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RESEARCH ARTICLE

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Macrophage extracellular traps are induced by *Mycoplasma bovis* in bovine macrophages through NADPH oxidase/ROS-dependent manner and their antibacterial efficacy

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Abstract

Mycoplasma bovis has emerged as a significant pathogen in cattle, leading to considerable economic losses in the cattle industry. It is associated with various clinical syndromes, including pneumoniae, mastitis, and arthritis. The innate immune response, particularly macrophages, plays a crucial role in combating infections caused by such pathogens. The release of macrophage extracellular traps (METs) represents a novel defense mechanism employed by macrophages; however, the impact of *M. bovis* on the formation of METs in bovine macrophages remains unknown. Therefore, the primary objective of this study is to investigate the mechanism by which *M. bovis* affects the formation of bovine METs and to evaluate their bactericidal efficacy. Our findings revealed that the bovine macrophage cell lines released DNA fibrils that colocalized with histones and cellular proteins in response to *M. bovis* infection, as visualized by confocal and scanning electron microscopy. Moreover, the formation of METs was found

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; ABAH, aminobenzoic acid hydrazide; BMDMs, bone marrow-derived macrophages; BSA, bovine serum albumin; Cath G, cathepsin G; CFU, colony-forming units; CitH3, citrullinated histone H3; Cyt D, cytochalasin D; DPI, diphenyleneiodonium; ESAT-6, early secreted antigenic target 6; FBS, fetal bovine serum; hMDMs, human monocyte-derived macrophages; HOCl, hypochlorous acid; IFN-γ, interferon-γ; IL-8, interleukin-8; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; *M. bovis*, *Mycoplasma bovis*; METs,, macrophage extracellular traps; MMPs, matrix metalloproteinases; MOI, multiplicity of infection; MPO, myeloperoxidase; NE, neutrophil elastase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PMA, phorbol-12-myristate-13-acetate; PPLO, pleuropneumonia-like organisms; ROS, reactive oxygen species; TNF-α, tumor necrosis factor.

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to depend on NADPH oxidase, which is crucial for reactive oxygen species generation. Importantly, the formation of METs led to cell lysis in response to *M. bovis* infection, as indicated by the release of lactate dehydrogenase, and this process was found to be independent of apoptosis and pyroptosis. Moreover, the release of METs in response to *M. bovis* infection was effective in both killing and restricting its growth. Overall, our study first described how METs were produced in bovine macrophages that responded to *M. bovis* and demonstrated their significant function in bacterial killing, which is helpful to improve our understanding of the host's immune defense against this pathogen.

KEYWORDS

bacterial killing, bovine, macrophage extracellular traps, macrophages, *Mycoplasma bovis*

1 | INTRODUCTION

Mycoplasma bovis is a small-sized microorganism belonging to the family *Mycoplasmataceae* within the class *Mollicutes*. The bacterium is associated with substantial economic losses indicating its impact on cattle industries.[1,2] *M. bovis* is characterized by a relatively smaller genome size of about 1 Mbp with low GC content.[3,4] Moreover, this pathogen is notable for lacking cell wall, making it unique among bacteria and contributing to its resistance to common antibiotics that target cell wall synthesis. The absence of a rigid cell wall also allows *M. bovis* to adopt various morphological forms, enhancing its survival and ability to colonize within different tissues of the host.[1,2,5] Over the last half-century, *M. bovis* has evolved as a significant pathogen affecting cattle worldwide, causing various clinical symptoms, including respiratory disease, mastitis, arthritis, otitis media, and conjunctivitis.[5–7]

Macrophages are key components of the immune system and serve as one of the first responders to infection. They have unique properties and versatile functions including phagocytosis, antigen presentation, secretion of inflammatory molecules, and tissue repair.[8,9] In addition to their classical roles in host defense, macrophages have a novel way of fighting bacterial infections by releasing extracellular traps. Macrophage extracellular traps (METs) were first reported in 2010[10] and significantly advanced our understanding of their critical role in the host immune defense against bacterial infections.[11–13] METs typically consist of a framework primarily made up of DNA, in conjunction with histones and various cytoplasmic proteins that exhibit antimicrobial characteristics.[14–16] The DNA structure within METs can derive from the nucleus[14] or mitochondria.[16] The structural histones play a critical role in the composition of METs. Notably, histones may undergo post-translational modifications, such as citrullination,[17,18] or may not be citrullinated.[16] Beyond DNA and histones, METs are enriched with a variety of protective enzymes that contribute to their antimicrobial properties. The key proteins included myeloperoxidase (MPO),[19–21] neutrophil elastase (NE),[15,21] lysozyme,[20] matrix metalloproteinases (MMPs),[15,22,23] and lactoferrin.[21]

METs formation is identified as a unique form of cell death termed METosis, which mainly involves the release of nuclear DNA from activated macrophages into the extracellular space, along with antimicrobial proteins.[10,12,15] Lactate dehydrogenase (LDH) is notable for either being released during this process or not, indicating that traditional forms of cell death such as apoptosis and necrosis do not primarily account for the observed DNA release.[14,15,20] The release of MET can be initiated by a range of mediators including interferon (IFN)-γ,[18] tumor necrosis factor (TNF)-α,[24] interleukin (IL)-8,[24] lipopolysaccharide (LPS),[25] phorbol-12-myristate-13-acetate (PMA),[24,25] nigericin,[16] zymosan,[25] statins,[10] calcium ionophores,[16,26] and hypochlorous acid (HOCl).[24] Moreover, the formation of METs can be induced by a variety of bacterial infections, which include Gram-positive bacteria, such as *Streptococcus agalactiae*[15] and *Clostridium perfringens*[27] as well as Gram-negative bacteria, such as *Escherichia coli*,[20] non-typeable *Haemophilus influenzae*,[23] and *Histophilus somni*.[28] METs can also be stimulated by *Mycobacterium tuberculosis*[29] and *Mycobacterium massiliense*.[30] Recent findings have also indicated that *Mycoplasma hyopneumoniae*, a respiratory pneumonic pathogen associated with swine, is capable of inducing MET formation in THP-1 cells.[31] This diverse range of pathogens underscores the potential role of METs as a significant function of the immune response.

While METs formation has been documented in various bacteria, the release of MET in response to *M. bovis* infection is still unknown. In this study, we present the mechanism of METs formation in response to *M. bovis*

infection and describe their features and involvement in combating bacterial infections. These findings will help our understanding of how the functional mechanisms of METs in the context of *M. bovis* infections.

2 | MATERIALS AND METHODS

2.1 | Bacterial strain and cell culture

M. bovis reference strain PG45 was inoculated into a modified pleuropneumonia-like organisms (PPLO, BD, USA) broth medium, which was supplemented with 10% horse serum (Gibco) as previously described.[32] *M. bovis* cell titer was conducted by enumerating colony-forming units (CFU) on PPLO agar plates following 3 days of incubation period at 37°C in a 5% CO_2 incubator. The cultures were subsequently preserved at −80°C until required for further use. Bovine macrophage cell lines (BoMac),[33] were kindly provided by Prof. Aizhen Guo from Huazhong Agricultural University. BoMac cells were cultured in a pre-warmed RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). The cells were incubated at 37°C in a 5% CO_2 incubator for 24 h before the co-culture experiments. The following day, bovine macrophage cells were observed under a microscope (Olympus CKX31, Japan) and counted using an automated cell counter (BodBoge, China) for further experiments.

