) Measurement of released ATP (M) and growth (OD_{600}) over time (hours) from PS, $\Delta nlpI$ and $\Delta tolB$. OMV collection time points are marked in purple. n = 2 measurements of N = 3 independent bacteria cultures. Means and standard deviations are shown.

(B) OMV before and after density gradient ultracentrifugation for 16h at 150'000g.

(C) Statistical parameters of OMV before electroporation as well as ATP-loaded and empty OMV after electroporation.

(D) Relative quantification of ATP in OMV 16 hours at 4°C after electroporation (0h = 100%). n = 2 measurements of N = 3 independent experiments. Means and individual values are shown.

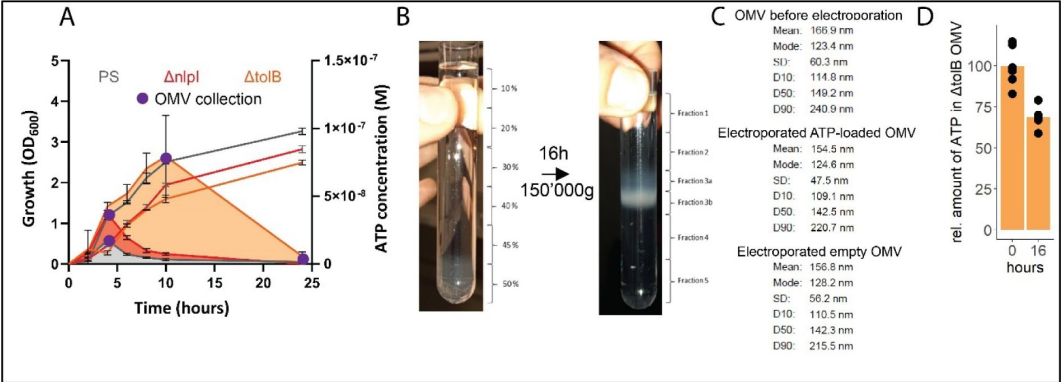
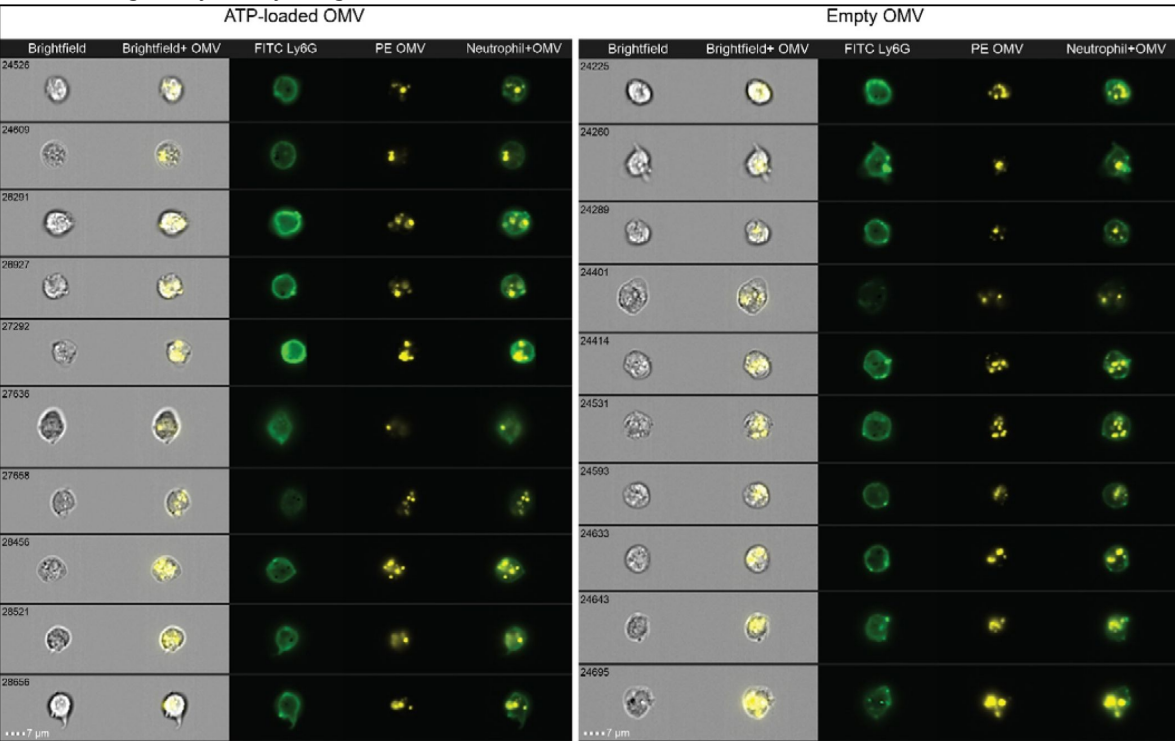


Figure 6-figure supplement 1

Uptake of OMV by neutrophils.

Representative images of OMV uptake by neutrophils in the abdominal cavity one hour after i.a. injection additionally assessed using flow cytometry (Image Stream).



