nature portfolio

Peer Review File

PDCD6 regulates lactate metabolism to modulate LC3-associated phagocytosis and antibacterial defense

Corresponding Author: Dr Tianliang Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Sun et al have conducted an extremely elegant study which implicates programmed cell death 6 (PDCD6) as a negative regulator of LC3-associated phagocytosis in macrophages. Ultimately, they link metabolic modifications including lactylation to the functional capacity of cells to induce LAP and to clear bacteria. Importantly, mice and macrophages which lack PDCD6 are protected from infection with Listeria monocytogenes, Salmonella typhimurium, and Francisella novicida. They identified that PDCD6 interacts with LDHA which downregulates lactate metabolism. Increased lactate results in lactylation of a critical regulator of LAP called rubicon at K33, which inhibits its interaction with Vps34. The study is extremely complete with extensive genetic and pharmacological evidence for their findings. It will likely have a broad impact on a variety of fields including immunology, immunometabolism, and host-pathogen interactions. I have several minor suggestions and comments to improve the manuscript:

Minor suggestions:

The in vivo work is quite convincing, but the methods did not include the mouse strain background and Salmonella typhimurium infection can be strongly affected by the NRAMP1 mutation in C57BL6/J mice. What is the strain of these mice? Can the authors address whether they are assessing the NRAMP1 mutation rather than PDCD6 deletion for this pathogen?

PDCD6 can also affect trafficking - have the authors considered that LAP could also be indirectly affected by changes in trafficking? How did they exclude this possibility?

Figure 1D - the lanes for PDCD6 for each pathogen look extremely similar - can you double check the full blots to see if there was a duplication?

Figure 7C - the rubicon blot following the IP for KLA is very convincing, but the other IPs almost look like blank white boxes and thus may have been too short of exposure to show in the manuscript. Can you show a higher exposure or the full blots to show that you didn't just pick a weak exposure? It is rare to see western blots this clean.

Discussion points:

The authors generalize the results from Lm, St and Fn but these pathogens have extremely different effects on signaling, on the metabolism of the cell and are sensed by different pathways due to different PAMPs (Gram positive, Gram-negative standard LPS and Gram-negative unique LPS) and thus it would be an important discussion point to speculate how all three pathogens are leading to or activating the same pathway of LAP. This could be expanded on in the discussion.

Since the manuscript focuses on PDCD6, I would suggest adding more about the protein and what is known about in the introduction to put the impact of the study in context. There were several sentences about it in the discussion but that was not sufficient to put the finding in context prior to the bulk of the results.

Typos:

Line 102 "Suplemmentary" instead of supplementary

Reviewer #2

(Remarks to the Author)
Manuscript Nr: NCOMMS-23-58207-T
Sun et al., “Title: PDCD6 modulates LC3-associated phagocytosis-mediated antimicrobial defense by regulating lactate metabolism”

The authors demonstrate that programmed cell death 6 (PDCD6) is a negative regulator of LC3-associated phagocytosis (LAP). The authors had identified PDCD6 as the most strongly down-regulated PDCD member upon Listeria monocytogenes infection of bone marrow derived macrophages (BMDM). This decrease was also seen with Salmonella typhimurium and Francisella novicida. Conditional deletion of PDCD6 in myeloid cells inhibited bacterial growth in these cells. This was also confirmed after RNA silencing of PDCD6 in human THP1 cells. PDCD6 deficiency in myeloid cells did not alter proinflammatory cytokine production upon bacterial infection. The autophagy protein LC3B that labels also single membranes of phagosomes during LAP colocalized more often with Listeria containing endosomes in PDCD6 deficient BMDM. Increased protection against bacterial infection in mice with PDCD6 deficiency in myeloid cells depended on Rubicon and Cybb of NOX2. Analysing metabolic changes the authors identified increased lactate levels in the absence of PDCD6. Lactate supplementation increased LAP and anti-bacterial activity. Accordingly, PDCD6 suppressed lactate dehydrogenase (LDH) activity. LDHA was also found as a direct interaction partner of PDCD6 by immunoprecipitation. Furthermore, LDHA deficiency increased bacterial growth in THP1 cells. LDH inhibitor treatment increased bacterial loads also in vivo. Next, lactylation of Rubicon was found, which is increased upon PDCD6 deficiency and after Listeria infection. Rubicon, lactylated at K33, showed increased incorporation in the VPS34 complex by immunoprecipitation and modelling suggested better interaction with VPS34. K33R mutant Rubicon is not able to support increased bactericidal activity in the absence of PDCD6. From these data the authors conclude that PDCD6 down-regulation or lactate supplementation might increase anti-bacterial activity via LAP.

This is an interesting study. However, the presented experiments do not show that LAP rather than efficient NOX2 assembly at phagosomes is anti-bacterial in the absence of PDCD6. Furthermore, it remains unclear how PDCD6 inhibits LDH activity, and why more efficient lactylated Rubicon interaction with VPS34 does not inhibit autophagosome maturation.

Major comments:
1. The authors demonstrate that the anti-bacterial activity of PDCD6 deficiency depends on Cybb. Increased NOX2 activity has been previously reported to attenuate phagosome acidification. Therefore, is the increased anti-bacterial function of LAP in PDCD6 deficient BMDM more dependent on ROS production by NOX2 or low pH due to lysosomal fusion? Anti-oxidant treatment could be compared with lysomotropic agents that elevate the pH to investigate this.
2. The authors exclude amphisome formation as a reason for Listeria co-localization with LC3 due to no influence of PDCD6 on mTOR and AMPK activation. However, they should also investigate if autophagy receptors localize with Listeria and if this changes upon PDCD6 deficiency. p62 is often used for this purpose.
3. In their metabolic studies the authors observed an influence of PDCD6 deficiency on lactate levels through regulation of LDHA by PDCD6. How does PDCD6 binding inhibit LDHA? Is LDHA differently localized by binding to PDCD6?
4. Rubicon and Cybb deficiency do not automatically demonstrate that LAP is required for more efficient anti-bacterial reactivity upon PDCD6 deficiency. These just indicate that NOX2 assembly at phagosomes is important. In order to demonstrate that LAP is required conditional PDCD6 deficiency should be paired with conditional deficiency of a core ATG protein involved in LC3B conjugation, e.g. ATG5 or ATG7.
5. It seems curious that no differences were found in classical macroautophagy upon PDCD6 deficiency. Rubicon was originally described as negative regulator of the UVRAG/VPS34 complex for autophagosome fusion with lysosomes. Would one not expect decreased autophagosome maturation in the absence of PDCD6, allowing lactylated Rubicon to be more efficiently incorporated into this complex and blocking fusion with lysosomes? Classical autophagosome turn-over, maybe again utilizing a macroautophagy receptor, such as p62, should be reported.