2.2 | Confocal laser scanning microscopy

BoMac cells were seeded onto 14-mm glass coverslips in a 24-well cell culture plate at a density of 2×10^5 cells/well and then infected with *M. bovis* at a multiplicity of infection (MOI) of 100. The co-cultured cells were incubated for 6 h at 37°C in a 5% CO_2 incubator. Some of the cells were pretreated with DNase I (180 U/mL, Coolaber, China) for 30 min before infection or PMA (500 nM, Invitrogen, USA) as a positive control. After 6 h of incubation, cells were fixed with 4.0% paraformaldehyde (PFA, Solarbio, Beijing, China) for 30 min at room temperature. Following fixation, cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for 5 min at room temperature, then blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) and 10% normal goat serum (ZSGB Biotechnology, Beijing, China) in phosphate-buffered saline (PBS) for 30 min at 37°C, and incubated overnight at 4°C with antibodies for histone H3 (1:50, Bioss), MPO (1:50, Abcam), NE (1:50, Abcam), MMP-1 (1:50, Bioss), MMP-2 (1:50, Solarbio), or MMP-11 (1:50, Bioss). Subsequently, the cells were washed three times with 1% BSA in PBS, followed by 1 h incubation with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (1:1000, Thermo Fisher), and washed three times with 1% BSA in PBS. The cells were stained with SYTOX Green (10 μM, Invitrogen) for double-stranded DNA and Hoechst 33342 (8 μM, Invitrogen) for condensed chromatin. The coverslips were then washed with PBS before mounting onto glass microscope slides with a mounting medium containing antifading (Solarbio, China). The cells were then observed with a Zeiss LSM 980 inverted laser scanning confocal microscope.

2.3 | Scanning electron microscopy

To perform scanning electron microscopy, BoMac cells were seeded onto 14-mm glass coverslips in a 24-well cell culture plate as indicated above. The cells were then washed with PBS and incubated with *M. bovis* at an MOI 100 for 6 h. Following this, coverslips were washed and subsequently fixed with 4.0% PFA and 2.5% glutaraldehyde (Solarbio, China) and processed at Chendu Lilai Biomedical Experiment Center. Examination of the samples was conducted utilizing a JSM-IT700HR scanning electron microscope (JEOL, Japan).

2.4 | Quantitative analysis of extracellular DNA release

Macrophage extracellular DNA was quantified as previously described.[14] BoMac cells were seeded in 96-well cell culture plates at a concentration of 1×10^5 cells/well. After *M. bovis* infection, the co-culture supernatant was collected, centrifuged at 300×*g* for 5 min, and subsequently transferred to a new 96-well plate. DNase I (180 U/mL) was added to some of the culture supernatant and incubated for 20 min at 37°C. A 1:200 dilution of PicoGreen reagent (YEASEN, Shanghai, China) in 10 mM Tris-base buffered with 1 mM EDTA was added to an equal volume of untreated or DNase I-treated mixtures, following the manufacturer's instructions. Fluorescence intensity was measured using a Tecan Infinite® M Plex microplate reader (excitation, 480 nm; emission, 520 nm) (Tecan, Australia). MET production was quantified as the fold increase of DNA released compared to uninfected cells.

2.5 | ELISA measurement of bovine CitH3, MPO, NE, and Cath G

BoMac cells were infected with various MOIs of *M. bovis* and incubated for indicated times, the supernatants from the co-cultures were collected and centrifuged to remove cell debris as earlier outlined.[15] The resulting supernatants

were then analyzed for the concentration of bovine citrullinated histone H3 (CitH3), MPO, NE, and cathepsin G (Cath G) using ELISA kits (MyBioSource, USA) according to the manufacturer's protocol.

2.6 | Monitoring intracellular ROS production

The production of intracellular ROS in BoMac cells was measured following the methodology described in a previous study,[27] using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime Biotechnology, China). Bovine macrophage cells were plated in 24-well cell culture plates and subsequently infected with *M. bovis*. In parallel experiments, the cells were preincubated with NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI, 10 μM, Cayman Chemical) and actin polymerization inhibitor cytochalasin D (Cyt D, 10 μg/mL, Cayman Chemical) for 30 min before infection. Following stimulation, the culture supernatant was harvested and centrifuged at 300×*g* for 5 min for further use, while the adherent cells were washed and incubated with diluted DCFH-DA (10 μM) for 20 min at 37°C in the dark. The positive control was PMA (500 nM), whereas the negative control was cells maintained in the RPMI 1640 medium. The samples were subsequently analyzed using a fluorescence microplate reader with excitation and emission set at 488 nm and 525 nm, respectively. Further confirmation for monitoring of intracellular ROS, treated and untreated cells were examined directly by confocal microscopy. Additionally, DNA released in the supernatant was assessed using the PicoGreen reagent, following the protocol mentioned above.

2.7 | LDH cytotoxicity assay

BoMac cells were initially cultured in RPMI 1640 medium devoid of serum and incubated for 6 h with *M. bovis*, aligned with another experiment in which *M. bovis* was incubated for different time points. The culture supernatants were collected and centrifuged to remove the cellular residues and the level of LDH released in the cell culture supernatant was detected using an LDH cytotoxicity assay detection kit (Beyotime Biotechnology), following the manufacturer's guidelines.

2.8 | Evaluation of the bactericidal activity of METs

The efficacy of bacterial killing was evaluated by plating BoMac cells in a 24-well cell culture plate as mentioned above, cells were primed with NADPH oxidase inhibitor DPI (10 μM, Cayman Chemical), phagocytosis inhibitor Cyt D (10 μg/mL Cayman Chemical), DNase I (180 U/mL, Coolaber), neutrophil elastase inhibitor sivelestat (1 mM, MedChemExpress), p38 MAPK inhibitor SB203580 (10 μM, Abcam), myeloperoxidase inhibitor aminobenzoic acid hydrazide (ABAH, 100 μM, Abcam), or store operating calcium entry inhibitor 2-aminoethoxydiphenyl borate (2-APB, 100 μM, Abcam) for 30 min at 37°C in a 5% CO_2 incubator, followed by incubation with *M. bovis* at MOI 100 for 6 h. Following stimulation, the supernatant was harvested and centrifuged for 5 min at 600×*g*. The bacterial pellets were resuspended in PBS and then plated on PPLO agar plates to quantify the bacterial numbers by counting CFU after 72 h of incubation. To further assess the bacterial killing, BoMac cells were incubated with *M. bovis* at MOI 100 for 24 h, with some cells pretreated with Cyt D or DNase I, and the number of viable bacteria was counted as previously described. The results were analyzed by comparing treated BoMac cells with untreated cells. Moreover, BoMac cells were seeded onto coverslips in a 24-well cell culture plate as described above and then infected with *M. bovis* at MOI 100 for 24 h for additional confirmation of bacterial killing. Following infection, the coverslips were stained with the Live/Dead BacLight bacterial viability kit (Invitrogen) according to the manufacturer's instructions before undergoing confocal laser scanning microscope analysis.

