Zinc-dependent histone deacetylases drive neutrophil extracellular trap formation and potentiate local and systemic inflammation

Highlights
- Zn-dependent lysine deacetylases govern neutrophil extracellular trap (NET) formation
- Class I/IIb HDACs deacetylase histone H3 allowing its citrullination by PAD4
- HDAC inhibition with ricolinostat protects against viral and bacterial pneumonia
- In a model of endotoxic shock, ricolinostat inhibits NETosis and dampens inflammation

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Zinc-dependent histone deacetylases drive neutrophil extracellular trap formation and potentiate local and systemic inflammation

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SUMMARY

Neutrophil extracellular traps (NETs) have been implicated in the pathogenesis of acute respiratory distress syndrome (ARDS) driven by viruses or bacteria, as well as in numerous immune-mediated disorders. Histone citrullination by the enzyme peptidylarginine deiminase 4 (PAD4) and the consequent decondensation of chromatin are hallmarks in the induction of NETs. Nevertheless, additional histone modifications that may govern NETosis are largely overlooked. Herein, we show that histone deacetylases (HDACs) play critical roles in driving NET formation in human and mouse neutrophils. HDACs belonging to the zinc-dependent lysine deacetylases family are necessary to deacetylate histone H3, thus allowing the activity of PAD4 and NETosis. Of note, HDAC inhibition in mice protects against microbial-induced pneumonia and septic shock, decreasing NETosis and inflammation. Collectively, our findings illustrate a new fundamental step that governs the release of NETs and points to HDAC inhibitors as therapeutic agents that may be used to protect against ARDS and sepsis.

INTRODUCTION

The immune system evolved to sense microbial and endogenous cues that alter cellular or organismal homeostasis. When infection and tissue damage coincide, the immune system reacts rapidly to avoid the spread of the infection, even at the cost of an overexuberant reaction that may cause further tissue damage. Neutrophils are key players in this process (Nathan, 2002). These polymorphonuclear cells orchestrate the spread of the infection, even at the cost of an overexuberant reaction that may cause further tissue damage.

Neutrophils and sepsis are characterized by an exaggerated elevation of pro-inflammatory cytokines in the lungs and/or bloodstream, and also by coagulopathy, thrombosis, and multiorgan dysfunction (Broggi et al., 2020; Channappanavar and Perlman, 2017; Giannis et al., 2020; Guan et al., 2020; Iba et al., 2019; Lee et al., 2020; Lucas et al., 2020; Mehta et al., 2020; Ruan et al., 2020; Shin et al., 2019; Short et al., 2014; Tang et al., 2019; Wu et al., 2019; Yang et al., 2019; Zhou et al., 2020). The central roles played by neutrophils and NETosis in driving these life-threatening immune disorders provide a strong mandate to reveal new steps in the regulation of NET induction that can be used as novel therapeutic targets.

Akin to transcriptional activation that is driven by local chromatin remodeling, epigenetic modifications of histones that decondense chromatin are a prerequisite for NET release (Kenny et al., 2017). In particular, histone citrullination by the peptidylarginine deiminase 4 (PAD4) has been shown to facilitate NET formation and release (Thiam et al., 2020; Wang et al., 2009). Granule-resident serine proteases, neutrophil elastase, as well as gasdermin D also participate to chromatin decondensation, nuclear expansion, and formation of NETs (Chen et al., 2018; Papayannopoulos et al., 2010; Sollberger et al., 2018). Whether other epigenetic modifications also participate in NETosis, and how they may be linked to the above-mentioned
processes that alter the structure of chromatin, remains largely overlooked. Based on the importance of histone acetylation in determining chromatin structure and accessibility, we hypothesized that enzymes involved in histone acetylation also contribute to NETosis. Histone acetylation is dynamically controlled by two counteracting protein families, the histone acetyltransferases and the histone deacetylases (HDACs) as “writers” and “erasers” (Grunstein, 1997; Zhao et al., 2018). HDACs belong to two families, based on the dependency on zinc or nicotinamide adenine dinucleotide (NAD+) for their activities (Seto and Yoshida, 2014). In this study, we found that class I and IIb HDACs, which belong to the family of zinc-dependent lysine deacetylases, regulate NETosis in human and mouse cells. Also, these zinc-dependent HDACs can be used as therapeutic targets in mouse models of ARDS and septic shock. In particular, the class I/IIb HDAC inhibitor ricolinostat dampens NET formation and pro-inflammatory cytokine production, reduced morbidity, and improved lung functionality in mouse models of microbial pneumonia and septic shock.

