Chapter 23
Repurposing of Doxycycline to Attenuate Influenza Virus Pathogenesis Via Inhibition of Matrix Metalloproteinases in Neutrophils

T. Narasaraju, Charlene Fong, Sunil K. Lal, and Vincent T. K. Chow

Abstract Influenza viruses constitute a significant threat to public health worldwide over many decades, and continue to inflict significant morbidity and mortality. Previous studies show that infection of mice with mouse-adapted influenza A/Aichi/2/1968(H3N2) passage 10 (P10) virus can elicit exuberant inflammatory responses in the lungs with extensive infiltration of macrophages and neutrophils (which are sources of gelatinases) that contribute to pulmonary damage. The lungs of mice with severe influenza pneumonitis also reveal extensive neutrophilic infiltration, neutrophil extracellular traps (NETs), alveolar damage, heightened viral load, and pathologic features of acute respiratory distress syndrome (ARDS). Excessive neutrophil and matrix metalloproteinase (MMP) activities are implicated in the pathogenesis of acute lung injury (ALI) and ARDS. Hence, an objective of this study was to investigate the production of MMP-2 and MMP-9 in neutrophils differentiated from the MPRO murine pro-myelocytic cell line, following infection with mouse-adapted influenza H3N2 virus. Another objective was to investigate the effects of doxycycline on expression of MMP-2 and MMP-9 at transcriptional and translational levels in neutrophils in the context of influenza virus infection. MMP-2 and MMP-9 production and gelatinase activity were found to be induced by infection of differentiated neutrophils with mouse-adapted influenza H3N2 virus.

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Furthermore, doxycycline treatment was able to abrogate this increase in MMP-2 and MMP-9 expression and gelatinase activity. Given that excessive neutrophil infiltration and uncontrolled neutrophil gelatinase activity in pulmonary tissues play pivotal roles in the pathogenesis of ALI and ARDS, this study offers insights into the mechanism of excessive MMP-2 and MMP-9 production and activity in neutrophils during influenza virus infection. Thus, repurposing doxycycline as an MMP inhibitor represents a potential therapeutic strategy to ameliorate influenza-associated pulmonary injury by targeting MMPs, gelatinase production and activity in neutrophils. Doxycycline therapy (alone or in combination with other drugs) has also been considered and explored for the management of other infectious diseases such as coronavirus infections and tuberculosis.

Keywords Influenza · H3N2 virus · Neutrophils · MPRO cell line · Gelatinases · Matrix metalloproteinases · MMP-2 · MMP-9 · MMP inhibitor · Doxycycline · Drug repurposing

Influenza constitutes a significant threat to public health worldwide over many decades. Influenza A viruses cause significant morbidity and mortality over widespread geographical distances (Ivan et al. 2020). The world has experienced several major pandemics caused by influenza A viruses, including the 1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1 strains. Moreover, outbreaks due to highly pathogenic avian influenza viruses (such as H5N1 and H7N9) have occurred in many countries all over the world (Chow et al. 2008; Sakharkar et al. 2009; Zhou et al. 2018).

23.1 Neutrophils and Influenza Virus-Induced Lung Injury

Influenza virus infection induces the mobilization of neutrophil effector systems—the virus and virus-infected cells are engulfed and phagocytosed by neutrophils to form a lysosome, leading to neutrophil activation. The neutrophil then undergoes a respiratory burst, leading to reactive oxygen species (ROS) generation, and release of contents from the azurophil and specific granules into the phagolysosome—thus creating a toxic microenvironment that kills the virus (Smith 1994; Hashimoto et al. 2007). In addition, neutrophils are also implicated in the inflammatory response that characterizes acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) during severe influenza virus infection (Abraham 2003; Quispe-Laime et al. 2010; Narasaraju et al. 2011).