2.9 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 software (Version 9.0g; GraphPad Software Inc., CA). All values are expressed as the means ± standard deviations. Three independent experiments were conducted in the study. ANOVA was used for multiple comparisons.*$p < .05$, **$p < .01$, ***$p < .001$. The ns, no significant difference ($p \geq .05$).

3 | RESULTS

3.1 | *M. bovis* triggers METs formation

To investigate the release of METs by bovine macrophages in response to *M. bovis*, BoMac cells were co-incubated with *M. bovis* for 6 h, after which the bovine macrophages cells were subjected to staining with SYTOX Green and Hoechst 33342, then subsequently analyzed using confocal laser scanning microscopy. This analysis revealed the presence of extracellular structures extended from the infected bovine macrophages, which were not visible when

the macrophages were treated with DNase I (Figure 1A). Further confirmation, cellular interaction between bovine macrophages and *M. bovis* was evaluated by scanning electron microscopy. This analysis revealed the presence of fibers or reticular structures released from the macrophages following 6 h of infection, which were not observed in uninfected cells (Figure 1B). Moreover, cells were then analyzed to determine the extent to which these structures contained commonly associated proteins, including histones, MPO, NE, and MMPs. Our results showed that each of these proteins colocalized with the extracellular DNA structures extending from bovine macrophages (Figure 2). The specificity of staining for MET-associated proteins was confirmed, as no fluorescent signal was detected when only a secondary conjugated antibody was employed to assess these structures

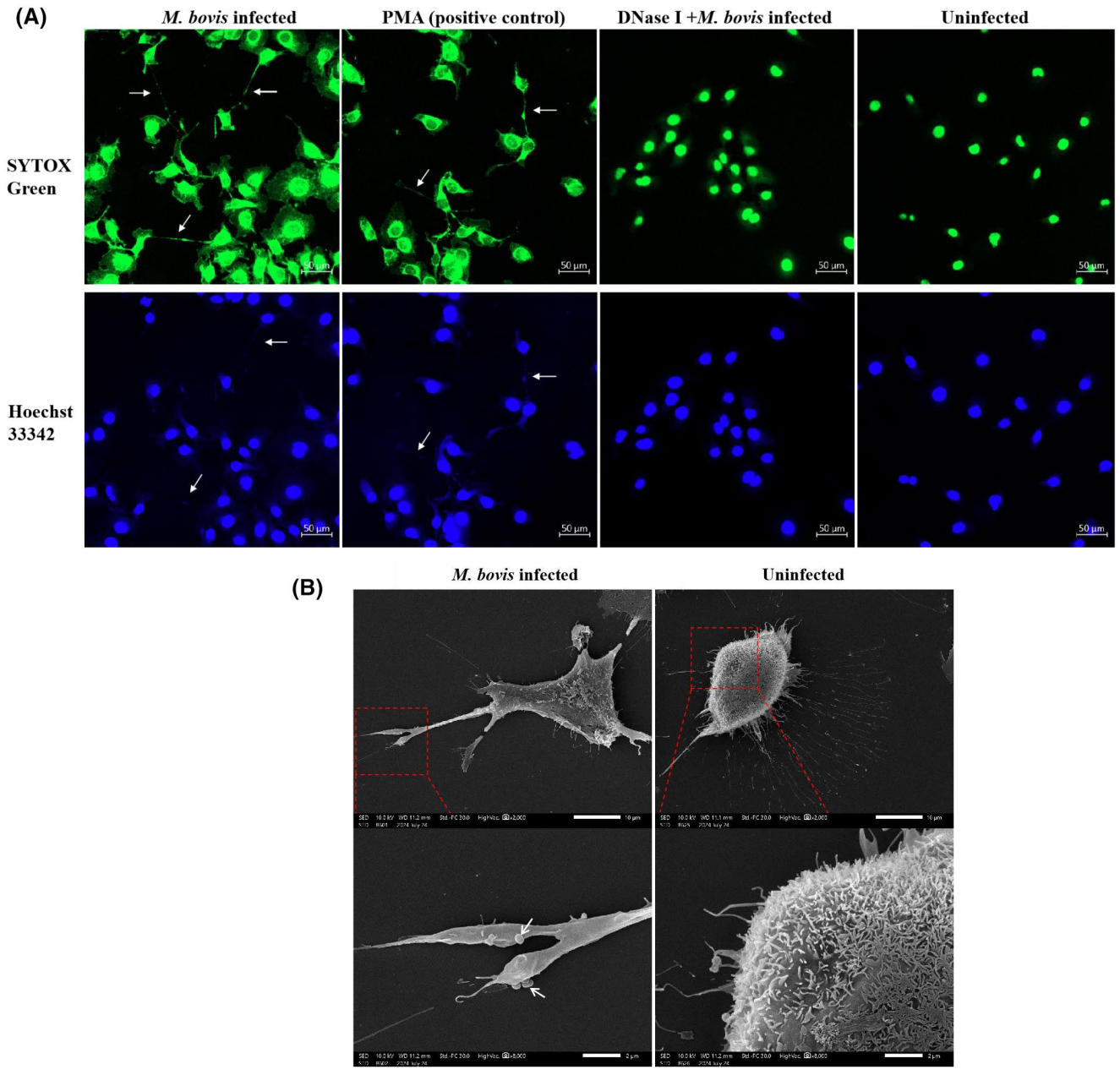

FIGURE 1 The release of METs from bovine macrophages in response to *M. bovis* infection. (A) BoMac cells were infected with *M. bovis* cells at an MOI 100 for 6 h. Some cells were pretreated with DNase I (180 U/mL) for 30 min before infection. PMA as a positive control. The cells were stained with SYTOX Green (green) for double-stranded DNA and Hoechst 33342 (blue) for condensed chromatin, then analyzed with confocal laser scanning microscopy. The results showed METs DNA structures (white arrow) in both infected and positive control groups while METs were not visible following DNase I treatment (scale bar = 50 µm). (B) Scanning electron microscopy demonstrated reticular extracellular structures (red square), released from *M. bovis*-infected bovine macrophages and appeared to trap *M. bovis* (white arrows), which were not observed in uninfected cells.