RESULTS

Class I/IIb HDACs drive NET formation

To test the capacity of HDACs to modulate NET formation, we assembled a focused collection of class and isoform specific inhibitors of HDACs with high structural and functional diversity (Park and Kim, 2020). This included pan-HDAC inhibitors that selectively target HDAC class I (HDAC1/2/3/8), class IIa (HDAC4/5/7/9), class IIb (HDAC6/10), and class III (sirtuins), as well as an inhibitor targeting bromodomain proteins, which recognize acetylated lysine on histones. Initially, we investigated the capacity of these inhibitors to alter NETosis by measuring extracellular DNA release by primary human neutrophils activated with phorbol myristate acetate (PMA), a well-known inducer of NETs. DNA release is often used as a proxy for NETosis and, indeed, inhibition of NET induction by using the PAD4 inhibitor GSK484 resulted in significant decrease of extracellular DNA by PMA-treated cells (Figure S1A). Compounds that inhibited either class I or class I/IIb HDACs also significantly diminished extracellular DNA secretion from PMA-treated neutrophils (Figure S1B), whereas class IIa, pan-HDAC inhibitors, other HDACs inhibitors, or bromodomain inhibition did not affect DNA release. These data demonstrate that inhibition of certain, but not all, classes of HDACs efficiently decreases the release of extracellular DNA from PMA-treated human neutrophils and suggest that targeting HDACs may effectively prevent NETosis. Thus, we focused on the compounds that significantly reduced DNA release to further assess their activity on NETosis. To confirm the capacity of specific HDAC inhibitors to interfere with NETosis, we investigated whether HDAC inhibition also prevented the release of citrullinated histone H3 (CitH3), a hallmark of NET formation (Thiam et al., 2020; Wang et al., 2009), that peaked 3–5 hours upon PMA administration (Figure S1C). In agreement with previous findings (Lewis et al., 2015), pretreatment with the NET-disrupting PAD4 inhibitor GSK484 dramatically reduced NET induction, measured as CitH3 staining, in PMA-stimulated human neutrophils (Figures 1A and S1D). Similar to extracellular DNA release, class I or class I/II HDAC inhibitors entinostat and ricolinostat prevented NET release in a dose-dependent manner with an efficacy comparable with GSK484 (Figures 1A and S1D). Next, we tested if HDACs regulate NETosis not only in human but also in mouse neutrophils. We found that, similarly to what we observed using human cells, the class I/IIb HDAC inhibitor ricolinostat efficiently decreased the release of CitH3 in PMA-treated mouse neutrophils (Figure 1B). In contrast to the human cell data, the class I HDAC-specific inhibitor entinostat had no effect on murine neutrophils. In keeping with a key role of class I/II HDACs in regulating NET release, we also found that ricolinostat significantly reduced the expansion of nuclei, another hallmark of NETosis in PMA-treated cells (Sollberger et al., 2018) (Figures 1C and 1D). Moreover, ricolinostat inhibited NET induction in response to nigericin, a bacterial toxin known to induce NETosis in neutrophils (Chen et al., 2018; Sollberger et al., 2018) (Figure S1E). Overall, these data imply that zinc-dependent lysine deacetylases represented by class I/IIb HDACs are required to induce NETosis both in human and mouse neutrophils, and that the class I/IIb HDAC inhibitor ricolinostat, a phase II clinical trial drug, efficiently inhibits NETosis both in humans and mouse neutrophils.

A previous study suggested that pan-HDAC inhibition switches neutrophil cell death from NETosis to apoptosis (Hamam and Palaniyar, 2019). To assess whether ricolinostat reduced NETosis by favoring other types of cell death, we tested the levels of apoptosis, pyroptosis, and/or necrosis induction in PMA-treated cells exposed to either ricolinostat or GSK484. We excluded the activation of apoptosis or necroptosis under our experimental conditions (Figure 1E). In agreement with previous reports (Chen et al., 2018; Sollberger et al., 2018), we found that PMA induced gasdermin D (GSDMD) cleavage, but, notably, this process was not altered by either ricolinostat or GSK484 (Figure 1E). Overall, these data confirm the capacity of ricolinostat, as well as GSK484, to prevent NETosis without affecting the induction of other forms of cell death.
Induced by exposure to viral ligands to RNA respiratory viruses and that we previously successfully used to unveil some features of RNA viral impacts viral pneumonia, we used a well-characterized mouse model that mimics the immune response.

HDAC6, the major class IIb HDAC, behaved similarly to wild-type cells and remained sensitive to ricolinostat administration (Figure S1G). Finally, human neutrophils derived from two independent donors as well as murine neutrophils were treated, or not, with ricolinostat or its chlorinated analog citarinostat (or GSK484, used as a positive control) and the level of CitH3 was assessed. Our data revealed both in humans and mice.

demonstrate that the capacity of zinc-dependent class I/IIb HDACs to drive NET formation is conserved in murine models. To determine if the capacity of ricolinostat to inhibit NETosis as assessed in vitro also impacts viral pneumonia, we used a well-characterized mouse model that mimics the immune response to RNA respiratory viruses and that we previously successfully used to unveil some features of RNA viral

Next, we assessed how HDAC inhibition prevents NETosis. Given that inhibition of class I/IIb HDACs decreased the release of citrullinated histone H3, we hypothesized that ricolinostat exerts its functions by increasing H3 acetylation, which in turn may prevent PAD4-dependent citrullination. Indeed, our data demonstrated that, upon ricolinostat, but not GSK484, administration, histone H3 acetylation was boosted in untreated as well as PMA-treated cells (Figures 1E and 1F). When cells were treated with PMA, and citrullination was increased to induce NETosis, ricolinostat significantly decreased H3 citrullination compared with cells that were treated with PMA only. As expected, the PAD4-inhibitor GSK484 completely abrogated H3 citrullination (Figures 1E and 1F). In keeping with the capacity of ricolinostat to affect neutrophil responses by preventing histone deacetylation, we found that ricolinostat treatment did not alter reactive oxygen species (ROS) production, another key step in NETosis (Douda et al., 2015; Fuchs et al., 2007; Remijen et al., 2011), either in human or mouse cells (Figure 1G). Similarly, we excluded that ricolinostat administration altered other important functions of neutrophils, such as bacterial phagocytosis (Figure 1H).