Minor comments:
1. The authors do not report the number of biological replicates for their Immunoblots nor quantify their Western blots, e,g. figures 2V and 3E. This should be done in comparison with the loading control, e.g. GAPDH.

Reviewer #3

(Remarks to the Author)
The authors demonstrate that PDCD6 acts as a negative regulator of LC3-associated phagocytosis (LAP) by inhibiting LDHA activity and subsequent lactylation of RUBCN at K33. Thus, loss of PDCD6 in macrophages and mice enhanced LAP formation and bactericidal activity, and increased host defense against intracellular bacterial infection. Overall, this is an interesting study on the role of PDCD6 on the LAP formation in macrophage, but the mechanism underlying PDCD6 inhibits LDHA activity needs further investigation.

Major comments:
1. How does the interaction of PDCD6 with LDHA inhibits the activity of LDHA without affecting the stability? Previous studies indicated that PDCD6 regulates the COPII vesicle transport and membrane repair process. Does PDCD6 inhibit LDHA by hijacking LDHA to a wrong compartment? Or Does PDCD6 block the kinase that catalyzes the phosphorylation of

LDHA to interact with LDHA? The authors may need to examine the localization of LDHA and PDCD6 in both WT and PDCD6-deficient macrophages.
2. The authors demonstrated that loss of PDCD6 in macrophages increased phagosome-lysosome fusion using immunostaining. It is necessary to confirm this using alternative techniques, such as FRET. Did the authors examine whether the degradation of phagocytosed bacteria in PDCD6-deficient macrophages is enhanced?
3. The authors demonstrated that loss of PDCD6 enhanced lactylation of RUBCN at K33 and RUBCNK33R failed to rescue the function of RUBCN in LAP formation. However, K33R mutation may block many types of posttranslational modifications in addition to lactylation of RUBCN. Can the authors find a K33 mutation of RUBCN that mimics lactylation and expression of this mutant can enhance LAP formation?
4. Does LDHAY10F or RUBCNK33R in macrophages inhibit the recruitment of PI3K complex and Nox2 to phagosomes? Does LDHAY10F or RUBCNK33R in macrophages restore the increased host defense of Pdcd6 conditional KO mice against intracellular bacterial infection?
5. The authors should carefully determine the capability of PDCD6-deficient macrophages to uptake intracellular bacteria.
Minor comments:
1. The authors measured ROS in macrophages stimulated with L. monocytogenes by exposing to DCFH-DA. The authors may need to measure the phagosomal ROS by challenge macrophages with DCFH-labelled L. monocytogenes.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Following careful review of the manuscript, the authors have addressed my comments and I think that the manuscript will be an exciting contribution to the field of autophagy, immunometabolism, and host-pathogen interactions.

Reviewer #2

(Remarks to the Author)
Manuscript Nr: NCOMMS-23-58207A
Sun et al., “PDCD6 modulates LC3-associated phagocytosis-mediated antimicrobial defense by regulating lactate metabolism”

The authors demonstrate that programmed cell death 6 (PDCD6) is a negative regulator of LC3-associated phagocytosis (LAP). The authors had identified PDCD6 as the most strongly down-regulated PDCD member upon Listeria monocytogenes infection of bone marrow derived macrophages (BMDM). This decrease was also seen with Salmonella typhimurium and Francisella novicida. Conditional deletion of PDCD6 in myeloid cells inhibited bacterial growth in these cells. This was also confirmed after RNA silencing of PDCD6 in human THP1 cells. PDCD6 deficiency in myeloid cells did not alter proinflammatory cytokine production upon bacterial infection. The autophagy protein LC3B that labels also single membranes of phagosomes during LAP colocalized more often with Listeria containing endosomes in PDCD6 deficient BMDM. Increased protection against bacterial infection in mice with PDCD6 deficiency in myeloid cells depended on Rubicon and Cybb of NOX2. Analysing metabolic changes the authors identified increased lactate levels in the absence of PDCD6. Lactate supplementation increased LAP and anti-bacterial activity. Accordingly, PDCD6 suppressed lactate dehydrogenase (LDH) activity. LDHA was also found as a direct interaction partner of PDCD6 by immunoprecipitation. Furthermore, LDHA deficiency increased bacterial growth in THP1 cells. LDH inhibitor treatment increased bacterial loads also in vivo. Next, lactylation of Rubicon was found, which is increased upon PDCD6 deficiency and after Listeria infection. Rubicon, lactylated at K33, showed increased incorporation in the VPS34 complex by immunoprecipitation and modelling suggested better interaction with VPS34. K33R mutant Rubicon is not able to support increased bactericidal activity in the absence of PDCD6. From these data the authors conclude that PDCD6 down-regulation or lactate supplementation might increase anti-bacterial activity via LAP.

In their revised manuscript version, the authors have addressed all of my concerns. Namely, they provide evidence that both ROS and acidification contribute to anti-bacterial function upon PDCD6 deficiency, they find no p62 co-localization, excluding canonical autophagy after endosomal damage as the anti-bacterial mechanism, they provide additional insights into how PDCD6 regulates lactate metabolism, they provide a deficiency as additional evidence that ATG8 lipidation (with LC3B as one mammalian orthologue) is required for the anti-bacterial function, and observe some evidence for decreased autophagosome maturation upon increased lactylated Rubicon incorporation into Vps34 complexes. Moreover, they have now quantified their Western blots and report the number of replicates. Therefore, I find the manuscript significantly improved.