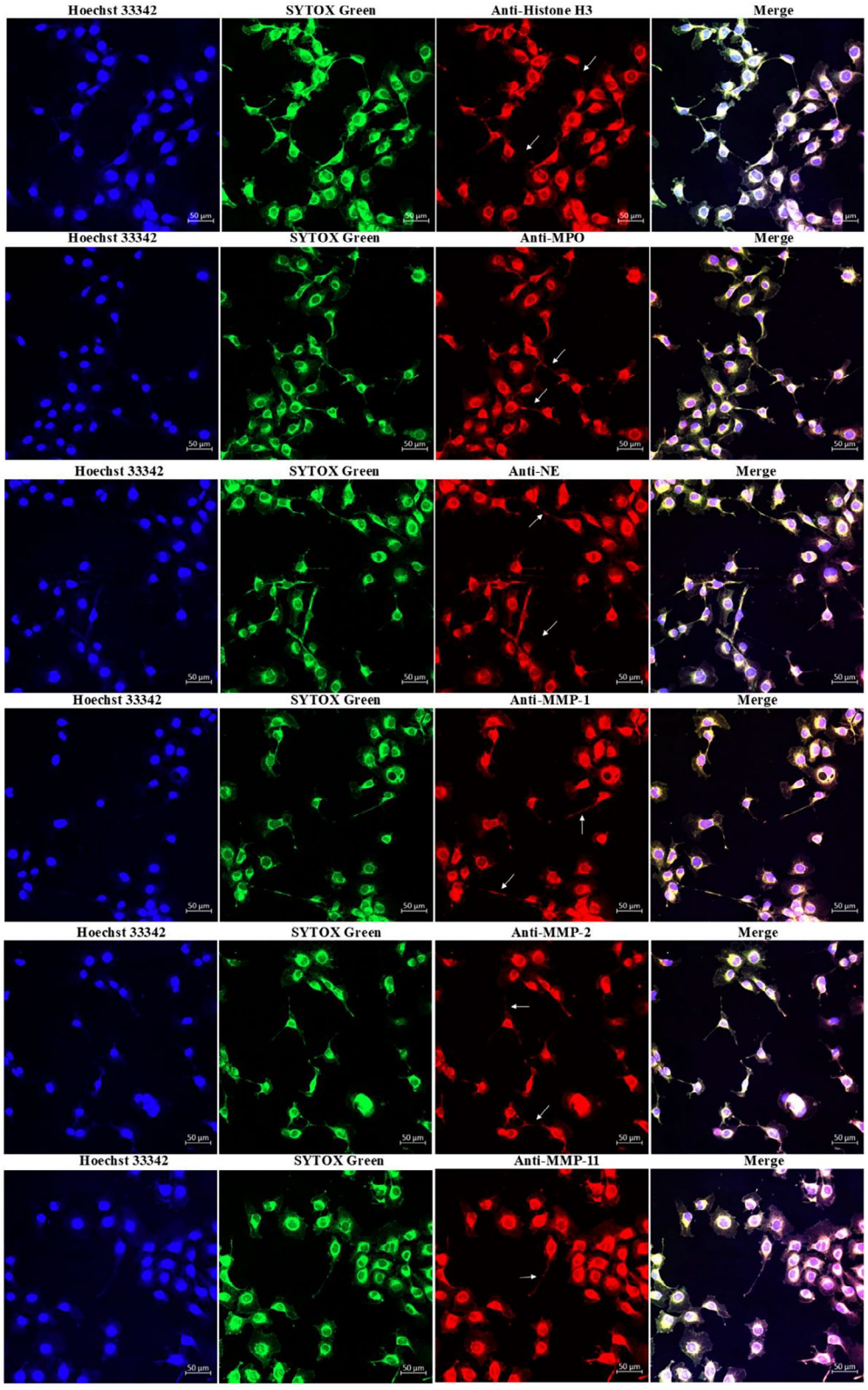

FIGURE 2 Confocal microscopy analysis of METs components formed in bovine macrophages following *M. bovis* infection. BoMac cells were incubated for 6 h with *M. bovis* at an MOI 100. Following incubation, the cells were stained with Hoechst 33342 (blue), SYTOX Green (green), and specific antibodies targeting histone H3, MPO, NE, MMP-1, MMP-2, or MMP-11, which were observed (red). The co-localization of histone H3, MPO, NE, MMP-1, MMP-2, and MMP-11 with extracellular DNA staining indicates the presence of these proteins within the MET structures (scale bar = 50 μm).

(Figure S1). Collectively, these findings imply that the observed structures were indeed METs released by bovine macrophages.

Additionally, quantifying MET release provides valuable insights into the immunological response of bovine macrophages upon exposure to *M. bovis*. Bovine macrophages were cocultured with *M. bovis* and the extent of METs associated-DNA was then quantified. The results indicated that the quantity of METs released was significantly higher in infected macrophage cells compared to uninfected ones, and treatment with DNase I resulted in the degradation of these extracellular structures (Figure 3A). The subsequent degradation of these structures further supports their extracellular nature and highlights the importance of DNA in the structural integrity of METs. Additionally, MET released by bovine macrophages was observed to be both dose-dependent and time-dependent in response to *M. bovis* infection (Figure 3B,C).

3.2 | Bovine METs contain CitH3, MPO, NE, and Cath G

The role of extracellular traps produced by macrophages is crucial for the innate immune response to infections, particularly for the trapping and killing of pathogens.[11–13] The concentrations of key proteins associated with these extracellular traps, specifically CitH3, MPO, NE, and Cath G, were quantitatively assessed using commercial ELISA kits. Notably, the results demonstrated that the concentration of these proteins in the supernatant from infected macrophages was significantly higher than that in uninfected controls, and this increase was dose and time-dependent (Figure 4). Such increases suggest that the macrophages were actively responding to *M. bovis* infection by releasing these proteins to form extracellular traps, which not only serve to immobilize the pathogens but also facilitate their degradation, thereby playing a pivotal role in the host's defense mechanism.

3.3 | METs formation dependent on NADPH oxidase

The formation of METs by macrophages can occur through two primary mechanisms: NADPH oxidase-dependent[14,15] and non-NADPH oxidase-dependent pathways.[20,26] To investigate whether the release of METs from bovine macrophages requires ROS generation, BoMac cells were pre-treated with DPI before infection, which resulted in a notable reduction in ROS production. In addition, treatment of uninfected cells with PMA led to a significantly higher production of ROS compared to their uninfected counterparts (Figure 5A). Additionally, the treatment of DPI also inhibited MET release from bovine macrophage cells, while PMA treatment of uninfected macrophages yielded MET release levels that were comparable to those observed in *M. bovis*-infected cells