These data support that the capacity of ricolinostat to prevent histone H3 deacetylation impedes its citrullination and the release of NETs. Therefore, our data support a model in which acetylation and citrullination modifications on histone H3 are divergently regulated in the context of NETosis.

We next focused on the specificity of action of ricolinostat. Ricolinostat inhibits both class I and class IIb HDACs, with a preference for the latter. We, thus, compared the activity of ricolinostat with other inhibitors specific for class IIb HDACs (tubastatin A and nexturastat) as well as with tubacin, that similarly to ricolinostat targets not only class IIb HDACs but also class I HDACs (Bergman et al., 2012; Butler et al., 2010; Haggarty et al., 2003). The siderophore deferoxamine and the hydroxamate-metalloprotease inhibitor batimastat were used as negative controls to exclude bystander activities of the drugs utilized on other divergent metals. We found that class IIb HDAC inhibition prevented NETosis, whereas selective class I targeting did not (Figure S1F). In agreement with these data, neutrophils that lack HDAC6, the major class IIb HDAC, behaved similarly to wild-type cells and remained sensitive to ricolinostat administration (Figure S1G). Finally, human neutrophils derived from two independent donors as well as murine neutrophils were treated, or not, with ricolinostat or its chlorinated analog citarinostat (or GSK484, used as a positive control) and the level of CitH3 was assessed. Our data revealed both compounds inhibited NET formation to a comparable extent (Figures S1H–S1L). Overall, these data demonstrate that the capacity of zinc-dependent class IIb HDACs to drive NET formation is conserved in humans and mice.

Inhibition of class IIb HDACs in vivo dampens NETosis and protects against pneumonia induced by exposure to viral ligands

The conserved activity of class IIb HDACs in regulating NETosis in human and mouse cells prompted us to assess how HDAC inhibition with ricolinostat affects the development of pathological inflammation in vivo in murine models. To determine if the capacity of ricolinostat to inhibit NETosis as assessed in vitro also impacts viral pneumonia, we used a well-characterized mouse model that mimics the immune response to RNA respiratory viruses and that we previously successfully used to unveil some features of RNA viral
Class I/IIb HDACs increase NETosis and ARDS in a mouse model of bacterial pneumonia

To further assess whether the capacity of ricolinostat to prevent NETosis can be used against ARDS, we employed a bacterial lung pneumonia mouse model based on the intratracheal injection of S. aureus (Figure 3A). As before, in this set of experiments we compared the activity of ricolinostat with the PAD4 inhibitor GSK484. When class I/IIb HDACs were inhibited with ricolinostat, morbidity was significantly decreased compared with mice injected with S. aureus only, and also with S. aureus and GSK484 (Figure 3B). Lung barrier alteration was prevented both by ricolinostat or GSK484 treatment, compared with mice exposed to S. aureus alone, whereas bacterial control was not affected by these treatments (Figures 3C and 3D). These data suggest the capacity of these drugs to affect tissue tolerance without changing immune resistance (Medzhitov et al., 2012). NETs are well known to alter tissue functionality and, in fact, have been shown to dampen tissue tolerance during lung infections (Iwasaki and Pillai, 2014; Pillai et al., 2016). Indeed, we found that administration of either ricolinostat or GSK484 potently decreased NETosis as measured by levels of citrullinated histone H3 as well as MPO-DNA complexes in the BALF of mice treated with S. aureus (Figures 3E and 3F). The capacity of these drugs to inhibit NETosis was further demonstrated by cytofluorimetric quantification of citrullinated histone H3-positive neutrophils in the BALF (Figure 3G).

In keeping with our previous findings in the poly(I:C)-driven pneumonia model, we found no major differences in the levels of neutrophils circulating in the blood, whereas reduction of NETosis led to the presence of increased numbers of neutrophils in the BALF (Figures 3H and 3I, and S3A). Pro-inflammatory cytokines IL-6 and IL-1β, but not TNF, were also significantly decreased in the BALF of mice administered ricolinostat and GSK484, compared with mice that received S. aureus only (Figures S3B–S3D). Collectively, these results demonstrate that zinc-dependent HDAC inhibition is as efficacious as PAD4 inhibition in preventing bacterial-induced NETosis and ARDS. Also, treatment with ricolinostat was more efficient in terms of morbidity reduction compared with GSK484.