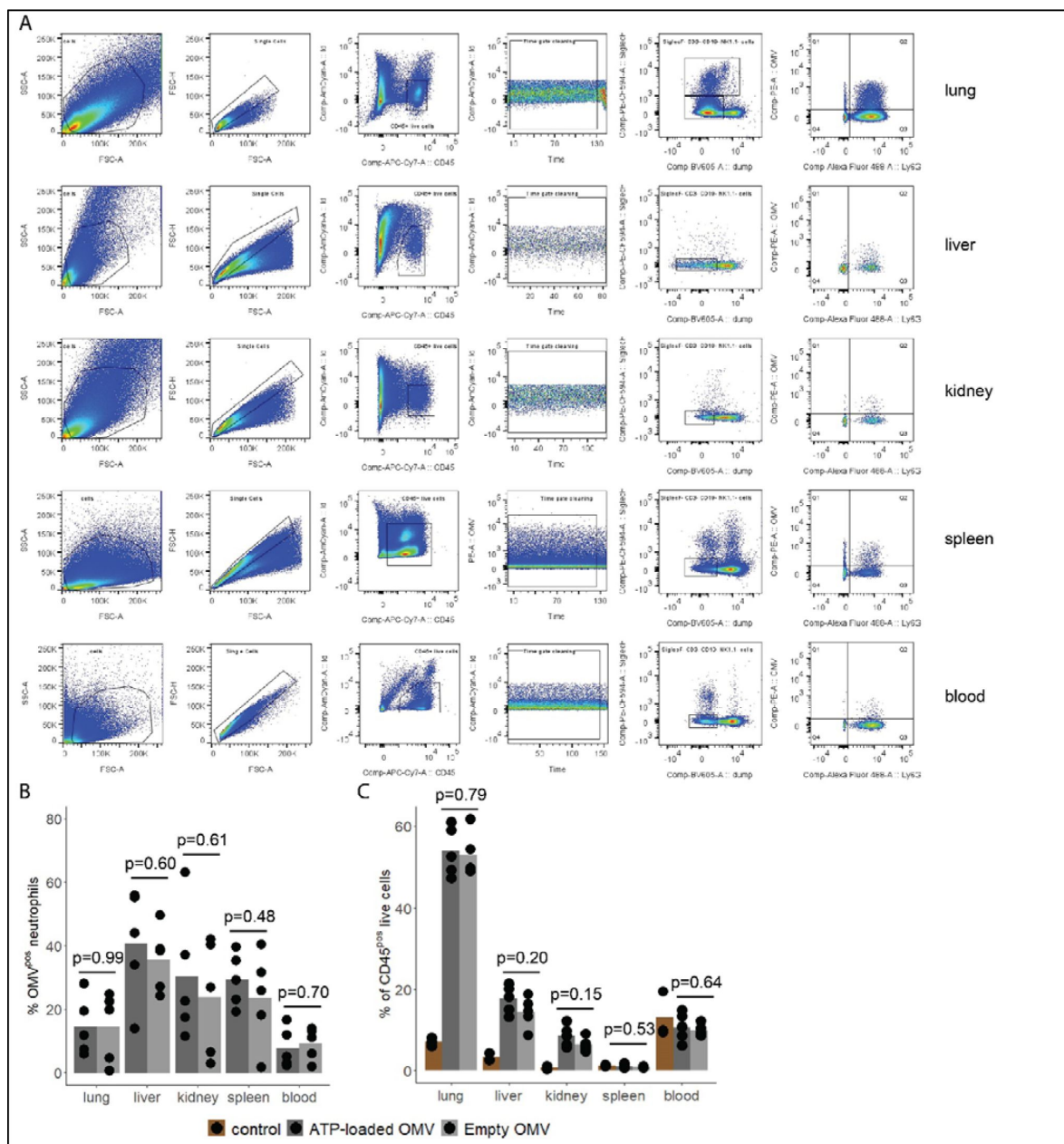


Figure 6-figure supplement 3

Assessment of OMV uptake by immune cells in remote organs.

(A) Gating strategy to identify total OMV^{pos} cells and specifically OMV^{pos} neutrophils in blood and remote organs (lung, liver, kidney, and spleen)

(B) Abundance of OMV^{pos} / (OMV^{pos}+OMV^{neg}) neutrophils one hour after i.a. injection of ATP-loaded or empty OMV. T-test, n = 5 animals per group of N = 2 independent experiments. Means and individual values are shown.

(C) Neutrophils as percentage of all CD45^{pos} live cells one hour after sham treatment or i.a. injection of either ATP-loaded or empty OMV. T-test, n = 5 animals for each treatment group, n = 3 animals for control group of N = 2 independent experiments. Means and individual values are shown.

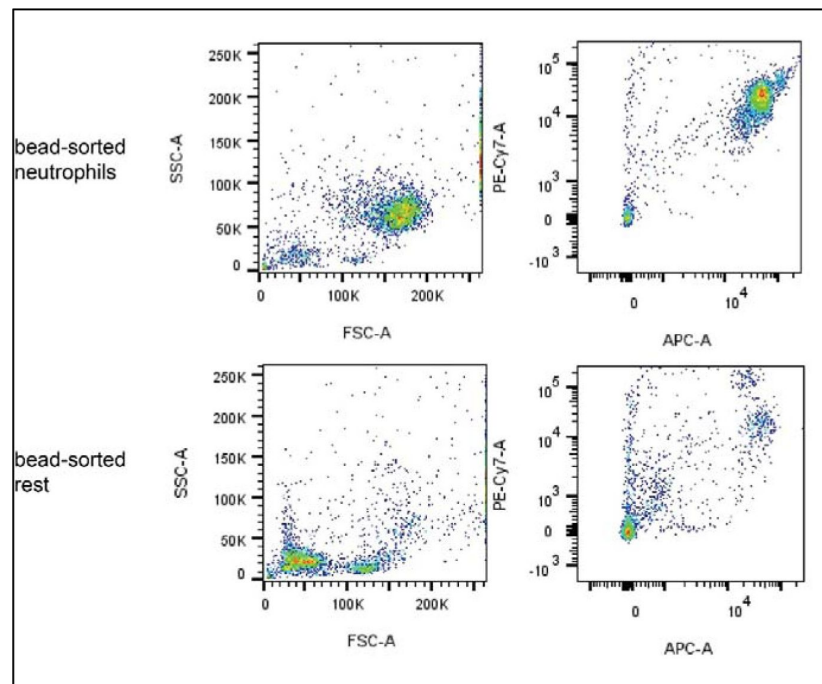


Figure 6-figure supplement 4

Assessment of the purity of bead-sorted pulmonary neutrophils.

Pulmonary neutrophils were isolated one hour after i.a. injection of ATPys-loaded or empty OMV, bead-sorted and assessed for purity by flow cytometry. A representative image is shown.