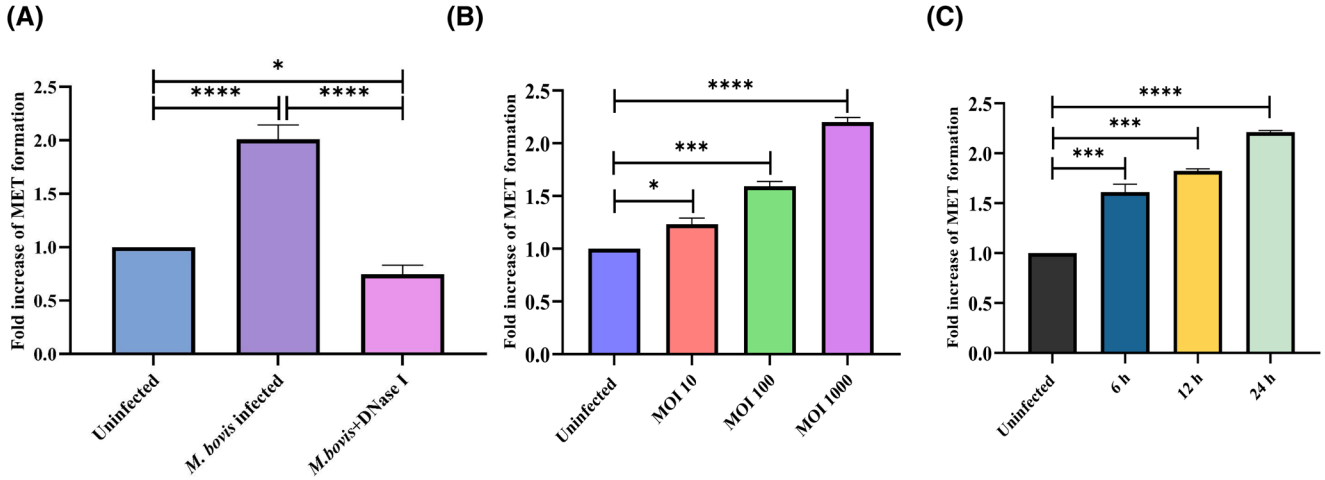

FIGURE 3 Quantification of METs released by bovine macrophage in response to *M. bovis* infection. (A) BoMac cells were co-cultured with *M. bovis* at an MOI 100 for 6 h. Following the incubation, the supernatant was analyzed using PicoGreen reagent to quantify DNA release in the presence of DNase I to degrade METs or without DNase I treatment. The results indicated obvious increase in the release of METs when compared to the uninfected control cells. Besides, the DNase I treatment effectively degraded the DNA-associated structures thereby confirming their nature as extracellular traps. (B) In a separate experiment, BoMac cells were co-cultured with *M. bovis* at varying MOIs (10, 100, and 1000) for 6 h, and the release of METs was increased in a dose-dependent manner. (C) BoMac cells were co-incubated with *M. bovis* at an MOI of 100 for different incubation times (6, 12, and 24 h), demonstrating the release of METs increased over time compared to uninfected control cells. Significances are indicated by asterisks: *$p < .05$, ***$p < .001$, ****$p < .0001$.

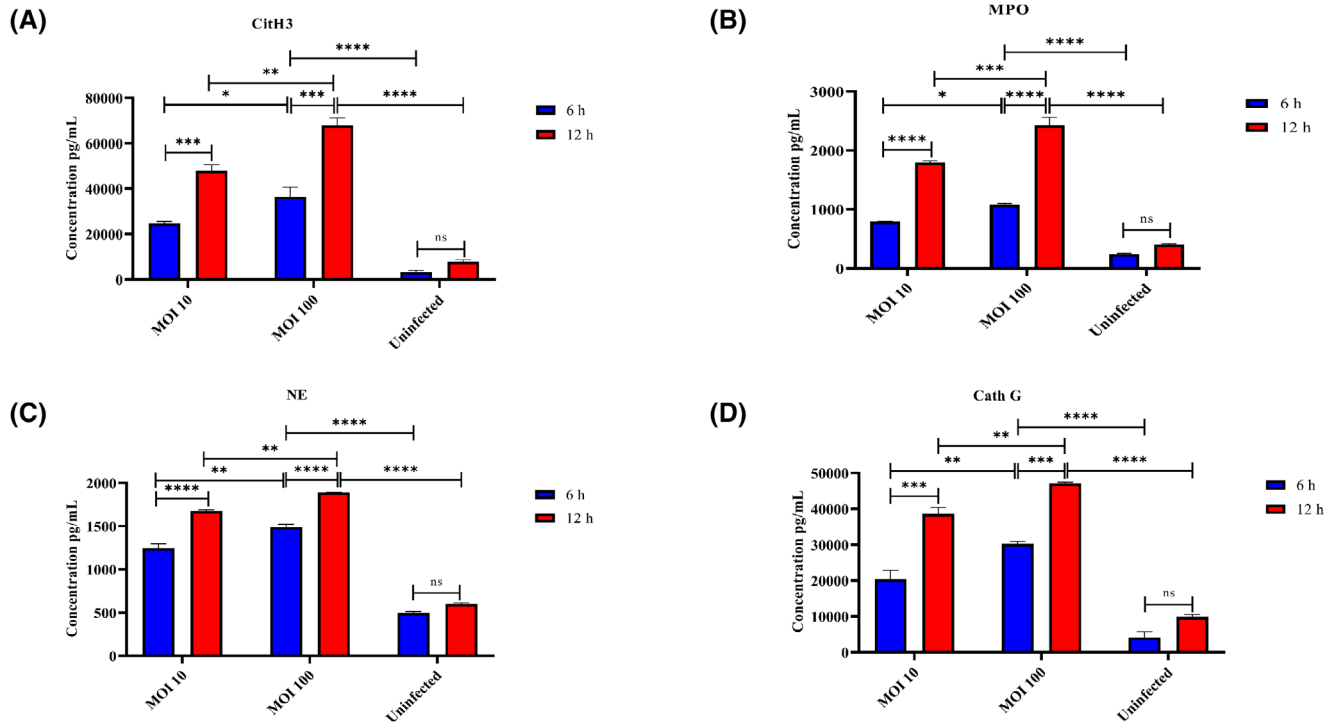

FIGURE 4 Quantitative assessment of proteins associated with extracellular traps in bovine macrophages following *M. bovis* infection. BoMac cells were co-cultured with *M. bovis* at various MOIs of 10, and 100 for 6 h, and 12 h. The concentrations of bovine CitH3 (A), MPO (B), NE (C), and Cath G (D) in the culture supernatant were measured using commercial ELISA kits. *M. bovis* infection increased the release of associated proteins compared to uninfected cells in dose and time manners. Significances are indicated by asterisks: *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. The ns, no significant difference ($p \geq .05$).