Class I/IIb HDAC inhibition protects against systemic inflammation

Finally, we investigated whether inhibition of class I/IIb HDACs dampens systemic inflammation in a mouse model of septic shock. Septic shock is characterized by systemic inflammation and disseminated intravascular coagulation (van der Poll et al., 2017), and neutrophils play a critical role in driving this lethal syndrome. We hypothesized that inhibition of NET formation using ricolinostat may yield beneficial effects in reducing sepsis-associated symptoms. Thus, we employed a well-established lipopolysaccharide (LPS)-driven model of septic shock (Zanoni et al., 2017) in mice treated, or not, with ricolinostat or GSK484, as a comparison (Figure 4A). Mice that received either ricolinostat or GSK484 showed no temperature loss, compared with untreated animals, and were significantly protected compared with mice treated with LPS only (Figure 4B). Previous studies demonstrated that decreased platelet counts are a hallmark of disseminated intravascular coagulation during sepsis (Yang et al., 2019). It is also worth noting that NETs are major drivers of disseminated coagulation in sepsis (Xu et al., 2009) and that recent studies underscore that interactions between platelets and neutrophils are critical in sustaining disseminated intravascular
Figure 2. Inhibition of class I/IIb HDACs protects against NETosis and ARDS in a mouse model of RNA viral lung infection

(A) Timeline of poly(I:C) instillation and drug treatments. Mice were intratracheally treated with 2.5 mg/kg poly(I:C) or saline daily for 4 days and euthanized 18 h post the last poly(I:C) instillation. Ricolinostat (30 mg/kg/dose) or GSK484 (25 mg/kg/dose) was administered intraperitoneally at day 2, day 4, and 3 h before the endpoint.

(B) Body temperature measurements at the endpoint.

(C) Lung permeability was measured as albumin content level in the BALF (n = 5).

(D) Measure of airway hyperreactivity (AHR) in the indicated groups in response to increasing concentration of methacholine. RI, responsiveness index.

(E–H) CitH3 and MPO-DNA levels were analyzed by ELISA in BALFs (E, F) and in lungs (G, H) of mice treated as indicated. The saline group represents mice that received saline instead of poly(I:C). NT group represents mice that were challenged with poly(I:C) without receiving any drug. Statistics were calculated using one-way ANOVA (B, C, E–H) and two-way ANOVA with Tukey’s analysis (D) (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. n.s., not significant). n = 10 (B, D) and 15 (E–H) for each group except for the GSK484-treated group (n = 5). Gray stars represent comparison of all experimental groups with the saline-treated control. Black stars represent comparison between drug-treated groups and mice treated with poly(I:C) alone. Violin plots represent median (dashed line) with quartiles (dotted line). See also Figure S2.
coagulation (Clark et al., 2007; McDonald et al., 2012, 2017). Of note, platelet counts were significantly higher in mice that received ricolinostat or GSK484, compared with mice treated with LPS alone (Figure 4C). Although more data will be needed to directly link HDAC inhibition to the reduction of disseminated coagulation during septic shock, these data suggest that immunothrombosis can be similarly prevented upon administration of inhibitors that target either HDAC or PAD4. Indeed, also in this septic shock mouse model we confirmed the efficacy of class I/IIb HDAC or PAD4 inhibition in preventing NETosis. We found not only reduced release of NETs by neutrophils in the peritoneal lavage (Figure 4D) but also increased neutrophils levels (Figures S4A–S4C). As before, these data confirm the efficacy of the used drugs in preventing NETosis and death of neutrophils. In keeping with the systemic nature of this sepsis model, and with the positive feedback loop recently described between NETosis and increased cytokine production (Apel et al., 2021), we found that NET inhibition by either ricolinostat or GSK484 significantly decreased pro-inflammatory cytokine production (Figures 4E–4G).

Since dexamethasone is widely used to dampen inflammation, we also compared the therapeutic effects of ricolinostat with dexamethasone. We did not observe a significant difference in platelet numbers when comparing the dexamethasone-treated group with the group treated with LPS alone (Figure S4C). Although more data will be needed to directly link HDAC inhibition to the reduction of disseminated coagulation during septic shock, these data suggest that immunothrombosis can be similarly prevented upon administration of inhibitors that target either HDAC or PAD4. Indeed, also in this septic shock mouse model we confirmed the efficacy of class I/IIb HDAC or PAD4 inhibition in preventing NETosis. We found not only reduced release of NETs by neutrophils in the peritoneal lavage (Figure 4D) but also increased neutrophils levels (Figures S4A–S4C). As before, these data confirm the efficacy of the used drugs in preventing NETosis and death of neutrophils. In keeping with the systemic nature of this sepsis model, and with the positive feedback loop recently described between NETosis and increased cytokine production (Apel et al., 2021), we found that NET inhibition by either ricolinostat or GSK484 significantly decreased pro-inflammatory cytokine production (Figures 4E–4G).

Since dexamethasone is widely used to dampen inflammation, we also compared the therapeutic effects of ricolinostat with dexamethasone. We did not observe a significant difference in platelet numbers when comparing the dexamethasone-treated group with the group treated with LPS only (Figure S4D), whereas both drugs efficiently reduced pro-inflammatory cytokine production (Figures S4E and S4F). Overall, our results demonstrate that inhibition of NETosis by ricolinostat protects against systemic inflammation in a mouse model of septic shock.