Reviewer #3

(Remarks to the Author)
The authors have included new data in the revised manuscript that address almost all the comments that were put forth in

the initial review. I have only a minor comment here. The authors proposed that PDCD6 inhibits LDHA activity by disrupting the FGFR1-LDHA interaction, leading to reduced FGFR1-mediated LDHA Y10 phosphorylation and LDHA activity. However, the competitive binding was examined in 293T overexpressing these three genes. How is the endogenous expression of FGFR1 and the interaction between LDHA and FGFR1 in PDCD6 KO BMDMs?

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments. Therefore, the manuscript is further improved.

PDCD6 modulates LC3-associated phagocytosis-mediated antimicrobial defense by regulating lactate metabolism

Lulu Sun, Sijin Wu, Hui Wang, Tianyu Zhang, Mengyu Zhang, Xuepeng Bai, Xiumei Zhang, Bingqing Li, Cai Zhang, Yan Li, Jun Zhou and Tianliang Li

Response to Reviewers' comments:

We thank the reviewers for their insightful comments. In response, we have incorporated new data and made extensive changes to the main text, figures, figure legends and supplemental information. These combined changes have further strengthened our conclusions and improved the clarity of this manuscript making it more accessible to a broader readership. Below please find the comments from the reviewers and our responses.

Reviewer #1 (Remarks to the Author):

1. The in vivo work is quite convincing, but the methods did not include the mouse strain background and Salmonella typhimurium *infection can be strongly affected by the NRAMP1 mutation in C57BL6/J mice. What is the strain of these mice? Can the authors address whether they are assessing the NRAMP1 mutation rather than PDCD6 deletion for this pathogen?*

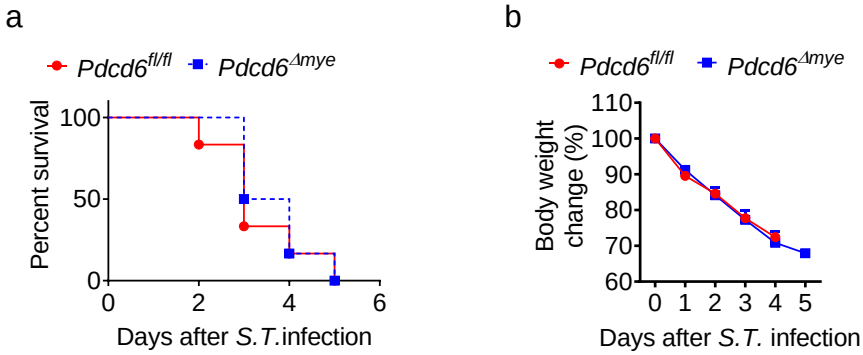
Reply: We appreciate the insightful feedback. We apologize for the omission of the background information relating to the mice used in our study. The mice belonged to the C57BL/6J strain and this detail has now been included in the Materials and Methods section. Furthermore, we have addressed this aspect in the Discussion section.

As noted by the reviewer, NRAMP1 (SlC11A1/Ity/Lsh/Bcg), a critical divalent cation transporter expressed in cells derived from the monocyte/macrophage lineage, plays a vital role in the innate immune response of mice to *Salmonella* (PMID: 12527228; PMID: 11909746; PMID: 8490962; PMID: 2182715; PMID: 8871656). We recognize the significance of the *Nramp1* mutation in C57BL/6 murine strains, with homozygous loss-of-function mutations in this gene impacting the susceptibility of the animals to *Salmonella* infection (PMID: 7650477; PMID: 27955815; PMID: 25350459; PMID: 8757814). However, in mice infected with *Salmonella*, the effects of *Nramp1* mutation significantly vary based on factors such as bacterial strain, the number of bacteria used for infection, and the method of infection. For instance, administering Strain C5 at 5×10^4 CFU per mouse via tail vein injection resulted in a survival period of 4–5 days (PMID: 7650477). Infecting *Nramp1*$^{-/-}$ mice with 1×10^8 CFU of *S.* Typhimurium (SL1344) via oral gavage led to clinical signs of salmonellosis, requiring euthanasia by day 10 (PMID: 27955815). C57BL/6 mice infected with 1×10^8 CFU of *S.* Typhimurium (strain SL1344) via oral gavage experienced mortality starting on day 10, with all mice succumbing by day 20 (PMID: 25350459). C57BL/6 mice carrying the *Nramp1*^{D169} mutation infected intravenously with 0.9×10^4 CFU of the highly virulent *S.* Typhimurium Keller strain quickly succumbed to this infectious inoculum, with no animal surviving more than 5 days (PMID: 8757814). These notable differences

in outcomes suggest that genetic variation, along with variation in the virulence of *S.* Typhimurium as well as laboratory and experimental conditions, collectively impact the spectrum of immune responses.

In this study, mice were infected with *S.* Typhimurium (ATCC 14028) by intraperitoneal injection, following which the antibacterial role of PDCD6 was evaluated. Our findings revealed distinct outcomes that were dependent on the infection dosage. Administering a higher dose of *S.* Typhimurium (ATCC 14028; 5×10^5 CFU per mouse) resulted in increased susceptibility in both *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ mice, leading to mortality within 5 days (**a**) along with significant weight loss (**b**). These findings are in line with the established *Nramp1* mutation phenotype (PMID: 7650477; PMID: 27955815). Meanwhile, using a lower dose of *S.* Typhimurium (1×10^5 CFU per mouse) resulted in a more moderate mortality rate in *Pdcd6*$^{fl/fl}$ mice, with most surviving up to 8 days post-infection. In contrast, *Pdcd6*$^{\Delta mye}$ mice displayed prolonged survival and a stronger antibacterial response compared with their *Pdcd6*$^{fl/fl}$ counterparts (Fig. 2f-i). These results were corroborated by our cellular experiments (Fig. 1f, Extended Data Fig. 1f and 1j) and previous studies involving *L.monocytogenes* and *F. novicida* (Fig. 2a-d, k-n), indicative of a clear association between PDCD6 deficiency and enhanced resilience to *S.* Typhimurium infection.

Our investigation on C57BL/6J mice using intraperitoneal injections of *S.* Typhimurium (ATCC 14028) highlights the notable effectiveness of PDCD6 deficiency in combating moderate bacterial infections while underscoring the increasing significance of NRAMP1 in scenarios involving infection with higher concentrations of *S.* Typhimurium.