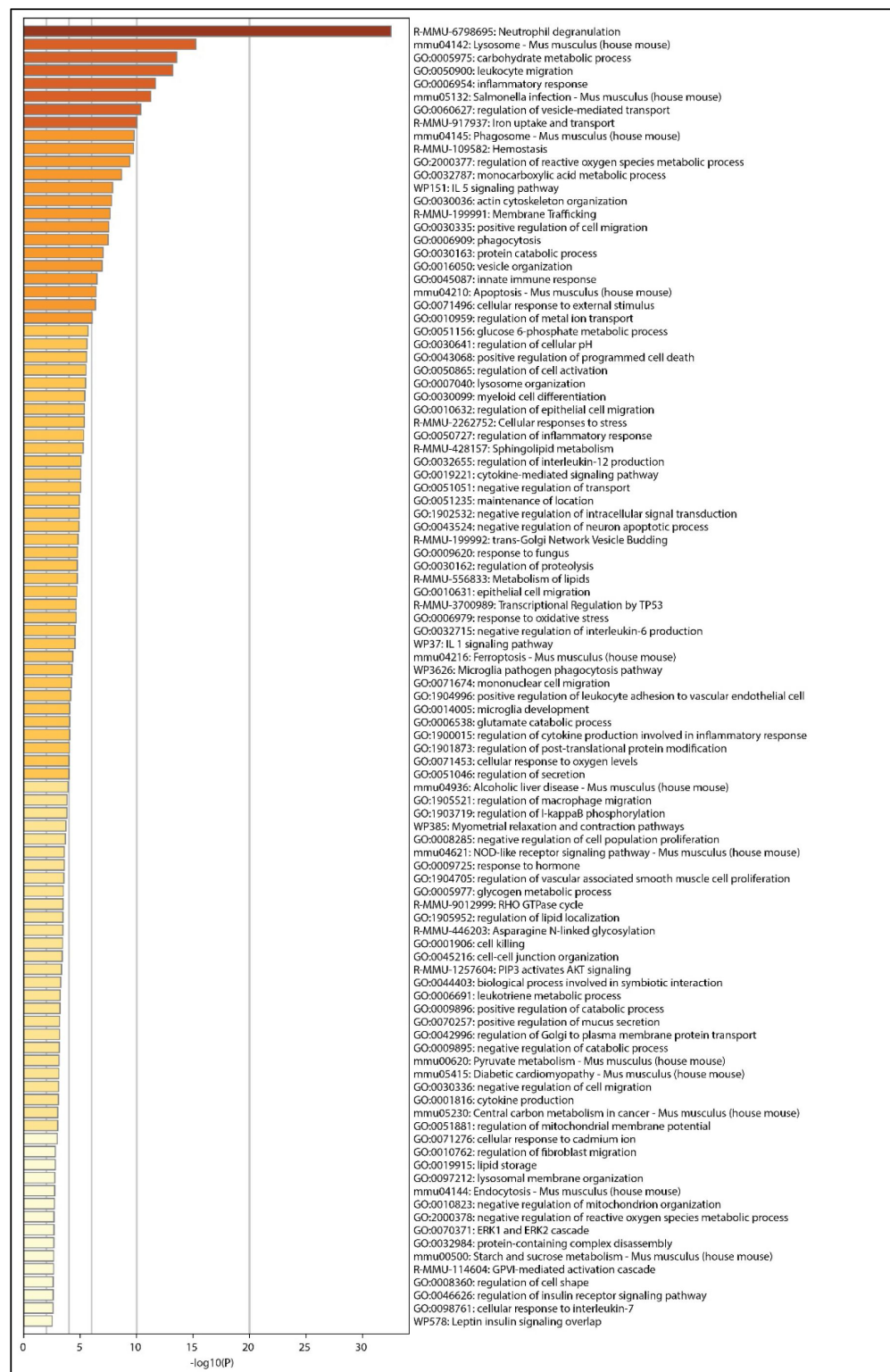


Figure 6-figure supplement 5

List of significantly different pathways after enrichment analysis of RNA sequencing results.

Pulmonary neutrophils were isolated one hour after i.a. injection of ATPys-loaded or empty OMV, bead-sorted and RNA sequencing was done. This resulted in these significantly different pathways between the groups after enrichment analysis. DESeq, n = 6 animals in the NE group, n = 5 animals in the NA group.

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (<i>Mus musculus</i> , female)	Wild-type mice	Inotiv, the Netherlands	C57Bl/6JRccHsd	
strain, strain background (<i>Enterococcus faecalis</i>)	Iso1	This paper	NA	See Materials and Methods: Human data
strain, strain background (<i>Escherichia coli</i>)	Iso2	This paper	NA	See Materials and Methods: Human data
strain, strain background (<i>Klebsiella pneumoniae</i>)	Iso3	This paper	NA	See Materials and Methods: Human data
strain, strain background (<i>Staphylococcus aureus</i>)	Iso4	This paper	NA	See Materials and Methods: Human data
strain, strain background (<i>E. faecalis</i>)	Iso5	This paper	NA	See Materials and Methods: CLP sepsis model
strain, strain background (<i>E. coli</i>)	Iso6	This paper	NA	See Materials and Methods: CLP sepsis model
strain, strain background (<i>S. aureus</i>)	Iso7	This paper	NA	See Materials and Methods: CLP sepsis model
strain, strain background (<i>E. coli</i>)	parental strain (PS)	Keio collection (Baba et al., 2006)	BW25113	
strain, strain background (<i>E. coli</i>)	$\Delta cyoA$	Keio collection (Baba et al., 2006)	JW0422-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta cyoB$	Keio collection (Baba et al., 2006)	JW0421-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta cyoC$	Keio collection (Baba et al., 2006)	JW0420-1	See Figure 2

strain, strain background (<i>E. coli</i>)	$\Delta cyoD$	Keio collection (Baba et al., 2006)	JW0419-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpA$	Keio collection (Baba et al., 2006)	JW3712-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpB$	Keio collection (Baba et al., 2006)	JW3716-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpC$	Keio collection (Baba et al., 2006)	JW3709-2	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpD$	Keio collection (Baba et al., 2006)	JW3710-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpE$	Keio collection (Baba et al., 2006)	JW3715-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpF$	Keio collection (Baba et al., 2006)	JW3714-2	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta atpH$	Keio collection (Baba et al., 2006)	JW3713-1	See Figure 2
strain, strain background (<i>E. coli</i>)	$\Delta ompF$	Keio collection (Baba et al., 2006)	JW0912-1	See Figure 3
strain, strain background (<i>E. coli</i>)	$\Delta ompC$	Keio collection (Baba et al., 2006)	JW2203-1	See Figure 3
strain, strain background (<i>E. coli</i>)	$\Delta lamB$	Keio collection (Baba et al., 2006)	JW3996-1	See Figure 3
strain, strain background (<i>E. coli</i>)	$\Delta phoE$	Keio collection (Baba et al., 2006)	JW0231-1	See Figure 3
strain, strain background (<i>E. coli</i>)	$\Delta mlaA$	Keio collection (Baba et al., 2006)	JW2343-1	See Figure 5

strain, strain background (<i>E. coli</i>)	$\Delta mlaE$	Keio collection (Baba et al., 2006)	JW3161-1	See Figure 5
strain, strain background (<i>E. coli</i>)	$\Delta nlpI$	Keio collection (Baba et al., 2006)	JW3132-1	See Figure 5
strain, strain background (<i>E. coli</i>)	$\Delta tolB$	Keio collection (Baba et al., 2006)	JW5100-1	See Figure 5
strain, strain background (<i>E. coli</i>)	$\Delta degP$	Keio collection (Baba et al., 2006)	JW0157-1	See Figure 5
strain, strain background (<i>E. coli</i>)	$\Delta rfaD$	Keio collection (Baba et al., 2006)	JW3594-1	See Figure 5
strain, strain background (<i>E. coli</i>)	$\Delta rodZ$	Keio collection (Baba et al., 2006)	JW2500-1	See Figure 5
antibody	Purified anti-Ms CD16/32, monoclonal	Biolegend	Cat# 101302; clone 93; Lot# B298973; RRID: AB_312801	(1:200)
antibody	Rat anti-Ms Ly-6G (FITC), monoclonal	BD Biosciences	Cat# 551460; clone 1A8; Lot# 9068981; RRID: AB_394207	(1:100)
antibody	Rat anti-Ms Ly-6C (PerCP-Cyanine5.5), monoclonal	Thermo Fisher Scientific	Cat# 45-5932-82; clone HK1.4; Lot# 2309273; RRID: AB_2723343	(1:100)
antibody	Rat anti-Ms/Hs CD11b (APC), monoclonal	Biolegend	Cat# 101212; clone M1/70; Lot# B312600; RRID: AB_312795	(1:800)
antibody	Rat anti-Ms CD206 (AF700), monoclonal	Biolegend	Cat# 141733; clone C068C2; Lot# B278058; RRID: AB_2629636	(1:300)
antibody	Armenian hamster anti-Ms CD11c (APC-eFluor780), monoclonal	Thermo Fisher Scientific	Cat# 47-0114-80; clone N418; Lot# 2133269; RRID: AB_1548652	(1:300)