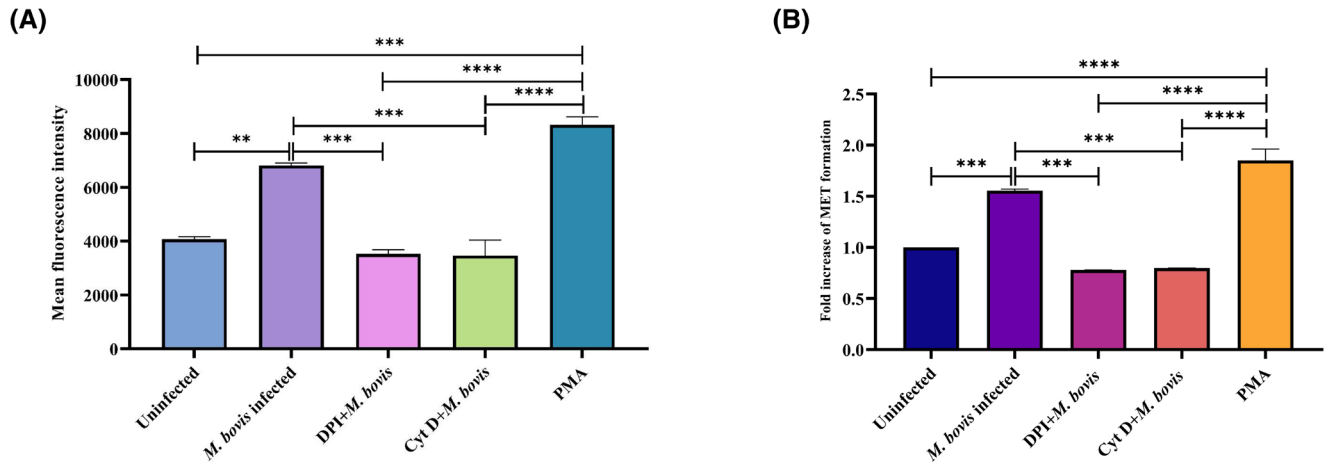

FIGURE 5 MET release from bovine macrophages requires ROS generation. BoMac cells were pretreated with NADPH oxidase inhibitor DPI, and Actin polymerization inhibitor Cyt D for 30 min, then infected with *M. bovis* at an MOI 100 for 6 h. Uninfected cells were also stimulated with 500 nM PMA to activate protein kinase C. (A) After incubation, treatment with DPI and Cyt D significantly inhibited ROS release, and the release of ROS from PMA-stimulated uninfected cells was notably elevated with some resemblance to the release observed in cells infected with *M. bovis*. (B) The MET release was quantified using PicoGreen as described in Legend 3 and treatment with DPI and Cyt D significantly inhibited MET release. Significances are indicated by asterisks: **$p < .01$, ***$p < .001$, ****$p < .0001$.

(Figure 5B). Moreover, it was observed that ROS production is increasing in correlation with both the dose of *M. bovis* and the time of exposure (Figure S2). To further clarify the connection between ROS production and MET release, bovine macrophages were pre-treated with Cyt D, which resulted in a decrease in intracellular ROS levels similar to DPI treatment (Figure 5A). Moreover, the confocal analysis revealed that untreated macrophages

exhibited high fluorescence intensity indicative of elevated ROS levels, while macrophages treated with DPI and Cyt D displayed substantially lower ROS fluorescence, confirming the effective inhibition of ROS release (Figure S3). This suggests that the use of these inhibitors may hinder MET release by obstructing ROS production and these results underscore the potential role of ROS in the host's defense mechanism against *M. bovis*, indicating that ROS production is essential for releasing METs from macrophages.

3.4 | *M. bovis* exposure influenced the cytotoxicity of bovine macrophages

To investigate whether *M. bovis* infection triggers cell death, BoMac cells were infected with *M. bovis* and evaluated for LDH release, which serves as an indicator of cellular death, alongside the use of a pan-caspase inhibitor (Z-VAD-FMK) and bovine IL-1β to assess apoptosis and pyroptosis respectively. After 6 h of infection, the supernatants from bovine macrophage co-cultured with *M. bovis* exhibited an increase in macrophage death in a dose-dependent manner, as evidenced by the elevated LDH levels (Figure 6A). Also, LDH levels exhibited a significant increase in a time-dependent manner following infection with *M. bovis* (Figure S4). This rise in LDH suggests enhanced cellular damage and cytotoxicity as the infection progresses, indicating that higher bacterial loads may lead to increased cellular stress or lysis. Notably, our results showed that MET formation in response to *M. bovis* infection was not inhibited by the pan-caspase inhibitor Z-VAD-FMK (Figure 6B), and there was no significant difference in bovine IL-1β release as measured by a commercial ELISA kit (Solarbio, China) between infected and uninfected cells (Figure 6C), indicating that these structures seem to be formed independently of apoptotic and pyroptotic processes. This suggests that METs formation is an alternative mechanism of cell death, separate from apoptosis, and pyroptosis.

3.5 | METs formation mediates *M. bovis* killing

To demonstrate the essential function of macrophage-derived extracellular traps in promoting bacterial killing, various inhibitors targeting NADPH oxidase, phagocytosis, NE, P38 MAPK, MPO, and calcium channels were employed in this experiment. Notably, following the pretreatment with these inhibitors, more bacterial colony-forming units were recovered from the treated cocultures following 6 h of infection, particularly in the presence of DNase I (Figure 7). Furthermore, the pretreatment with Cyt D or DNase I indicated a significant increase in the number of viable *M. bovis* cells over

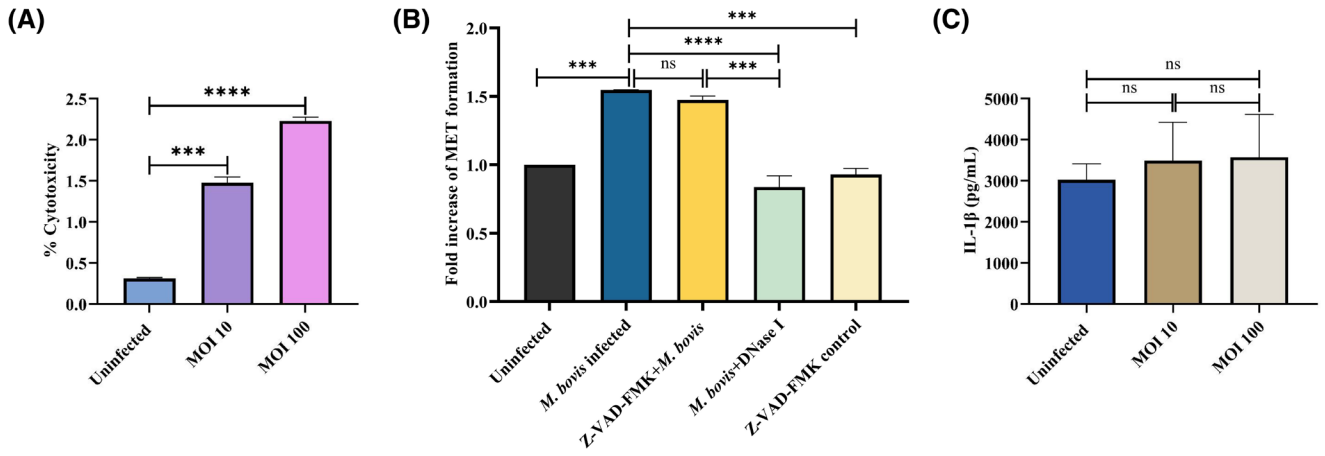

FIGURE 6 *M. bovis* induces cytotoxicity independent of apoptosis and pyroptosis. BoMac cells were maintained and co-cultured with *M. bovis* at an MOI of 10 and 100 for 6 h. Supernatants from co-culture were assayed for LDH release to calculate the percentage of cytotoxicity. (A) Cell death in bovine macrophage cells after 6 h of *M. bovis* infection, with the extent of cell death escalating concerning the dosage compared to uninfected cells. (B) Bovine macrophages were preincubated with 200 μM the pancaspase inhibitor Z-VAD-FMK for 30 min at 37°C before *M. bovis* infection at an MOI 100. Following 6 h of infection, the release of METs was measured as described in Legend 3. The results obtained with Z-VAD-FMK showed no significant effect on MET release when compared to the untreated infected cells. (C) bovine macrophages were maintained and cocultured with *M. bovis* at an MOI of 10 and 100 for 6 h. Supernatants from co-culture were assayed to calculate the concentration of IL-1β release to indicate pyroptosis. The results showed no significant difference in IL-1β release between infected and uninfected groups. Significances are indicated by asterisks: ***$p < .001$, ****$p < .0001$. The ns, no significant difference ($p \geq .05$).