2. PDCD6 can also affect trafficking - have the authors considered that LAP could also be indirectly affected by changes in trafficking? How did they exclude this possibility?

Reply: We acknowledge and appreciate the insightful comments from the Reviewer. We evaluated the potential indirect impact of trafficking changes on LAP and have excluded the possibility of trafficking changes influencing this process based on the following observations:

It has been reported that PDCD6 participates in vesicular trafficking through its interaction with SEC31, which facilitates ER-to-Golgi transport by coat complex type II (COPII) vesicles (PMID: 25006245), and TRPML1, which promotes retrograde endosome/lysosome transport (PMID: 26950892). However, COPII vesicles mediate anterograde ER-Golgi trafficking, during which they bud from specialized domains known as ER exit sites (ERES), and transport the embedded cargos to the Golgi

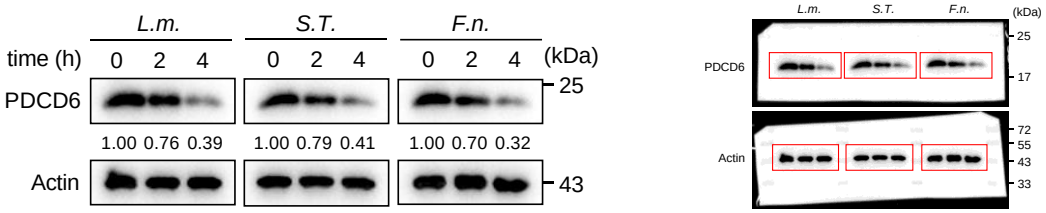
(PMID: 21172817). While retrograde transport delivers cargo from endosomes to the trans-Golgi network (TGN), LAP delivers phagocytosed cargo to the lysosome. Thus, they are spatially distinct biological processes.

The basic COPII vesicle is composed of five cytosolic proteins—SAR1, SEC23, SEC24, SEC13, and SEC31, with SEC23/SEC24 and SEC13/SEC31 forming the inner and outer COPII coat, respectively (PMID: 21172817). Retromer, responsible for sorting cargo from endosomes to the Golgi, consists of a stable VPS35/VPS29/VPS26 heterotrimer. VPS35 forms an extended α-helical solenoid that associates with VPS26A or VPS26B and VPS29 at its amino-terminal and carboxy-terminal ends, respectively. At steady state, retromer is enriched on the cytosolic face of the early and late endosomal membrane. Its association with the late endosome is mediated through binding to RAS-related protein RAB7-GTP, whereas its association with the early endosome is governed through interaction with sorting nexin 3 (SNX3), which binds PtdIns(3)P (PMID: 30194414). None of the proteins mentioned above is involved in LAP.

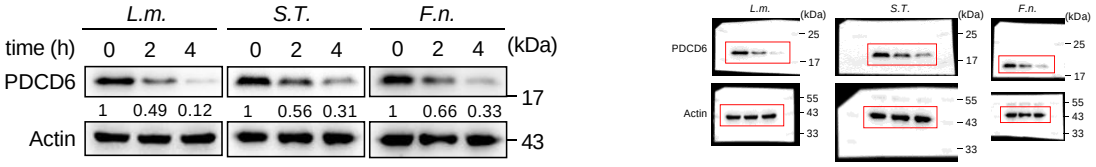
In addition, there is no documented evidence regarding the cargo delivered through COPII vesicles or retrograde transport being associated with LAP. Collectively, these observations imply that there is no correlation between LAP and PDCD6-associated trafficking processes.

3. Figure 1D - the lanes for PDCD6 for each pathogen look extremely similar - can you double check the full blots to see if there was a duplication?

Reply: We apologize for the confusion caused by running multiple samples on a single SDS-PAGE gel and not providing raw blot data at the time of the initial submission. We have thoroughly reviewed both the full blots (left) and raw blots (right) and can confirm that there was no duplication.



In addition, to prevent similar confusion, we have replaced the blots in new Figure 1d (left), with the related raw blots shown on the right.



4. Figure 7C - the rubicon blot following the IP for KLA is very convincing, but the other IPs almost look like blank white boxes and thus may have been too short of exposure to show in the manuscript. Can you show a higher exposure or the full blots

to show that you didn't just pick a weak exposure? It is rare to see western blots this clean.

Reply: We thank the Reviewer for the excellent suggestion. We have selected higher-exposure blots detected with the provided antibodies and replaced the blots in new Figure 7c.

Discussion points:

5. The authors generalize the results from Lm, St and Fn but these pathogens have extremely different effects on signaling, on the metabolism of the cell and are sensed by different pathways due to different PAMPs (Gram positive, Gram-negative standard LPS and Gram-negative unique LPS) and thus it would be an important discussion point to speculate how all three pathogens are leading to or activating the same pathway of LAP. This could be expanded on in the discussion.

Reply: We appreciate the Reviewer's insightful suggestion. The relevant discussion has been included in the revised manuscript.

6. Since the manuscript focuses on PDCD6, I would suggest adding more about the protein and what is known about in the introduction to put the impact of the study in context. There were several sentences about it in the discussion but that was not sufficient to put the finding in context prior to the bulk of the results.

Reply: We thank the Reviewer for this feedback and have incorporated additional information about PDCD6 into the Introduction section.

Typos:

Line 102 "Suplemmentary" instead of supplementary

Reply: Thank you for pointing out the spelling error. It has been corrected to "Supplementary."

Reviewer #2 (Remarks to the Author):

1. The authors demonstrate that the anti-bacterial activity of PDCD6 deficiency depends on Cybb. Increased NOX2 activity has been previously reported to attenuate phagosome acidification. Therefore, is the increased anti-bacterial function of LAP in PDCD6 deficient BMDM more dependent on ROS production by NOX2 or low pH due to lysosomal fusion? Anti-oxidant treatment could be compared with lysomotropic agents that elevate the pH to investigate this.