antibody	Rat anti-Ms CD45 (efluor450), monoclonal	Thermo Fisher Scientific	Cat# 48-0451-82; clone 30-F11; Lot# 2005853 RRID: AB_1518806	(1:600)
antibody	Rat anti-Ms CD19 (Super Bright 600), monoclonal	Thermo Fisher Scientific	Cat# 63-0193-82; clone eBio1D3; Lot# 2366423; RRID: AB_2637308	(1:150)
antibody	Rat anti-Ms CD3 (BV 605), monoclonal	Biolegend	Cat# 100237; clone 17A2; Lot# B389899; RRID: AB_2562039	(1:100)
antibody	Mouse anti-Ms NK1.1 (BV605), monoclonal	Biolegend	Cat# 108739; clone PK-136; Lot# B389899; RRID: AB_2562273	(1:150)
antibody	Rat anti-Ms CCR2 (BV650), monoclonal	Biolegend	Cat# 150613; clone SA203G11; Lot# B294599; RRID: AB_2721553	(1:100)
antibody	Rat anti-Ms I-A/I-E (BV711), monoclonal	Biolegend	Cat# 107643; clone M5/114.15.2; Lot# B299330; RRID: AB_2565976	(1:600)
antibody	Mouse anti-Ms CX3CR1 (BV785), monoclonal	Biolegend	Cat# 149029; clone SA011F11; Lot# B304744; RRID: AB_2565938	(1:300)
antibody	Rat anti-Ms Siglec F (PE), monoclonal	Thermo Fisher Scientific	Cat# 12-1702-80; clone 1RNM44N; Lot# 2252684; RRID: AB_2637129	(1:300)
antibody	Armenian hamster anti-Ms FcεR1α (PE/Dazzle 594), monoclonal	Biolegend	Cat# 134331; clone MAR-1; Lot# B280348; RRID: AB_2687240	(1:300)
antibody	Rat anti-Ms CD115 (PE-Cy7), monoclonal	Biolegend	Cat# 135523; clone AFS98; Lot# B268547; RRID: AB_2566459	(1:600)
antibody	Rat anti-Ms F4/80 (BUV395), monoclonal	BD Biosciences	Cat# 565614; clone T45- 2342; Lot# 1104580; RRID: AB_2739304	(1:150)

antibody	Rat anti-Ms CD45 (APC-Cy7), monoclonal	Biolegend	Cat# 103115; clone 30-F11; Lot# NA; RRID: AB_312980	(1:150)
antibody	Rat anti-Ms Ly-6C (PE-Cy7), monoclonal	Biolegend	Cat# 128017; clone HK1.4; Lot# B331355; RRID: AB_1732093	(1:600)
antibody	Rat anti-Ms Ly-6G (Biotin), monoclonal	Biolegend	Cat# 127604; clone 1A8; Lot# B314606; RRID: AB_1186108	(1:600)
antibody	Rabbit anti- <i>E. coli</i> ftsZ, polyclonal	Agrisera	Cat# AS10715; RRID: AB_10754647	(1:200)
antibody	Rabbit anti- <i>E. coli</i> ompF, polyclonal	Biorbyt	Cat# orb308741; RRID: NA	(1:500)
antibody	Goat anti-rabbit, polyclonal	Licor Biosciences	Cat# 925-68021; RRID: AB_2713919	(1:10'000)
recombinant DNA reagent	pBAD28 == pEMPTY (plasmid)	(Proietti et al., 2019b)	Ampicillin resistance	(Santapaola et al., 2006; Scribano et al., 2014)
recombinant DNA reagent	pHND10 == pAPY (plasmid)	(Proietti et al., 2019b)	Ampicillin resistance	(Santapaola et al., 2006; Scribano et al., 2014)
sequence-based reagent	fD1	(Weisburg et al., 1991)	PCR primers	5'-AGA-GTT- TGA-TCC- TGG-CTC-AG- 3'
sequence-based reagent	fD2	(Weisburg et al., 1991)	PCR primers	5'-AGA-GTT- TGA-TCA-TGG- CTC-AG-3'
sequence-based reagent	rP1	(Weisburg et al., 1991)	PCR primers	5'-ACG-GTT- ACC-TTG-TTA- CGA-CTT-3'

commercial assay or kit	QIAquick Gel Extraction Kit	Qiagen	Cat# 28706	
commercial assay or kit	ATP Kit SL	BioThema	Cat# 144-041	
commercial assay or kit	Cell viability kit with BD liquid counting beads	BD Biosciences	Cat# 349480	
commercial assay or kit	Microbial ATP Kit HS	BioThema	Cat# 266-112	
commercial assay or kit	Intracellular ATP Kit HS	BioThema	Cat# 266-111	
commercial assay or kit	ReliaPrep RNA Cell Miniprep System	Promega	Cat# Z6011	
chemical compound, drug	Bacto Yeast Extract	Gibco	Cat# 212750	
chemical compound, drug	Bacto Tryptone	Gibco	Cat# 211699	
chemical compound, drug	Agar	Sigma-Aldrich	Cat# 05039-500G	
chemical compound, drug	GoTaq G2 Green Master Mix	Promega	Cat# M782A	
chemical compound, drug	Collagenase I (Col I)	Sigma-Aldrich	Cat# C0130-100MG	
chemical compound, drug	Collagenase IV (Col IV)	Worthington	Cat# LS004189	

chemical compound, drug	Collagenase D (Col D)	Roche	Cat# 11088858001	
chemical compound, drug	DNase I	Roche	Cat# 63792800	
chemical compound, drug	Adenosine 5'-triphosphate disodium salt hydrate (ATP)	Sigma-Aldrich	Cat# 2383-1G	
chemical compound, drug	Adenosine-5'-(γ-thio)-triphosphate, Tetralithium salt (ATPys)	Jena Bioscience	Cat# NU-406-50	
software, algorithm	Graphpad Prism v9.5.1	Prism Graphpad software	https://www.graphpad.com/	
software, algorithm	FlowJo v10.8.1	FlowJo software	https://www.flowjo.com	
software, algorithm	fastqc v0.11.9	(Babraham Bioinformatics - FastQC A Quality Control Tool for High Throughput Sequence Data, n.d.)	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	
software, algorithm	fastp v0.19.5	(Chen et al., 2018)	https://github.com/OpenGenome/fastp	
software, algorithm	STAR v2.7.10a_alpha_220818	(Dobin et al., 2013)	https://github.com/alexdobin/STAR	
software, algorithm	subread v2.0.1	(Liao et al., 2014)	https://github.com/ShiLab-Bioinformatics/subread	
software, algorithm	R v4.2.2	The R Project for Statistical Computing	https://cran.r-project.org	
software, algorithm	RStudio v2022.07.2	RStudio Desktop	https://www.rstudio.com	