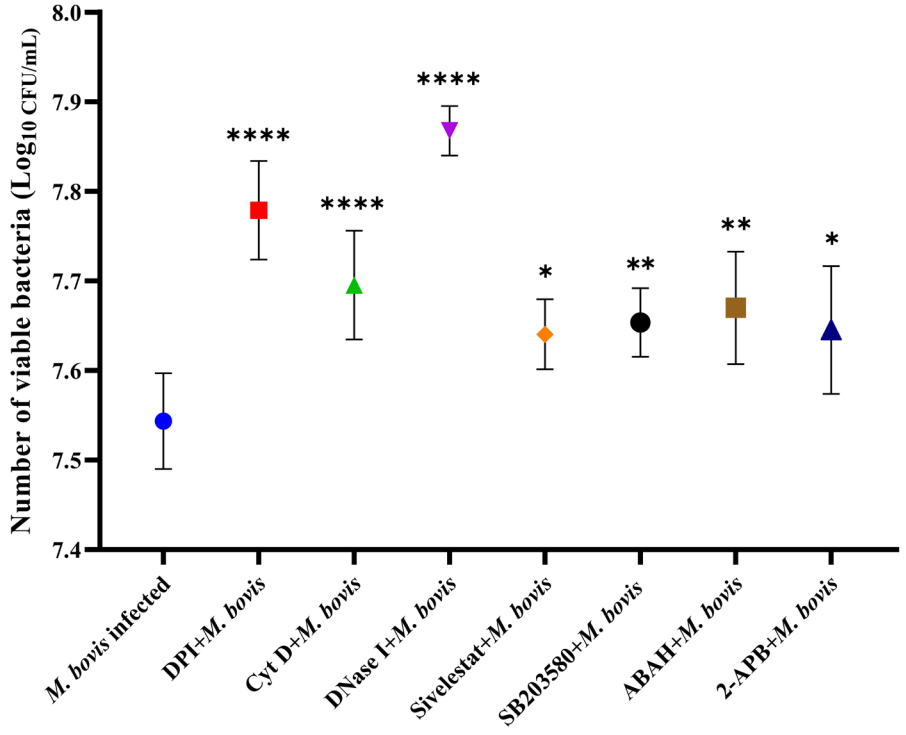

FIGURE 7 Assessment of the role of METs in *M. bovis* killing. BoMac cells were preincubated with various inhibitors that target NADPH oxidase, phagocytosis, DNA structure, neutrophil elastase, p38 MAPK, myeloperoxidase, and calcium channels for 30 min. Following this, cells were co-cultured with *M. bovis* at an MOI 100 for 6 h. Supernatant from the co-culture was collected, and the resultant pellet was resuspended and plated onto PPLO agar plates. The subsequent incubation revealed an increased number of colony-forming units (CFUs) from the treated co-cultures, suggesting obvious reduction in bacterial killing efficiency which may be attributed to the disruption of extracellular traps. Significances are indicated by asterisks: *$p < .05$, **$p < .01$, ****$p < .0001$.

24 h of the co-incubation (Figure S5), whereas a greater reduction in the viable *M. bovis* cells was observed after 24 h compared to 6 h post-infection. Additionally, live–dead bacterial staining of BoMac infected with *M. bovis* revealed dead bacterial cells close to MET fibrils (Figure S6). Together, these data highlight the role of macrophage-derived extracellular traps in killing *M. bovis* infection.

4 | DISCUSSION

M. bovis presents a complex challenge to animal health because of its unique characteristics and ability to cause severe disease in cattle. As a bacterium free of a cell wall, it resists many conventional antibiotics, making treatment and disease control particularly challenging.[5,7] The complex nature of *M. bovis* infection is further underscored by the multifaceted immune response mounted by the host. Macrophages play a crucial role in the innate immune response, METs have recently been reported and recognized for their potential immune functions, which encompass the trapping and killing of microorganisms as well as the degradation of bacterial products.[10–13]

In the current study, we present the characterization of METs against *M. bovis* infection. Our results demonstrated that bovine macrophages can effectively form METs in response to *M. bovis*, which was characterized by the release of DNA fibrils intertwined with cellular proteins including histones H3, MPO, NE, and various MMPs, such as MMP-1, MMP-2, and MMP-11. The present results are supported by earlier studies demonstrating that bovine macrophages generate a network of DNA fibrils associated with histones following exposure to *Mannheimia haemolytica*.[14] In another study, human placental macrophages released METs composed of DNA along with histones, NE, MPO, and MMPs in response to *Streptococcus agalactiae*.[15] Similarly, bone marrow-derived macrophages (BMDMs) and J774.A1 macrophages have been shown to release METs containing histones H3, MPO, and NE when infected with *Clostridium perfringens*.[27] Comparable compositions of METs have also been observed in THP-1 macrophage-like cells infected with *Mycobacterium massiliense*.[30] This process highlights a crucial aspect of the innate immune response in which macrophages utilize METs as a novel mechanism to combat bacterial infections. Moreover, we examined the extracellular DNA content in the supernatant and observed a significant increase in extracellular DNA in macrophages infected with *M. bovis*. Collectively, these

findings imply that *M. bovis* could promote the generation of METs, highlighting a potential mechanism by which this pathogen interacts with the host immune response. This phenomenon not only reflects the bovine macrophage's ability to adapt their immune responses to various pathogens but also points to the potential role of METs in the broader context of host-pathogen interactions. It is worth noting that METs formation in response to *M. bovis* in vivo models and physiological contexts necessitates further research.