Reply: We appreciate this insightful comment. Given that NOX2 has been reported to diminish the acidification of phagosomes, we conducted experiments to determine whether the augmented antibacterial efficacy of LAP following PDCD6 deletion relies on NOX2 generation or the acidic environment resulting from lysosome fusion. Following the reviewer's suggestions, we pre-treated *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs with the ROS scavenger N-acetylcysteine (NAC) (PMID: 35760871) (5 mM) and the lysosome inhibitor ammonium chloride (NH_4Cl; prevents lysosomal protease activity by raising vesicular pH) (PMID: 30737478; PMID: 32662244) for 4 hours, followed by challenge with *L. monocytogenes*. The results of gentamicin protection assays showed that following treatment with NAC or NH_4Cl, significant increases in intracellular bacterial growth were observed in both *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs. The bacterial count in *Pdcd6*$^{\Delta mye}$ BMDMs was comparable to that in *Pdcd6*$^{fl/fl}$ BMDMs across the various treatments (Extended Data Fig. 4f). Considering the crucial involvement of NOX2-generated ROS in directing LC3 to phagosomes (PMID: 19339495; PMID: 23584039; PMID: 29544096; PMID: 33686057; PMID: 35511089) and elevating phagosomal pH to activate the V-ATPase/ATG16L1 axis for LC3 conjugation to phagosomal membranes (PMID: 35511089; PMID: 36288298), NAC treatment is likely to impede LAP, thus hindering the clearance of *L. monocytogenes* via this mechanism. This indicates that the inhibitory effect of NAC treatment parallels the outcomes observed when lysosomes are obstructed with multiple lysosome inhibitors. These results provide additional evidence supporting that enhanced LAP mediates the improved bactericidal effect resulting from the absence of PDCD6.

2. The authors exclude amphisome formation as a reason for Listeria co-localization with LC3 due to no influence of PDCD6 on mTOR and AMPK activation. However, they should also investigate if autophagy receptors localize with Listeria and if this changes upon PDCD6 deficiency. p62 is often used for this purpose.

Reply: This is an excellent suggestion by the Reviewer. To explore the impact of PDCD6 deletion on the co-localization of *L. monocytogenes* with autophagy receptors, we employed immunofluorescence to examine the co-localization of *L. monocytogenes* and p62. We found that the lack of PDCD6 does not impact p62 co-localization with *L. monocytogenes*, indicating that there is no discernible difference

in the autophagic clearance of *L. monocytogenes* between wild-type and PDCD6-deficient cells (Extended Data Fig. 4d, e).

3. In their metabolic studies the authors observed an influence of PDCD6 deficiency on lactate levels through regulation of LDHA by PDCD6. How does PDCD6 binding inhibit LDHA? Is LDHA differently localized by binding to PDCD6?

Reply: We greatly appreciate these insightful questions. In our study, we observed that PDCD6 deficiency led to an elevation in LDHA phosphorylation at Y10 (Fig. 6a, b), suggestive of heightened LDHA activity (PMID: 21969607; PMID: 28218905). Given the interaction between PDCD6 and LDHA (Fig. 6e–g, Extended Data Fig. 6e–g), we hypothesized that PDCD6 blocks the interaction between LDHA and the kinase that catalyzes its phosphorylation. FGFR1 interacts with LDHA and functions as a kinase for the Y10 site of LDHA (PMID: 21969607; PMID: 28218905). To investigate the influence of PDCD6 on FGFR1-LDHA interaction, we transfected 293T cells with varying concentrations of PDCD6, FGFR1, and LDHA expression plasmids, and then performed immunoprecipitation assays. The results indicated that an increase in PDCD6 dosage leads to a significant weakening of the interaction between FGFR1 and LDHA, resulting in a marked decrease in LDHA phosphorylation in both the IP and Input samples. This suggested that PDCD6 hinders the binding of LDHA to FGFR1, possibly through competitive binding with LDHA (Extended Data Fig. 6h). Additionally, no interaction between PDCD6 and FGFR1 was observed (Extended Data Fig. 6i).

LDHA is primarily located in the cytoplasm of cells, where it catalyzes the conversion of lactate to pyruvate during glycolysis. In addition to the cytoplasm, LDHA has also been found in mitochondria and nuclei (PMID: 9927705; PMID: 30356100). To investigate the impact of PDCD6 on the cellular localization of LDHA, we analyzed LDHA localization in wild-type and PDCD6-deficient BMDMs challenged with *L. monocytogenes* or left untreated. Mitochondria were labeled with Mitotracker Red and nuclei with DAPI. As shown in new Extended Data Fig. 6j, k, LDHA was found to be predominantly localized in the cytoplasm. There was no significant difference in the cellular localization of LDHA between wild-type and PDCD6-deficient cells, irrespective of *L. monocytogenes* challenge, indicating that PDCD6 does not influence the cellular location of LDHA.

4. Rubicon and Cybb deficiency do not automatically demonstrate that LAP is required for more efficient anti-bacterial reactivity upon PDCD6 deficiency. These just indicate that NOX2 assembly at phagosomes is important. In order to demonstrate that LAP is required conditional PDCD6 deficiency should be paired with conditional deficiency of a core ATG protein involved in LC3B conjugation, e.g. ATG5 or ATG7.

Reply: We thank the Reviewer for the comment. We completely agree that genetic deletion of Rubicon or NOX2 from *PDCD6*-KO cells may not be sufficient to

conclude that LAP is responsible for the increased bacterial killing response in *PDCD6*-KO cells. Accordingly, we genetically deleted *ATG7* (encoding another molecule required for LAP formation) from *PDCD6*-KD THP-1 cells, as suggested by the Reviewer. The deletion of *ATG7* completely disrupted *L. monocytogenes*-induced LAPosome formation and abolished the augmented bacterial killing capacity of *PDCD6*-KD cells (Fig. 4l, m). These results provide additional evidence to support that enhanced LAP is responsible for the improved bactericidal effect observed in the absence of PDCD6.

5. It seems curious that no differences were found in classical macroautophagy upon PDCD6 deficiency. Rubicon was originally described as negative regulator of the UVRAG/VPS34 complex for autophagosome fusion with lysosomes. Would one not expect decreased autophagosome maturation in the absence of PDCD6, allowing lactylated Rubicon to be more efficiently incorporated into this complex and blocking fusion with lysosomes? Classical autophagosome turn-over, maybe again utilizing a macroautophagy receptor, such as p62, should be reported.