software, algorithm	R package ggplot2 v3.4.3	(Wickham, 2016)	https://ggplot2.tidyverse.org	
software, algorithm	R package readxl v1.4.3	(Wickham, Bryan, et al., 2023)	https://CRAN.R-project.org/package=readxl	
software, algorithm	R package ggbreak v0.1.2	(Xu et al., 2021)	https://github.com/YuLab-SMU/ggbreak	
software, algorithm	R package rstatix v0.7.2	(Kassambara, 2023)	https://CRAN.R-project.org/package=rstatix	
software, algorithm	R package dplyr v1.0.10	(Wickham, François, et al., 2023)	https://CRAN.R-project.org/package=dplyr	
software, algorithm	R package DESeq2 v1.38.3	(Love et al., 2014)	https://github.com/mikelove/DESeq2	
software, algorithm	R package ggrepel v0.9.3	(Slowikowski et al., 2023)	https://CRAN.R-project.org/package=ggrepel	
software, algorithm	R package vegan v2.6-4	(Oksanen et al., 2022)	https://github.com/vegandevs/vegan	
software, algorithm	R package pairwiseAdonis v0.4	(Arbizu, 2017/2023)	https://github.com/pmartinezarbizu/pairwiseAdonis	
software, algorithm	R package gprofiler2 v0.2.2	(Kolberg & Raudvere, 2023)	https://cran.r-project.org/web/packages/gprofiler2/index.html	
software, algorithm	R package gplots v3.1.3	(Warnes et al., 2022)	https://CRAN.R-project.org/package=gplots	
software, algorithm	R package viridis v0.6.3	(Garnier et al., 2021)	https://sjmgarnier.github.io/viridis/	
other	Fixable Viability Dye eFluor506	Thermo Fisher Scientific	Cat# 65-0866-18	(1:600)
other	Nalgene Rapid-Flow PES Filter Units	Thermo Scientific	Cat# 168-0045, 165-0045, 124-0045PK	

other	Vybrant Dil Cell-Labeling Solution	Invitrogen	Cat# V22885	(1:100)
other	DAPI	Sigma-Aldrich	Cat# D9542-5MG	(1:5000)
other	Hoechst33342	Thermo Fisher Scientific	Cat# H3570	(1:1000)
other	LysoTracker	Invitrogen	Cat# L12492	(1:1000)

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Reviewer #1 (Public Review):**Summary:**

Extracellular ATP represents a danger-associated molecular pattern associated to tissue damage and can act also in an autocrine fashion in macrophages to promote proinflammatory responses, as observed in a previous paper by the authors in abdominal sepsis. The present study addresses an important aspect possibly conditioning the outcome of sepsis that is the release of ATP by bacteria. The authors show that sepsis-associated bacteria do in fact release ATP in a growth dependent and strain-specific manner. However, whether this bacterial derived ATP play a role in the pathogenesis of abdominal sepsis has not been determined. To address this question, a number of mutant strains of *E. coli* has been used first to correlate bacterial ATP release with growth and then, with outer membrane integrity and bacterial death. By using *E. coli* transformants expressing the ATP-degrading enzyme apyrase in the periplasmic space, the paper nicely shows that abdominal sepsis by these transformants results in significantly improved survival. This effect was associated to the reduction of small peritoneal macrophages and CX3CR1⁺ monocytes, and increase in neutrophils. To extrapolate the function of bacterial ATP from the systemic response to microorganisms, the authors exploited bacterial OMVs either loaded or not with ATP to investigate the systemic effects devoid of living microorganisms. This approach showed that ATP-loaded OMVs induced degranulation of neutrophils after lysosomal uptake, suggesting this mechanism could contribute to sepsis severity.

Strengths:

The most compelling part of the study is the analysis of *E. coli* mutants to address different aspects of bacterial release of ATP that could be pathogenically relevant during systemic dissemination of bacteria in the host.

Weaknesses:

As pointed out in the limitations of the study whether ATP-loaded OMVs could provide a mechanistic proof of the pathogenetic role of bacteria-derived ATP independently of live microorganisms in sepsis is interesting but not definitively convincing. It could be useful to see whether degranulation of neutrophils is differently induced also by apyrase-expressing vs control *E. coli* transformants. Also, the increase of neutrophils in bacterial ATP-depleted abdominal sepsis, which has better outcome than "ATP-proficient" sepsis, seems difficult to correlate to the hypothesized tissue damage induced by ATP delivered via non-infectious OMVs. Is neutrophils count affected by ATP delivered via OMVs? Probably a comparison of cytokine profiles in the abdominal fluids of *E. coli* and OMV treated animals could be helpful in defining the different responses induced by OMV-delivered vs bacterial-released ATP.

The analyses performed on OMV treated versus *E. coli* infected mice are not immediately related and difficult to combine when trying to draw a pathogenetic hypothesis for bacterial ATP in sepsis.

It's not clear why lung neutrophils were used for RNAseq.

<https://doi.org/10.7554/eLife.96678.2.sa2>

Reviewer #2 (Public Review):**Summary:**

In their manuscript, Daniel Spari et al. explored the dual role of ATP in exacerbating sepsis, revealing that ATP from both host and bacteria significantly impacts immune responses and

disease progression.

Strengths:

The study meticulously examines the complex relationship between ATP release and bacterial growth, membrane integrity, and how bacterial ATP potentially dampens inflammatory responses, thereby impairing survival in sepsis models. Additionally, this compelling paper implies a concept that bacterial OMVs act as vehicles for the systemic distribution of ATP, influencing neutrophil activity and exacerbating sepsis severity.

Weaknesses:

(1) The researchers extracted and cultivated abdominal fluid on LB agar plates, then randomly picked 25 colonies for analysis. However, they didn't conduct 16S sequencing on the fluid itself. It's worth noting that the bacterial species present may vary depending on the individual patients. It would be beneficial if the authors could specify whether they've verified the existence of unculturable species capable of secreting high levels of Extracellular ATP.

(2) Do mice lacking commensal bacteria show a lack of Extracellular ATP following cecal ligation puncture?

(3) The authors isolated various bacteria from abdominal fluid, encompassing both Gram-negative and Gram-positive types. Nevertheless, their emphasis appeared to be primarily on the Gram-negative *E. coli*. It would be beneficial to ascertain whether the mechanisms of Extracellular ATP release differ between Gram-positive and Gram-negative bacteria. This is particularly relevant given that the Gram-positive bacterium *E. faecalis*, also isolated from the abdominal fluid, is recognized for its propensity to release substantial amounts of Extracellular ATP.

(4) The authors observed changes in the levels of LPM, SPM, and neutrophils in vivo. However, it remains uncertain whether the proliferation or migration of these cells is modulated or inhibited by ATP receptors like P2Y receptors. This aspect requires further investigation to establish a convincing connection.

(5) Additionally, is it possible that the observed in vivo changes could be triggered by bacterial components other than Extracellular ATP? In this research field, a comprehensive collection of inhibitors is available, so it is desirable to utilize them to demonstrate clearer results.

(6) Have the authors considered the role of host-derived Extracellular ATP in the context of inflammation?

(7) The authors mention that Extracellular ATP is rapidly hydrolyzed by ectonucleotases in vivo. Are the changes of immune cells within the peritoneal cavity caused by Extracellular ATP released from bacterial death or by OMVs?

(8) In the manuscript, the sample size (n) for the data consistently remains at 2. I would suggest expanding the sample size to enhance the robustness and rigor of the results.