**DISCUSSION**

When infection and tissue damage coincide, the immune system potently reacts to avoid the spread of the infection, even at the cost of further damage. Neutrophils are central to this process, thanks to the release of NETs that contain and restricts viral, bacterial, as well as fungal infections (Brinkmann et al., 2004; Saitoh et al., 2012; Thiam et al., 2020). Nevertheless, excessive tissue damage caused by NET release has been often associated with immune-driven disorders (Thiam et al., 2020). Moreover, NETosis has been shown to play both protective as well as detrimental roles during tissue repair and resolution (Hahn et al., 2019; Kang et al., 2020; Schauer et al., 2014). Thus, a better understanding of the mechanisms that regulate NET induction is fundamental to develop future possible therapeutic ways of intervention to either potentiate or block the induction of NETs.

Herein, we show that class I/IIb HDACs drive the release of NETs by human and mouse neutrophils. Their inhibition in mice dampens inflammation and protects against microbial pneumonia or septic shock. These data establish a new biological feature of a group of HDACs whose activity is well known to regulate gene transcription and open to the use of drugs that inhibit HDACs to restrain the tissue-damaging functions of neutrophils. Although targeting of PAD4 with the chemical compound GSK484 was previously shown to be effective in inhibiting CitH3 and NET formation, we demonstrated that ricolinostat, a drug currently utilized in a phase II clinical trial, can also be used to efficiently inhibit NETosis and inflammation. Using multiple in vivo mouse models mimicking bacterial- or viral-induced infections and septic shock, we show that
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**A**

-24h | -7h | -5h | 0h | +2h

**Pre-treatment**
1. 30 mg/kg Rico
2. 25 mg/kg GSK
3. 10 mg/kg Dex

**Pre-treatment**
1. 30 mg/kg Rico
2. 25 mg/kg GSK
3. 10 mg/kg Dex

**Priming**
1 mg/kg LPS

**Challenge**
2 mg/kg LPS

**Endpoint**
Peritoneal lavage, blood collection and analysis

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**B**

Temperature (°C)

- Saline
- NT
- Ricollinostat
- GSK444

LPS+LPS

---

**C**

Platelets/µl

- Saline
- NT
- Ricollinostat
- GSK444

LPS+LPS

---

**D**

CtH3+ neutrophils (%)

- Saline
- NT
- Ricollinostat
- GSK444

LPS+LPS

---

**E**

IL-1β (pg/ml)

- Saline
- NT
- Ricollinostat
- GSK444

LPS+LPS

---

**F**

IL-6 (ng/ml)

- Saline
- NT
- Ricollinostat
- GSK444

LPS+LPS

---

**G**

TNF (ng/ml)

- Saline
- NT
- Ricollinostat
- GSK444

LPS+LPS
pharmacological inhibition of HDACs ameliorates lung functionality, reduces systemic inflammation, and decreases thrombosis via inhibition of NETosis.

Viral or bacterial-induced pneumonia, as well as COVID-19, have a strong correlation to elevated levels of circulating neutrophils, NETs, and inflammatory cytokines that hint at a chronic inflammatory storm in patients (Mehta et al., 2020). NET formation is intimately linked to thrombosis that drives microvasculature damages in lung and other organs (Ackermann et al., 2020; Al-Samkari et al., 2020; Tang et al., 2020). Neutrophils and NETosis also participate in the most severe cases of COVID-19 (Zuo et al., 2020a, 2020b). NETs infiltrate lungs of patients with severe COVID-19, and SARS-CoV-2 has been shown to trigger the release of NETs that mediate lung pathology (Middleton et al., 2020; Radermecker et al., 2020; Veras et al., 2020). As dysregulated production of NETs is known to exacerbate inflammation and accelerate the pathogenesis of ARDS and sepsis caused by other coronaviruses (Blondonnet et al., 2016; Perlman and Dandekar, 2005), these findings support the hypothesis that targeting NET formation represents a viable strategy to alleviate tissue damage and hyperinflammation. Therefore, our results imply that pharmacological inhibition of HDAC activity may be beneficial for patients with COVID-19 by blocking NETosis.

In our mouse models, we show that drugs that inhibit class I/IIb HDACs can partially reduce the levels of pro-inflammatory cytokines including IL-1β, TNF, and IL-6. Whether in our model IL-1β levels are directly controlled by HDAC activity, as recently shown in a different LPS-driven sepsis model (Magupalli et al., 2020), or they are a consequence of NET formation will require further studies.

Overall, our data demonstrate that zinc-dependent lysine deacetylation inhibition prevents NET formation by neutrophils, which in turn reduces systemic inflammation. This strongly suggests that HDAC inhibition represents a new potential therapeutic strategy for the treatment of a variety of diseases mediated by the overproduction of NETs such as COVID-19, pulmonary diseases, autoimmune diseases (e.g., rheumatoid arthritis and systemic lupus erythematosus), diabetes, and cancers (Arpinati et al., 2020; Lee et al., 2017; Porto and Stein, 2016; Wong et al., 2015).