Reply: We appreciate the Reviewer's insightful suggestion. In response, we investigated the impact of PDCD6 deficiency on autophagosome maturation, assessing p62 degradation through immunoblotting assays. Following the induction of autophagy in $Pdcd6^{fl/fl}$ and $Pdcd6^{\Delta mye}$ BMDMs with EBSS for varying durations, we observed the levels of p62 protein were notably higher in $Pdcd6^{\Delta mye}$ BMDMs than in $Pdcd6^{fl/fl}$ BMDMs (new Extended Data Fig. 7h). Furthermore, no obvious difference in the activation of both AMPK and the AKT/mTOR axis in $Pdcd6^{fl/fl}$ and $Pdcd6^{\Delta mye}$ BMDMs upon *L. monocytogenes* challenge (new Extended Data Fig. 7i), both of which are critical markers of autophagy induction (PMID: 21258367). To further explore whether the increased p62 levels in PDCD6-deficient BMDMs were due to enhanced autophagy induction or blocked autophagy maturation, we utilized LY294002, a well-known PI3K inhibitor, to suppress the formation of autophagosomes and chloroquine (CQ) to impair autophagosome fusion with lysosomes. No discernible differences in p62 protein levels were observed between $Pdcd6^{fl/fl}$ and $Pdcd6^{\Delta mye}$ BMDMs with LY294002 treatment (new Extended Data Fig. 7j), consistent with the results showing no differences in AMPK and mTOR pathway activation (new Extended Data Fig. 7i). However, the levels of p62 protein were markedly higher in $Pdcd6^{\Delta mye}$ BMDMs than in $Pdcd6^{fl/fl}$ BMDMs with chloroquine treatment (new Extended Data Fig. 7k). These results indicated that PDCD6 deficiency leads to a decrease in autophagosome maturation rather than autophagy induction.

Minor comments:
1. The authors do not report the number of biological replicates for their Immunoblots nor quantify their Western blots, e,g. figures 2V and 3E. This should be done in comparison with the loading control, e.g. GAPDH.

Reply: We apologize for this oversight. The statement “In all the panels, data are from one experiment representative of three independent experiments with similar results” has been included in the corresponding figure legends. Densitometric analyses were performed on immunoblots of three repeats using independent biological samples (new Fig. 2s and 3e).

Reviewer #3 (Remarks to the Author):

1. How does the interaction of PDCD6 with LDHA inhibits the activity of LDHA without affecting the stability? Previous studies indicated that PDCD6 regulates the COPII vesicle transport and membrane repair process. Does PDCD6 inhibit LDHA by hijacking LDHA to a wrong compartment? Or Does PDCD6 block the kinase that catalyzes the phosphorylation of LDHA to interact with LDHA? The authors may need to examine the localization of LDHA and PDCD6 in both WT and PDCD6-deficient macrophages.

Reply: We appreciate these insightful questions. LDHA is primarily localized to the cytoplasm, where it catalyzes the conversion of lactate to pyruvate during glycolysis. While predominantly cytoplasmic, LDHA has also been observed in mitochondria and the nuclei (PMID: 9927705; PMID: 30356100). To assess the impact of PDCD6 on the cellular localization of LDHA, we analyzed LDHA localization in wild-type and PDCD6-deficient BMDMs challenged or not with *L. monocytogenes*. Mitochondria were labeled with Mitotracker Red and nuclei with DAPI. The findings demonstrated that LDHA is mainly cytoplasmic following *L. monocytogenes* challenge or left untreated. Notably, there were no significant differences in LDHA cellular localization between wild-type and PDCD6-deficient cells (new Extended Data Fig. 6j, k), indicating that PDCD6 does not influence the subcellular distribution of LDHA.

FGFR1 interacts with LDHA and serves as a kinase for the Y10 site of LDHA (PMID: 28218905). To explore the effect of PDCD6 on FGFR1-LDHA interaction, we transfected 293T cells with varying concentrations of PDCD6, FGFR1, and LDHA expression plasmids, and then conducted immunoprecipitation assays. The results revealed that an increase in the PDCD6 dosage significantly weakened FGFR1-LDHA interaction, resulting in decreased LDHA phosphorylation in both the IP and Input samples. This indicated that PDCD6 impedes the binding of LDHA to FGFR1, potentially through competitive binding with LDHA (new Extended Data Fig. 6h). Moreover, no interaction was observed between PDCD6 and FGFR1 (new Extended Data Fig. 6i). These results suggested that PDCD6 inhibits LDHA activity by disrupting FGFR1-LDHA interaction, leading to reduced FGFR1-mediated LDHA activity.

LDHA degradation involves both the proteasome and lysosome and is associated with various post-translational modifications. LDHA interacts with the E3 ligase FBW7, resulting in LDHA ubiquitination and subsequent proteasomal degradation (PMID: 35359405). Additionally, carnitine palmitoyltransferase 1A (CPT1A), acting as a lysine succinyltransferase, succinylates LDHA at K222. While this modification does not affect LDHA ubiquitination, it reduces the binding of ubiquitinated LDHA to SQSTM1, leading to decreased lysosomal degradation (PMID: 32859246). The acetylation of LDHA at lysine 5 (K5) marks it for recognition by the HSC70 chaperone, thereby facilitating LDHA delivery to lysosomes for degradation (PMID: 23523103).

Considering the association of LDHA degradation with ubiquitination, succinylation, and acetylation, our investigation focused on these modifications in LDHA under conditions of increased PDCD6 expression. The results indicated that elevated expression of PDCD6 enhances its interaction with LDHA without affecting ubiquitination, succinylation (Ksn), or acetylation (Ac-K) levels (new Extended Data Fig. 6n). This suggested that the interplay between PDCD6 and LDHA does not influence ubiquitination, CPT1A-mediated succinylation, or acetylation-related LDHA degradation. In conclusion, these observations demonstrate that PDCD6 inhibits LDHA activity by disrupting FGFR1-LDHA interaction, leading to reduced FGFR-mediated LDHA activity without a concomitant effect on LDHA stability.