<https://doi.org/10.7554/eLife.96678.2.sa1>

Author response:

The following is the authors' response to the original reviews.

The points raised let us critically rethink our approach, our results, and our conclusions. Furthermore, it gave us the chance to elaborate on some critical aspects that were mentioned. With the help of the reviewers, we made some clarifications in the point-by-point responses and implemented them in the manuscript. Furthermore, we modified the figures as suggested:

- The colors in Figure 1C, D, G and H have been adapted as suggested
- We added a Figure2-figure supplement 1, which strengthens our conclusion in Figure 2
- As asked by reviewer #1 (weaknesses #3), we added the data about neutrophil numbers in the different organs (Figure 6-figure supplement 3C).

Reviewer #1 (Public Review):

Summary:

- Extracellular ATP represents a danger-associated molecular pattern associated to tissue damage and can act also in an autocrine fashion in macrophages to promote proinflammatory responses, as observed in a previous paper by the authors in abdominal sepsis. The present study addresses an important aspect possibly conditioning the outcome of sepsis that is the release of ATP by bacteria. The authors show that sepsis-associated bacteria do in fact release ATP in a growth dependent and strain-specific manner. However, whether this bacterial derived ATP play a role in the pathogenesis of abdominal sepsis has not been determined. To address this question, a number of mutant strains of E. coli has been used first to correlate bacterial ATP release with growth and then, with outer membrane integrity and bacterial death. By using E. coli transformants expressing the ATP-degrading enzyme apyrase in the periplasmic space, the paper nicely shows that abdominal sepsis by these transformants results in significantly improved survival. This effect was associated with a reduction of peritoneal macrophages and CX3CR1+ monocytes, and an increase in neutrophils. To extrapolate the function of bacterial ATP from the systemic response to microorganisms, the authors exploited bacterial OMVs either loaded or not with ATP to investigate the systemic effects devoid of living microorganisms. This approach showed that ATP-loaded OMVs induced degranulation of neutrophils after lysosomal uptake, suggesting that this mechanism could contribute to sepsis severity.

Strengths:

- A strong part of the study is the analysis of E. coli mutants to address different aspects of bacterial release of ATP that could be relevant during systemic dissemination of bacteria in the host.

We want to thank the reviewer for recognizing this important aspect of our experimental approach.

Weaknesses:

- As pointed out in the limitations of the study whether ATP-loaded OMVs provide a mechanistic proof of the pathogenetic role of bacteria-derived ATP independently of live microorganisms in sepsis is interesting but not definitively convincing. It could be useful to see whether degranulation of neutrophils is differentially induced by apyrase-expressing vs control E. coli transformants.

We thank the reviewer for raising several important points. In our study, we assessed local and systemic effects of released bacterial ATP. The consequences of local bacterial ATP release

were assessed using an apyrase-expressing *E. coli* transformant. Locally, bacterial ATP resulted in a decrease in neutrophil numbers and we hypothesize that directly released bacterial ATP either leads to neutrophil death (e.g. via P2X7 receptor (Proietti et al., 2019)) or interferes with the recruitment of neutrophils (e.g. via P2Y receptors (Junger, 2011)).

The systemic consequences were assessed using ATP-loaded and empty OMV. We have shown that degranulation is induced by OMV-derived bacterial ATP. ATP-containing OMV are engulfed by neutrophils, reach its endolysosomal compartment and might activate purinergic receptors, which then lead to aberrant degranulation. This concept, that needs to be explored in future studies, is fundamentally different from classical purinergic signaling via directly released bacterial ATP into the extracellular space.

It is possible that neutrophil degranulation is also modulated by directly released bacterial ATP. We agree that this should be assessed in future studies. Also, the role of OMV-derived bacterial ATP should be assessed locally as well as the importance of directly released vs. OMV-mediated bacterial ATP dissected locally. Based on our measurements (Figure 4-figure supplement 1A and Figure 5C), we estimate that the effect of OMV-derived bacterial ATP might be much smaller than the effects of directly released bacterial ATP. Thus, direct ATP release might predominate locally. However, we fully agree that this has to be investigated in a future study to reconcile the different aspects of bacterial ATP signaling. A paragraph will be added to the manuscript, in which we discuss this particular issue.

- Also, the increase of neutrophils in bacterial ATP-depleted abdominal sepsis, which has better outcomes than "ATP-proficient" sepsis, seems difficult to correlate to the hypothesized tissue damage induced by ATP delivered via non-infectious OMVs.

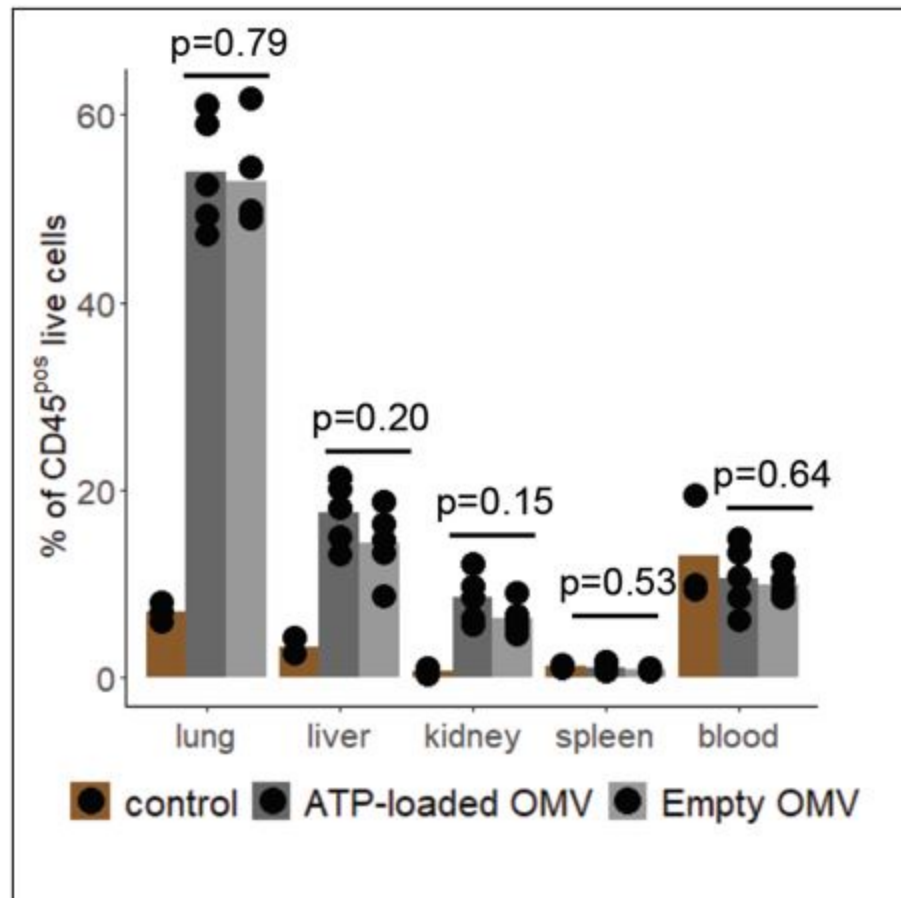
We fully acknowledge the mentioned discrepancy. What we propose is that bacterial ATP exhibits different functions that are dependent on the release mechanism (see above). Locally, in the peritoneal cavity, neutrophil numbers are decreased by directly released bacterial ATP. Remotely, ATP is delivered via OMV and impacts on neutrophil function. We agree that, in particular, in the peritoneal cavity, both effects may play a role. However, the impact of directly released bacterial ATP seems to be dominant (see above).