Limitations of the study
A previous study suggested that abrogation of HDAC activities using pan-HDAC inhibitors may impact NETosis by favoring neutrophil apoptosis (Hamam and Palaniyar, 2019). Our data excluded that class I/IIb HDAC inhibition alters other forms of cell death in neutrophils and instead point to deacetylation of histone H3 as a step necessary to allow histone citrullination and NET release. In another report, the same group suggested pan-HDAC inhibition increases NETosis (Hamam et al., 2019); however, it failed to address whether inhibition of a specific HDAC class has a differential impact and if in vitro data can be translated in vivo. In our hands, pan-HDAC inhibition does not alter the release of NET compared with specific targeting of class I/IIb HDAC activity. Whether this is due to cross talk between and/or opposing effects of different classes of HDACs or specific HDAC complexes, as well as the dose and/or type of drugs utilized, remains to be investigated.

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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103256.

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AUTHOR CONTRIBUTIONS
V.P., M.D.G., A.B., M.B., and Q.C. designed, performed, and analyzed the experiments; I.Z., V.P., and V.P-Y.M. wrote the paper; T.A.C., R.M., and S.J.H. contributed to design the experiments; I.Z. and J.M.K. conceived the project, designed the experiments, and supervised the study.

DECLARATION OF INTERESTS
V.P., V.P-Y.M., R.M., S.J.H., and I.Z. submitted a patent related to the findings of this work. J.M.K. has been a paid consultant and/or equity holder for companies including Stempeutics, Sanofi, Celltex, LifeVaultBio, Camden Partners, Stengent, Biogen, Pancryos, Element Biosciences, Frequency Therapeutics, and Mesoblast. J.M.K. is also an inventor on a patent that was licensed to Mesoblast. J.M.K. holds equity in Frequency Therapeutics, a company that has licensed IP generated by J.M.K. that may benefit financially if the IP is further validated. J.M.K. has been a paid consultant and or equity holder for multiple companies (listed here https://www.karlab.net/team/jeff-karp). The interests of J.M.K. were reviewed and are subject to a management plan overseen by his institutions in accordance with its conflict-of-interest policies. S.J.H. was or is a member of the scientific advisory board and equity holder in Rodin Therapeutics, Psy Therapeutics, Frequency Therapeutics, and Vesigen Therapeutics and has received speaking or consulting fees from AstraZeneca, Amgen, Merck, Biogen, Syros Pharmaceuticals, Regency Pharmaceuticals. S.J.H.‘s laboratory has received sponsored research funding from the Tau Consortium, F-Prime Biomedical Research Initiative, AstraZeneca, JW Pharmaceuticals, ATA1, and Stealth BioTherapeutics, although none of these sponsors were involved in the present study. R.M. is a scientific advisory board (SAB) member and equity holder of Regency Pharmaceuticals, ERX Pharmaceuticals, and Frequency Therapeutics.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Antibodies          |        |            |
| Anti-citrullinated histone H3 | Abcam | Cat# ab5103 RRID:AB_304752 |
| Anti-rabbit Alexa 568 | Invitrogen | Cat# A11011 RRID:AB_143157 |
| Anti-Ly6G APC       | BioLegend | Cat# 127613 RRID:AB_1877163 |
| Anti-CD11b FITC     | BioLegend | Cat# 101206 RRID:AB_312789 |
| Anti-CD45 Brilliant Violet 510 | BioLegend | Cat# 103138 RRID:AB_2563061 |
| Anti-acetyl-histone H3 | Sigma/Millipore | Cat# 06-599 RRID:AB_2115283 |
| Anti-histone H3     | Cell Signaling Technology | Cat# 4499 RRID:AB_10544537 |
| Anti-caspase 8      | Cell Signaling Technology | Cat# 4790 RRID:AB_10545768 |
| Anti-cleaved caspase 8 | Cell Signaling Technology | Cat# 9496 RRID:AB_561381 |
| Anti-caspase 3      | Cell Signaling Technology | Cat# 9662 RRID:AB_331439 |
| Anti-phospho-MLKL   | Cell Signaling Technology | Cat# 91689 RRID:AB_2732034 |
| Anti-MLKL           | Cell Signaling Technology | Cat# 14993 RRID:AB_2721822 |
| Anti-GSDM-D         | Cell Signaling Technology | Cat# 93709 RRID:AB_2800210 |
| Anti-p-actin        | Sigma | Cat# A2228 RRID:AB_476697 |
| Anti-neutrophil     | Abcam | Cat# ab2557 RRID:AB_303154 |
| **Bacterial and virus strains** | | |
| Staphylococcus aureus subsp. aureus Rosenbach | ATCC | Cat# 25904 |
| *Escherichia coli* (O111) | ATCC | Cat# BAA-2440 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Ricolinostat (ACY-1215) | Cayman Chemical | Cat# 21531 |
| Citarinostat        | Cayman Chemical | Cat# 26173 |
| GSK848 (PAD4 inhibitor) | Cayman Chemical | Cat# 17488 |
| Entinostat          | Sellickchem | Cat# S1053 |
| Givinostat          | Sellickchem | Cat# S2170 |
| TMP195              | Sellickchem | Cat# S8502 |
| Belinostat          | Sellickchem | Cat# S1085 |
| Panobinostat        | Sellickchem | Cat# S1030 |
| FK866               | Cayman Chemical | Cat# 13287 |
| Apabetalone         | Sellickchem | Cat# S7295 |
| Deferoxamine        | Cayman Chemical | Cat# 14595 |
| Batimastat          | Cayman Chemical | Cat# 14742 |
| Tubastatin A        | Cayman Chemical | Cat# 10559 |
| Nexturastat A       | Cayman Chemical | Cat# 16874 |
| Tubacin             | Cayman Chemical | Cat# 13691 |
| Phorbol 12-myristate 13-acetate | Cayman Chemical | Cat# 10008014 |
| Poly(C) HMW         | Invivogen | Cat# tlr-pic |
| LPS from *E. coli*, Serotype O55.85 (TLRGRADE™) | Enzo Lifesciences | Cat# ALX-581-013-L002 |
| Nigericin           | Invivogen | Cat# tlr-nig |
| Peroxidase from horseradish (HRP) | Sigma | Cat# P8375-1KU |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ivan Zanoni (ivan.zanoni@childrens.harvard.edu; @Lo_Zanzi).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
6–8 week-old female C57BL/6J (Jax 000664) and C57BL/6J-Hdac6m2Ltzy/J (Jax 029318) mice were purchased from The Jackson Laboratory. Mice were housed under specific pathogen-free conditions at Boston Children’s Hospital, and all the procedures were approved under the Institutional Animal Care and Use Committee (IACUC) and operated under the supervision of the department of Animal Resources at Children’s Hospital (ARCH). Sex as a biological variable was not analyzed in this work.
Neutrophil purification