We acknowledge that the molecular mechanisms involved are intricate and further detailed investigations are warranted. Regrettably, due to constraints on revision time, we have completed this phase and intend to delve deeper into this aspect in future studies.

2. The authors demonstrated that loss of PDCD6 in macrophages increased phagosome-lysosome fusion using immunostaining. It is necessary to confirm this using alternative techniques, such as FRET. Did the authors examine whether the degradation of phagocytosed bacteria in PDCD6-deficient macrophages is enhanced?

Reply: We appreciate the Reviewer's excellent suggestion for using alternative techniques. We followed the Reviewer's suggestion and employed FRET to further investigate the impact of PDCD6 on phagosome-lysosome fusion. Wild-type and PDCD6-deficient BMDMs were prelabeled with 100 μg/ml Alexa Fluor-594 hydrazide, a red-fluorescent dye commonly used for lysosome labeling and visualization, followed by treatment with Alexa Fluor-488 Zymosan. Subsequently, the dynamics underlying the fusion between phagosomes and lysosomes were assessed using FRET. Our findings revealed that the absence of PDCD6 led to an increase in phagosome/lysosome fusion compared with that seen in wild-type BMDMs, indicating that PDCD6 deficiency enhances the fusion of phagosomes with lysosomes (new Extended Data Fig. 3c).

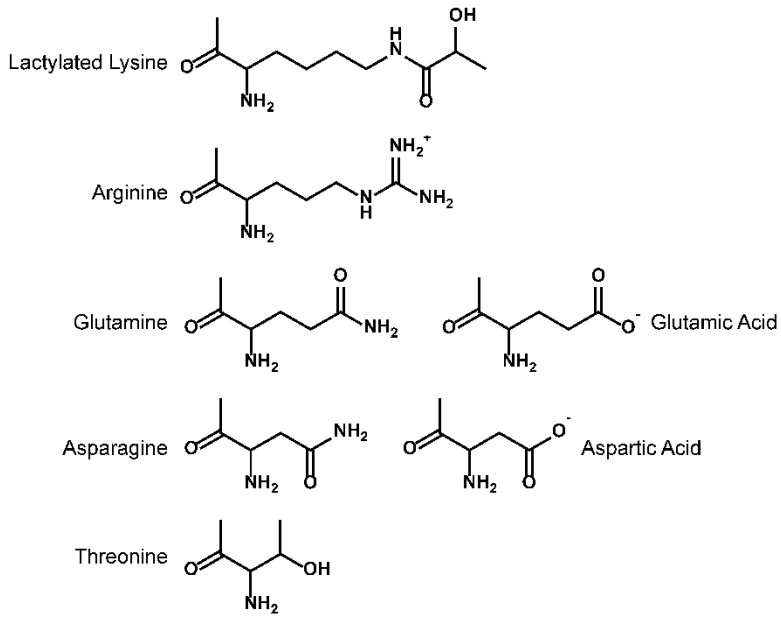
Regarding the Reviewer's question, we initially did not specifically address the impact of PDCD6 deficiency on the degradation of phagocytosed bacteria in macrophages. While data from the gentamycin protection assays (Fig. 1e–g, Extended Data Fig. 1e–g), fluorescence imaging analysis (new Fig. 1l), and transmission electron microscopy (new Fig. 1n) indicated that intracellular bacterial growth in PDCD6-deficient macrophages was significantly suppressed compared with that in wild-type macrophages, these results did not conclusively establish a direct involvement of PDCD6 in the degradation of phagocytosed bacteria within macrophages. To determine whether attenuated intracellular bacterial growth in *Pdcd6*$^{\Delta mye}$ BMDMs was due to improved bacterial killing, we analyzed the intracellular growth of *L. monocytogenes* Δhly strains (a deletion in the *hly* gene, encoding listeriolysin O (LLO), a critical factor implicated in damaging cell

membranes), which is confined within the phagosome. As shown in new Extended Data Fig. 1h, *Pdcd6*$^{\Delta mye}$ BMDMs exhibited a notable reduction in live bacteria compared to *Pdcd6*$^{fl/fl}$ BMDMs, suggesting that phagosomal bacterial killing was enhanced. In conclusion, these results indicate that the degradation of phagocytosed bacteria is enhanced in PDCD6-deficient macrophages.

3. The authors demonstrated that loss of PDCD6 enhanced lactylation of RUBCN at K33 and RUBCN K33R failed to rescue the function of RUBCN in LAP formation. However, K33R mutation may block many types of posttranslational modifications in addition to lactylation of RUBCN. Can the authors find a K33 mutation of RUBCN that mimics lactylation and expression of this mutant can enhance LAP formation?

Reply: We thank the Reviewer for this excellent suggestion. Following an exhaustive review of the literature and databases on post-translational modifications (PTMs), we determined that lysine is currently the only amino acid known to undergo lactylation. This relatively novel PTM was first identified in histones in 2019 (Nature. 2019;574(7779):575-580. PMID: 31645732).

We apologize but, upon examining the natural amino acids, we find it difficult to identify alternative residues that can serve as mimics for lysine (K) lactylation owing to the physical properties of the post-translationally modified residue and its structural characteristics (Extended data Fig.7g). Arginine (R) may be the most plausible candidate given its similar molecular size; however, our previous studies suggested that arginine cannot restore the function of lysine and also possesses distinct charge properties. Furthermore, the physical and chemical properties of the guanidinium group in the sidechain of arginine largely differ from the amine group in lysine, rendering it unable to restore the full function of lysine. Glutamic acid (E) and aspartic acid (D) may also be proposed as potential candidates; however, their charges in proteins are usually negative, which differs from the neutral nature of lactylated lysine. Residues with neutral polar side chains, such as glutamine (Q), asparagine (N), and threonine (T), are more similar to lactylated lysine. Nonetheless, owing to their small molecular size, their ability to mimic the properties of lactylated lysine is limited.



4. Does LDHAY10F or RUBCNK33R in macrophages inhibit the recruitment of PI3K complex and Nox2 to phagosomes? Does LDHAY10F or RUBCNK33R in macrophages restore the increased host defense of Pdcd6 conditional KO mice against intracellular bacterial infection?