We propose that neutrophils are decreased locally because of directly released bacterial ATP, which prevents efficient infection control and, therefore, impairs sepsis survival. In addition, these fewer neutrophils might even be dysregulated by the engulfment of bacterial ATP delivered via OMV, which leads to an upregulated and possibly aberrant degranulation process worsening local and remote tissue damage. We agree that in addition to neutrophil numbers, the function of local neutrophils should be assessed with and without the influence of OMV-delivered bacterial ATP. This could be done by RNA sequencing of primary neutrophils from the peritoneal cavity or neutrophil cell lines as well as degranulation assays.

- Are the neutrophils counts affected by ATP delivered via OMVs?

This is difficult to show in the peritoneal cavity where we have both, directly released bacterial ATP and OMV-derived bacterial ATP. We assessed such putative difference, however, for the systemic organs and the blood, where we did not find any differences in neutrophil numbers.

Author response image 1.



- A comparison of cytokine profiles in the abdominal fluids of *E. coli* and OMV treated animals could be helpful in defining the different responses induced by OMV-delivered vs bacterial-released ATP. The analyses performed on OMV treated versus *E. coli* infected mice are not closely related and difficult to combine when trying to draw a hypothesis for bacterial ATP in sepsis.

We fully agree that there are several open questions that remain to be elucidated, in particular, to differentiate the local role of directly released versus OMV-delivered bacterial ATP. In this study, we laid the foundation for future in vivo research to examine the specific role of bacterial ATP in sepsis. Such future research avenues might be to investigate the local effects of OMV-delivered bacterial ATP, and how neutrophil migration, apoptosis and degranulation are altered. We agree that exploration of the local secretory immune response and cytokine profiles are relevant to understand the different mechanisms of how bacterial ATP alters sepsis. However, such experiments should be ideally performed in systems where the source and the delivery of ATP can be modulated locally.

- Also it was not clear why lung neutrophils were used for the RNAseq data generation and analysis.

Thank you for this remark. We have chosen primary lung neutrophils for four reasons:

- (1) Isolation of primary lung neutrophils allowed us to assess an in vivo response that would not have been possible with cell lines.
- (2) The lung and the respiratory system are among the clinically most important organs affected during sepsis resulting in a significant cause of mortality.
- (3) We show in Figure 6C that specifically in the lung, OMV are engulfed by neutrophils, which shows the relevance of the lung also in our study context.
- (4) And finally, lung neutrophils were chosen to examine specifically distant and not local effects.

Reviewer #2 (Public Review):

Summary:

- In their manuscript "Released Bacterial ATP Shapes Local and Systemic Inflammation during Abdominal Sepsis", Daniel Spari et al. explored the dual role of ATP in exacerbating sepsis, revealing that ATP from both host and bacteria significantly impacts immune responses and disease progression.

Strengths:

- The study meticulously examines the complex relationship between ATP release and bacterial growth, membrane integrity, and how bacterial ATP potentially dampens inflammatory responses, thereby impairing survival in sepsis models. Additionally, this compelling paper implies a concept that bacterial OMVs act as vehicles for the systemic distribution of ATP, influencing neutrophil activity and exacerbating sepsis severity.

We thank the reviewer for mentioning these key points and supporting the relevance of our study.

Weaknesses:

(1) The researchers extracted and cultivated abdominal fluid on LB agar plates, then randomly picked 25 colonies for analysis. However, they did not conduct 16S rRNA gene amplicon sequencing on the fluid itself. It is worth noting that the bacterial species present may vary depending on the individual patients. It would be beneficial if the authors could specify whether they've verified the existence of unculturable species capable of secreting high levels of Extracellular ATP.

Most septic complications are caused by a limited spectrum of bacteria, belonging mainly either to the Firmicutes or the Proteobacteria phyla, including *E. coli*, *K. pneumoniae*, *S. aureus* or *E. faecalis* (Diekema et al., 2019; Mureşan et al., 2018). We validated this well documented existing evidence by randomly assessing 25 colonies. For the planned experiments, it was crucial to work with culturable bacteria; otherwise, ATP measurements, the modulation of ATP generation or loading of OMV would not have been possible. Using such culturable bacteria allowed us to describe mechanisms of ATP release.

We fully agree that hard-to-culture or unculturable bacteria might contribute significantly to septic complications. This, however, would need to be explored in future studies using extensive culturing methods (Cheng et al., 2022).

(2) Do mice lacking commensal bacteria show a lack of extracellular ATP following cecal ligation puncture?

ATP is typically secreted by many cells of the host in active and passive manners in the case of any injury, including cecal ligation and puncture (Burnstock, 2016; Dosch et al., 2018; Eltzschig et al., 2012; Idzko et al., 2014). We hypothesize that bacterial ATP is a potential priming agent at early stages of sepsis, and indeed, at such early time points, a comparison of peritoneal ATP levels between germfree and colonized mice could support our hypothesis. Future studies addressing this question must, however, correct for the different immune responses between germ-free and colonized mice. This is of utmost importance, especially for the cecal ligation and puncture model, since the cecum of germ-free mice is extremely large, making such experiments hard to control.

(3) The authors isolated various bacteria from abdominal fluid, encompassing both Gram-negative and Gram-positive types. Nevertheless, their emphasis appeared to be primarily on the Gram-negative E. coli. It would be beneficial to ascertain whether the mechanisms of Extracellular ATP release differ between Gram-positive and Gram-negative bacteria. This is particularly relevant given that the Gram-positive bacterium E. faecalis, also isolated from the abdominal fluid, is recognized for its propensity to release substantial amounts of Extracellular ATP.

We fully agree with this comment. In this paper, we used E. coli as our model organism to determine the principles of sepsis-associated bacterial ATP release and therefore focused on gram-negative bacteria. In addition to the direct, growth-dependent release, we found a relevant impact of OMV-delivered bacterial ATP. For this latter purpose, a gram-negative strain, in which OMV generation has been well described (Schwechheimer & Kuehn, 2015), was chosen. Recently, gram-positive bacteria have been shown to secrete ATP and OMV as well (Briaud & Carroll, 2020; Hironaka et al., 2013; Iwase et al., 2010). Given the fundamental differences in the structure of the cell wall of gram-positive bacteria and the mechanisms of OMV generation and release, future studies are required to assess the relevance of directly released and OMV-delivered ATP in gram-positive bacteria.

(4) The authors observed changes in the levels of LPM, SPM, and neutrophils in vivo. However, it remains uncertain whether the proliferation or migration of these cells is modulated or inhibited by ATP receptors like P2Y receptors. This aspect requires further investigation to establish a convincing connection.