Human neutrophils from blood of healthy donors were purified with Polymorphoprep (Progen) according to manufacturer’s instruction. Murine neutrophils were purified over a 62.5% Percoll gradient (GE Healthcare) as previously described (Broggi et al., 2017). Purified neutrophils were resuspended in DMEM medium (ThermoFisher) and plated in a 96 multi-well plate previously coated with poly-D-lysine (ThermoFisher). Plate was spin at 200 x g for 1 minute without brake to facilitate neutrophils seeding.

METHODS DETAILS

Extracellular DNA quantification

Neutrophils were treated with indicated drugs for 1 hour and then stimulated with 1 μM PMA for 3 hours. Extracellular DNA was quantified in the supernatant with Quant-iT PicoGreen (Invitrogen).

Citrullinated histone H3 in vitro quantification and imaging

Neutrophils pretreated with indicated HDAC inhibitors, or protein-arginine deaminase (PAD4) inhibitor GSK484, and then stimulated with 1 μM PMA for 3 hours, were fixed with equal volume of 10% formalin for 1 h at room temperature. Samples were blocked with 2% BSA in PBS for 1 hour at room temperature and stained with the anti-citrullinated histone H3 antibody overnight at 4°C, followed by anti-rabbit Alexa 568 secondary antibody. Nuclei were stained with DAPI. NETs formation was measured as induction of citrullinate histone H3 positivity and quantified with a plate-reader (Ex 568 nm, Em 603 nm). Percentage of NET induction was calculated using signal in un-treated cells as 0% of induction and signal in PMA-treated cells as 100% of induction. Alternatively, NETosis induction was quantified by imaging. Images were acquired with EVOS M7000 Imaging System (ThermoFisher Scientific). Number of citrullinated histone H3 positive cells and area of DAPI signal were quantified in multiple fields of view (FOV) using ImageJ software (NIH). For 3D nuclei reconstruction images were acquired with ZEISS 880 Fast Airyscan and analyzed with ZEISS ZEN Blue Software.

Immunoblotting

For western blotting, 2x10⁶ neutrophils pretreated with indicated HDAC inhibitor, or protein-arginine deaminase (PAD4) inhibitor GSK484, and then stimulated with 1 μM PMA for 3 hours were lysed using RIPA buffer with protease and phosphatase inhibitors (T-2494, A.G. Scientific) and diisopropylfluorophosphate (D0879, Sigma). Immunoblotting was performed using standard molecular biology techniques. Band intensity was quantified using ImageJ software.

ROS quantification

Neutrophils plated in a white 96 multi-well plate were pretreated with indicated HDAC inhibitor, or protein-arginine deaminase (PAD4) inhibitor GSK484 for 1 hour and then were stimulated with 1 μM PMA in presence of luminol (Cat# A8511, Sigma) and HRP (Sigma). ROS production was monitored in real-time by luminescence. Luminescence was measured every 3 minutes for 3 hours in a plate-reader analyzer (Molecular Device).

Phagocytosis assay

E. coli (O111) was stained with Cell Proliferation Dye eFluor™ 670 (cat# 65-0840-85, Invitrogen) for 30 minutes and then opsonized with 20% mouse serum for 10 minutes. Neutrophils were stimulated with the indicated MOI for 1 hour at 37°C, washed, and then stained with anti-Ly6G PE antibody and DAPI. Ultimately, samples were acquired with BD FACS Fortessa and percentage of neutrophils positive for E. coli was quantified.