Reply: We appreciate these insightful questions. To investigate the potential inhibitory effect of LDHA Y10A in macrophages on the recruitment of the PI3K complex and NOX2 to phagosomes, a comprehensive approach was employed. Initially, CRISPR/CAS9 was utilized to knockout LDHA in wild-type THP-1 cells, followed by reconstitution with either wild-type LDHA or LDHA Y10A. Subsequent immunoblotting of purified phagosomes revealed that reconstitution with wild-type LDHA, but not LDHA Y10F, in LDHA-knockdown cells restored the levels of VPS34 and NOX2 to those observed in the Ctrl sample (new Extended Data Fig. 6q). Furthermore, gentamicin protection assays confirmed that reconstitution with LDHA Y10A in LDHA-knockdown cells was ineffective in restoring the antibacterial effects when compared with that in LDHA knockdown cells reconstituted with wild-type LDHA (new Extended Data Fig. 6r).

Additionally, to determine the impact of RUBCN K33R in macrophages on the recruitment of the PI3K complex and NOX2 to phagosomes, wild-type and *RUBCN* KO THP-1 cells were employed, followed by reconstitution with wild-type RUBCN or RUBCN K33R in *RUBCN* KO THP-1 cells. Immunoblotting of purified phagosomes indicated that reconstitution with wild-type RUBCN, but not RUBCN K33R, in *RUBCN* KO THP-1 cells restored the levels of VPS34 and NOX2 to those observed in wild-type THP-1 cells (new Extended Data Fig. 7d). Subsequent gentamicin protection assays confirmed that reconstitution with RUBCN K33R in *RUBCN* KO THP-1 cells failed to restore the antibacterial effects to levels

comparable to those observed in *RUBCN* KO THP-1 cells reconstituted with wild-type RUBCN (new Extended Data Fig. 7e).

5. The authors should carefully determine the capability of PDCD6-deficient macrophages to uptake intracellular bacteria.

Reply: We thank the reviewer for the suggestion. Initially, we used the pHrodo Green *E. coli* BioParticles Conjugate as a surrogate model for bacteria, a commonly employed method for detecting phagocytosis. However, it has been noted that this model does not accurately replicate live bacterial infections.

To further investigate the impact of PDCD6 on bacterial phagocytosis, we conducted a phagocytosis assay following the protocol provided by Dr. Jae U. Jung's lab (PMID: 22423966). Briefly, *L. monocytogenes* was fluorescently labeled with N, N-dimethylaniline N-oxide (DMAO). *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs were then infected with DMAO-labeled *L. monocytogenes* or GFP-labeled *S.* Typhimurium (1×10^8 CFU) in a 12-well plate for different durations (20, 40, 60, 90 and 120 minutes) at 37 °C. Partially attached but non-ingested bacteria were eliminated by treatment with 50 μg/ml gentamycin for 1 hour, using trypan blue as a quenching agent to eliminate fluorescence originating from the cell surface. Subsequently, the samples were fixed in 4% paraformaldehyde and analyzed using flow cytometry (BD LSR Fortessa flow cytometer; a minimum of 10,000 cells per sample) to determine the proportion of cells associated with fluorescent bacteria, serving as a marker for phagocytosis. The data is presented in new Fig. 1j, k.

6. The authors measured ROS in macrophages stimulated with L. monocytogenes by exposing to DCFH-DA. The authors may need to measure the phagosomal ROS by challenge macrophages with DCFH-labelled L. monocytogenes.

Reply: We greatly appreciate the excellent suggestion from the Reviewer. Following the Reviewer's suggestion, we measured phagosomal ROS levels by challenging *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs with DCFH-labeled *L. monocytogenes*. Time-lapse analysis of ROS production in phagosomes revealed that ROS levels within the phagosomes of *Pdcd6*$^{\Delta mye}$ BMDMs were notably higher than those in the phagosomes of *Pdcd6*$^{fl/fl}$ BMDMs (new Fig. 3 n, o and Extended Data Video 1 and 2).

PDCD6 modulates LC3-associated phagocytosis-mediated antimicrobial defense by regulating lactate metabolism

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Response to Reviewers' comments:

We sincerely thank the reviewer for the insightful and constructive comments. In response, we have incorporated new data and made corresponding updates to the main text, figures and figure legends. Below please find the comments from the reviewers and our responses.

Reviewer #3 (Remarks to the Author):

The authors have included new data in the revised manuscript that address almost all the comments that were put forth in the initial review. I have only a minor comment here. The authors proposed that PDCD6 inhibits LDHA activity by disrupting the FGFR1-LDHA interaction, leading to reduced FGFR1-mediated LDHA Y10 phosphorylation and LDHA activity. However, the competitive binding was examined in 293T overexpressing these three genes. How is the endogenous expression of FGFR1 and the interaction between LDHA and FGFR1 in PDCD6 KO BMDMs?

Reply: We greatly appreciate the insightful suggestions from the Reviewer. Following the Reviewer's suggestion, we assessed the endogenous expression levels of FGFR1 in *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs following exposure to *L. monocytogenes* or *S.* Typhimurium. The results indicated that there were no significant changes in FGFR1 expression during the challenges at different time points in both *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs (new Supplementary Fig. 6h).

Furthermore, as suggested by the Reviewer, we conducted an immunoprecipitation assay using the anti-LDHA antibody to further investigate the interaction between LDHA and FGFR1 in *Pdcd6*$^{fl/fl}$ and *Pdcd6*$^{\Delta mye}$ BMDMs challenged with *L. monocytogenes* or left untreated. The findings revealed that the interaction between LDHA and FGFR1 was significantly enhanced in *Pdcd6*$^{\Delta mye}$ BMDMs compared to *Pdcd6*$^{fl/fl}$ BMDMs, while the interaction between LDHA and PDCD6 decreased in *Pdcd6*$^{fl/fl}$ BMDMs upon *L. monocytogenes* challenge. No noticeable changes in the endogenous expression of FGFR1 were observed in the input samples (new Supplementary Fig. 6k). These results further confirm that PDCD6 interferes with the binding of LDHA to FGFR1 by competitively binding to LDHA.