We fully agree with this comment. The decrease in LPM and the consequential predominance of SPM have been well described after inflammatory stimuli in the context of the macrophage disappearance reaction (Ghosh et al., 2010). Also, it has been shown that purinergic signaling modulates infiltration of neutrophils and can lead to cell death as a consequence of P2Y and P2X receptor activation (Junger, 2011; Proietti et al., 2019). In our study, we propose that intracellular purinergic receptors contribute to neutrophil function during sepsis. After introducing the general principles and fundamentals of bacterial ATP with our studies, we fully agree that additional experiments need to address downstream purinergic receptor activation. That, however, would go beyond the scope of our study.

(5) Additionally, is it possible that the observed in vivo changes could be triggered by bacterial components other than Extracellular ATP? In this research field, a comprehensive collection of inhibitors is available, so it is desirable to utilize them to demonstrate clearer results.

This question is of utmost importance and defined the choice of our model and experimental approach. When we started the project, we used two different E. coli mutants that release low (ompC) and high (eaeH) amounts of ATP. However, the limitation of this approach is that these are different bacteria, which may also differ in the components they secrete or the

surface proteins they express. We, therefore, decided against that approach. With the approach we finally used (same bacterium, just with and without ATP), we aimed to minimize the influence of non-ATP bacterial components.

(6) Have the authors considered the role of host-derived Extracellular ATP in the context of inflammation?

Yes, the role of host-derived extracellular ATP in inflammation and sepsis is well-established with contradictory results (Csóka et al., 2015; Ledderose et al., 2016). This conflicting data was the rationale to test the relevance of bacterial ATP. We suggest that bacterial ATP is essential in the early phase of sepsis when bacteria invade the sterile compartment and before efficient host response, including the eukaryotic release of ATP, is established.

(7) The authors mention that Extracellular ATP is rapidly hydrolyzed by ectonucleotases in vivo. Are the changes of immune cells within the peritoneal cavity caused by Extracellular ATP released from bacterial death or by OMVs?

This is a relevant question that was also asked by reviewer #1, and we answered it in detail above (weaknesses comment #1 and #2). From our ATP measurements (Figure 4-figure supplement 1A and Figure 5C), we conclude that locally, the role of directly released bacterial ATP (extracellular) predominates over OMV-derived bacterial ATP. Furthermore, the mechanisms between directly released and OMV-derived bacterial ATP (within OMV, engulfed and transported to the endolysosomal compartment) are different, and especially extracellular ATP has been described to lead to apoptosis via P2X7 signaling.

(8) In the manuscript, the sample size (n) for the data consistently remains at 2. I would suggest expanding the sample size to enhance the robustness and rigor of the results.

Two biological replicates (independent cultures) were only used for the bacteria cultures in Figure 1, Figure 2, and Figure 3, which achieved similar results and the standard deviation remained very small, indicating its robustness. In the in vitro experiments in Figure 5 we used a sample size of 6 (three biological replicates measured in technical duplicates), since we saw bigger deviations in our measurements. For the in vivo experiments, we always used 5 or more animals in at least two independent experiments.

Reviewer #2 (Recommendations For The Authors):

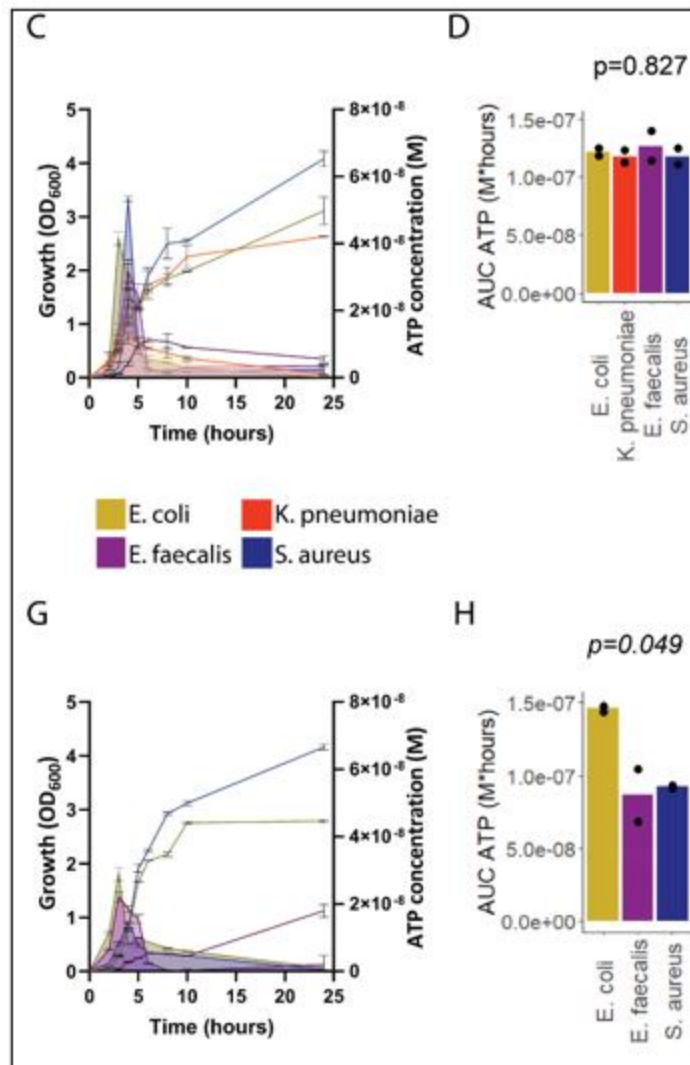
(9). Line 37: 11 million sepsis-related deaths were reported "in" 2017.

The passage has been corrected as suggested.

(10) By the way, the similar colors used in Figure 1C and G are too chaotic, making it difficult to distinguish.

We agree, the colors have been adapted.

Author response image 2.



(11). All "in vivo" and "in vitro" should be italicized.

We italicized all of them.

(12). The title of Figure 4 is confusing: "Impairs sepsis outcome in vivo?" Could you make it more specific?

We agree, the title has been rephrased:

"Bacterial ATP reduces neutrophil counts and reduces survival in a mouse model of abdominal sepsis."

(13) Line 314-316: The sentence "Potentially, despite the lack of a transporter, ATP may similarly to eukaryotic cells leak (Yegutkin et al., 2006) across the inner membrane into the periplasmic space that lacks the enzymes for ATP generation." sounds odd.

This passage was reformulated in the manuscript.

“Despite the lack of a transporter, ATP may leak across the inner membrane into the periplasmic space. Such leakage may be similar to baseline leakage in eukaryotic cells (Yegutkin et al., 2006).”

(14) The numerical notation in the paper is odd: sometimes it uses a prime symbol as a superscript (such as line 504), and sometimes it does not (such as line 421). Should it be standardized to "3,200" and "150,000"?

Thank you for this remark. The numbers have been standardized throughout the manuscript.

(15) Line "0.4 mm EP cuvettes" should be "0.4 cm EP cuvettes"

The specified passage has been corrected as suggested.

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