NADPH oxidase is a crucial enzyme complex that plays a significant role in the immune response, leading to the generation of ROS through the activation of NADPH oxidase.[11,12] Recent studies indicated that some bacterial stimuli could trigger the release of METs, which is dependent on the activation of NADPH oxidase. This process appears to involve an increase in intracellular ROS concentrations, which serves as a signaling mechanism for the release of these traps.[14,15,23,34,35] Thus, we examined whether the NADPH oxidase-ROS pathway was involved in *M. bovis*-triggered METs formation. As a result, increased ROS production was detected in *M. bovis*-stimulated macrophages. Furthermore, pretreatment with DPI significantly decreased bacteria-triggered ROS production and MET release, suggesting that NADPH oxidase-ROS contributed to METs formation. The findings align with other studies revealing the formation of METs in response to other bacterial infections, reinforcing the importance of ROS acts as key signaling molecules in the context of METs formation and pathogen interaction.[14,15,23]

In contrast, previous reports described the formation of METs-like structures in murine J774A.1 and murine peritoneal macrophages in response to *E. coli* and *Candida albicans* in an NADPH oxidase-independent manner, in which the application of the NADPH oxidase inhibitor DPI did not result in a significantly reduced production of METs.[20] Another study demonstrated that cord-forming *Mycobacterium tuberculosis* was capable of inducing METs in human monocyte-derived macrophages (hMDMs) in a manner reliant on Early Secreted Antigenic Target 6 (ESAT-6), a key virulence factor of *M. tuberculosis*.[29] This finding highlights the specific role of ESAT-6 in triggering METs formation in response to *M. tuberculosis* infection, indicating the strategic utilization of virulence factors by the pathogen to modulate host immune responses. Furthermore, MET formation was enhanced in *Porphyromonas gingivalis*-pretreated murine peritoneal macrophages, which might be related to the increased levels of intracellular Ca^{2+} and independent of ROS, and the inhibition of intracellular Ca^{2+} resulted in a decrease in the levels of extracellular DNA.[26] Further confirmation demonstrated that pretreatment of bovine macrophage cells with the actin cytoskeletal inhibitor Cyt D significantly inhibited MET release. This inhibition is likely due to disruption of the actin cytoskeleton, which plays a crucial role in the trafficking of the cellular NADPH oxidase complex. Impeding the proper trafficking of this complex can lead to a notable decrease in ROS production. These findings align with the existing literature that highlights the importance of actin cytoskeleton inhibitors in preventing the release of MET.[14,15] The inhibition of MET release and its subsequent impact on ROS production suggest a potential mechanism whereby cytoskeletal dynamics influence redox signaling in bovine macrophage cells. In contrast, exposure to *H. somni* cells induced METs formation in bovine monocyte-derived macrophages and was unaffected by the presence of Cyt D.[28] Moreover, RAW 264.7 murine macrophages and THP-1-derived human macrophages displayed the ability to produce METs in response to *E. coli* hemolysin (*E. coli* HLY), and the METs formation in both macrophage types was not inhibited by the addition of Cyt D, suggesting that the process of MET formation in response to *E. coli* HLY may not rely on actin cytoskeletal dynamics.[14] These observations suggest a mechanism that overrides the influence of the actin cytoskeleton on MET formation, indicating that the process may involve alternative pathways or signaling mechanisms that do not require actin remodeling.

Moreover, our findings revealed a slight increase in LDH during *M. bovis* infection in both time and dose-dependent, which is consistent with previous reports indicating that the release of MET is associated with cell death.[10,14,15] Conversely, other studies have indicated that METs formation is acknowledged without a substantial impact on LDH release, implying that METs may occur independently of cellular lysis.[20,27] Interestingly, the formation of METs was observed to be independent of apoptosis, and pyroptosis as demonstrated by the ineffectiveness of the pan-caspase inhibitor Z-VAD-FMK, where the DNA release appeared to be negligible in both treated and untreated cells after 6 h of infection, and these results showed to be similar with another finding.[14] Additionally, there were no significant differences observed in the concentration of bovine IL-1β release between the infected and uninfected cells after 6 h of co-incubation, further suggesting the exclusion of the pyroptotic pathway, a result that aligns with previous findings.[15] This suggests that the mechanisms leading to MET formation differ from those involved in apoptosis and pyroptosis. This independence may provide insights into how macrophages can maintain their bactericidal functions while simultaneously undergoing the processes that lead to MET formation. While *M. bovis* can induce the formation of METs, it employs different mechanisms to induce cytotoxicity. The cytotoxicity associated with *M. bovis* primarily arises from its metabolic byproducts, such as hydrogen peroxide, rather than direct contact with host cells, it can lead to the activation of apoptosis, depending on the context of infection and immune response and subsequently

the release of LDH. Moreover, our observation was characterized by low LDH from activated macrophages into the extracellular environment in response to *M. bovis* infection. This little LDH release is also described in a previous study, which showed that *Mannheimia haemolytica* induces METs formation in the cytotoxicity test.[14]

Additionally, our study highlighted the intricate relationship between MET formation and their bactericidal functions in response to *M. bovis* infection. In our study, using inhibitors targeting NADPH oxidase, phagocytosis, DNA structure, NE, P38 MAPK, MPO, and calcium channels led to a significant decrease in both MET production and the overall bactericidal activity against *M. bovis*. The considerable increase in the number of viable bacteria recovered from the treated co-cultures, particularly in the presence of DNase I, effectively dismantles the structural framework of METs, thereby allowing for the resurgence of viable bacteria. Furthermore, the number of *M. bovis* cells was significantly increased in the co-incubated cultures that pretreated with phagocytosis inhibitor (Cyt D) or DNase I over 24 h, which further emphasized the critical function of METs in bacterial killing. This function was further validated by confocal microscopy analysis, which revealed dead *M. bovis* cells adjacent to MET fibers. This suggests that the processes of MET formation and bacterial elimination are closely linked, potentially representing a coordinated response to infection. Furthermore, these results align with previous research demonstrating the ability of macrophages to engage in trap formation for their protective function.[27] Previous reports also have found significantly more bacterial-forming colonies were recovered from co-cultures treated with DNase I, suggesting that METs structure has bactericidal activity and eliminating these structures with DNase I impaired bacterial killing.[15,36] This indicates that the bactericidal efficacy of METs is not merely a byproduct of macrophage activation but is integral to the overall immune response against *M. bovis*. The compromised ability of bovine macrophages to form METs when their bactericidal functions are inhibited indicates that these two processes are interconnected, emphasizing the importance of METs as a defense mechanism against *M. bovis*.

In conclusion, our study described that bovine macrophages are activated in response to *M. bovis* infection through the release of METs, highlighting their critical role in the host defense mechanism. These MET structures contain cellular DNA and associated proteins including histones, MPO, NE, and various MMPs. The formation of METs was found to be dependent on the activity of NADPH oxidase, which is essential for the effective killing of *M. bovis*. Understanding the mechanisms underlying MET formation and their bactericidal properties could pave the way for novel interventions aimed at controlling *M. bovis* infection and improving cattle health.

AUTHOR CONTRIBUTIONS

Shengli Chen conceived and designed this research. Yuefeng Chu supervised the study. Ahmed Adel Baz and Shengli Chen performed research and drafted the manuscript. Huafang Hao contributed to reagents, materials, and instrumentality. Xiangrui Jin, Shimei Lan, Zhangcheng Li, Shanyu Jin, Yifan Zhang contributed to reagents and materials. All the authors read and approved the final manuscript.

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DISCLOSURES

The authors have no competing interests to declare.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the articles and the supplementary material.

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SUPPORTING INFORMATION

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

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