In vivo NET quantification

NETosis was measured in bronco-alveolar lavage fluid or lung tissue homogenates as quantity of citrullinated histone H3 with Citrullinated histone H3 ELISA kit (Cayman) and as quantity of myeloperoxidase (MPO)-DNA complexes by using a modified Cell Death Detection ELISA (Roche). Briefly, anti-myeloperoxidase antibody (ThermoFisher) was used to coat a high-binding 96 multi-well plate overnight at 4°C. Following a 1h blocking with 2% BSA/PBS solution, samples were added for an overnight incubation at 4°C. Finally, DNA was detected with Cell Death Detection kit according to manufacture instruction. Absorbance was measured at 405 nm and MPO-DNA complex levels were quantified as normalized relative to saline.
Alternatively, neutrophils positive for citrullinated histone H3 were quantified by cytofluorimetry. Briefly, neutrophils from broncho-alveolar lavage fluid or peritoneal lavage fluid were stained for Ly6G, CD11b, CD45 and Zombie dye for 20 minutes at room temperature and then fixed with BD FACS Lysing Solution (cat# 349202, BD Biosciences). Samples were washed twice with Intracellular Staining Permeabilization Wash buffer (cat# 421002, Biolegend), incubated with anti-citrullinated histone H3 antibody (Abcam) 1 hour at room temperature and subsequently with PE anti-rabbit antibody for 30 minutes. Finally, samples were acquired with BD FACS Fortessa and neutrophils (CD45+/Ly6G+/CD11b+) positive for citrullinated histone H3 were quantified with FlowJo software.

**Poly(I:C) pulmonary NET induction**

6-8 weeks old female mice were treated daily with 2.5 mg/kg of poly (I:C) for 4 days and euthanized 18 hours post the last poly(I:C) intra-tracheal instillation, as previously described (Broggi et al., 2020). Temperatures were monitored with a rectal probe. NETosis was measured in broncho-alveolar lavage fluid and lung homogenates as quantity of citrullinated histone H3 and quantity of MPO-DNA complexes. Ricolinostat (30 mg/kg/dose) and GSK484 (25 mg/kg/dose) were administered intraperitoneally at day 2, day 4 and 3 hours prior the end point. To assess lung permeability, albumin in BALF was measured with Albumin Assay Kit (ab235628, Abcam). Number of neutrophils (CD45+/Ly6G+/CD11b+) in blood, BALF and lung homogenate was quantified by cytofluorimetry using CountBright Absolute Cell Counting Beads (Thermo Scientific). Paraffin-embedded lung sections were stained with DAPI for nuclei and with anti-neutrophil antibody (Abcam) for the presence of neutrophils.

**Measurement of airway functional responses**

Airway hyperreactivity (AHR) was measured, as previously described (Harb et al., 2020). Anesthetized mice were exposed to doubling concentrations of aerosolized acetyl-β-methacholine (Sigma-Aldrich) by using a Buxco small-animal ventilator (Data Sciences International). The relative peak airway resistance for each methacholine dose, normalized to the saline baseline (Score = 1), was calculated.

**S. aureus pulmonary infection model**

6-8 week-old female mice were pretreated with indicated HDAC inhibitors at −1 day and −3 hours prior in vivo NETs induction by intra-tracheal instillation of 6×10⁶ CFU of Staphylococcus aureus subsp. aureus Rosenbach (ATCC 25904). Ricolinostat was used at 30 mg/kg/dose and GSK484 at 25 mg/kg/dose. Temperatures were monitored with a rectal probe. NETosis was measured in broncho-alveolar lavage fluid as quantity of citrullinated histone H3 and quantity of MPO-DNA complexes by ELISA. Alternatively, neutrophils positive for citrullinated histone H3 were analyzed by cytofluorimetry in BALF samples. Albumin in BALF was measured with Albumin Assay Kit (ab235628, Abcam). Number of neutrophils (CD45+/Ly6G+/CD11b+) in blood and BALF was quantified by flow cytometry using CountBright Absolute Cell Counting Beads (Thermo Scientific).

**In vivo LPS-induced septic shock**

8-week-old female mice were primed with 1 mg/kg of LPS intra peritoneum (i.p.) for 5 hours and then challenged with 2 mg/kg of LPS i.p. Ricolinostat was used at 30 mg/kg/dose, GSK484 at 25 mg/kg/dose and dexamethasone at 10 mg/kg/dose. Drugs were administered i.p. at day −1 and 2 hours prior priming. Blood samples were collected 2 hours post-challenge and cytokines were measured by ELISA. The platelet concentrations and neutrophils numbers in the blood and peritoneal cavity were calculated by cytofluorimetry using CountBright Absolute Cell Counting Beads (Thermo Scientific) according to the manufacturer’s instructions. NETosis was measured as quantity of neutrophils in the peritoneal lavage positive for citrullinated histone H3 by cytofluorimetry.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Results were analyzed with GraphPad Prism statistical software (version 8). One-way ANOVA and Two-way ANOVA were used to analyze statistically significant differences between the means of two or more independent groups, as indicated in the figure legends. Sample sizes for each experiment are provided in the figures and the respective legends. Asterisks were used as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.