ALI is a complex clinical syndrome which is characterized by pulmonary edema, capillary leakage, and pneumonia. ARDS is the most severe form of ALI, and is characterized by diffuse alveolar damage and leukocytic inflammation of the lung parenchyma, epithelial damage, and hypoxemia. Neutrophil-predominant host inflammatory responses are essential for the development of ALI and ARDS. The

inappropriate release of the proteolytic enzymes contained in the neutrophil granules into the extracellular space can cause host tissue injury via proteolytic activity and release of ROS. This occurs in the case of excessive neutrophil infiltration, premature activation of neutrophils during migration, and/or formation of neutrophil extracellular traps or NETs (Narasaraju et al. 2011). During neutrophil migration into the lung airways, uncontrolled activation of neutrophils may occur in response to certain microbial or host-derived stimuli—excessive release of the proteolytic enzymes then culminates in damage and sloughing of pulmonary epithelial and endothelial cells (Ware and Matthay 2000; Xu et al. 2006).

In the early phase of ALI and ARDS, the release of pro-inflammatory mediators from monocytes, alveolar macrophages, and vascular endothelial cells results in neutrophil migration and sequestration. Activated neutrophils release terminal effectors such as ROS, neutrophil elastases, and matrix metalloproteinases (MMPs) to cause lung tissue injury, leading to leakage of proteinaceous fluid into the alveolar spaces and airways. The intense inflammatory response leads to pulmonary endothelial and epithelial cell damage, and disruption of the capillary-alveolar barrier function (Taubenberger and Morens 2008).

The MPRO cell line is derived from murine bone marrow cells via the transduction of a dominant-negative retinoic acid receptor. The MPRO line is dependent on granulocyte-macrophage colony-stimulating factor (GM-CSF), is arrested at a pro-myelocytic stage, and can morphologically differentiate into neutrophils following treatment with 10 μM all-*trans* retinoic acid (ATRA)—rendering it a useful cell line for in vitro neutrophil studies (Lawson et al. 1998; Johnson et al. 1999).

23.2 Functions of Matrix Metalloproteinases

MMPs carry out essential functions in the form of extracellular matrix (ECM) degradation, which is necessary for ECM turnover and tissue remodeling in various processes such as cell migration, embryonic development, and angiogenesis. MMPs have a wide range of targets, including non-ECM proteins such as growth factors and various cytokines—thus also affecting various processes in cellular proliferation, cell migration, and apoptosis. MMPs are naturally regulated at various levels including gene expression, zymogen activation, mRNA stability, enzyme inactivation, and compartmentalization. Most MMPs are inducibly transcribed, with the exception of MMP-2 which is constitutively expressed (Sternlicht and Werb 2001; Parks et al. 2004; Snoek-van Beurden and Von den Hoff 2005).

Within the MMP family, gelatinases are considered an integral subclass due to their ability to degrade major constituents of the basement membrane, including type IV collagen, laminin, and gelatin. Gelatinases include MMP-2 (gelatinase A) and MMP-9 (gelatinase B). MMP-2 can also digest type I, II, and III collagens, while MMP-9 can digest type V collagen (Murphy and Crabbe 1995; Sternlicht and Werb 2001; Parks et al. 2004). However, the two gelatinases differ in certain ways. MMP-2 is synthesized by a broad range of cells, including alveolar epithelial cells, endothelial cells, fibroblasts, macrophages, and dendritic cells. MMP-9 is mainly

produced by inflammatory cells such as neutrophils, monocytes, macrophages, and lymphocytes (Murphy and Crabbe 1995; Corbel et al. 2000). Both gelatinases are differentially regulated at transcriptional and extracellular levels. MMP-9 is transcriptionally regulated by cytokines and growth factors, whereas MMP-2 is only mildly responsive to these molecules.

Given that various MMPs, especially MMP-2 and MMP-9, are involved in pulmonary pathology, MMP inhibitors may be therapeutically exploited for ameliorating influenza-induced immunopathology. MMP inhibitors function in several ways, such as by inhibiting RNA synthesis, chelating zinc, and binding to the MMP active site.

MMP inhibitors have been shown to be effective therapeutic agents in animal models in which they can prevent pathologic changes of emphysema and ARDS (Carney et al. 2001). However, the nonspecificity of MMP inhibitors may lead to dose-limiting adverse effects. Periostat® or doxycycline hyclate is an example of MMP inhibitor which is clinically approved for use in periodontal disease (Corbitt et al. 2007; Fingleton 2007).

23.3 Repurposing Doxycycline to Mitigate Influenza-Induced Tissue Injury

Doxycycline is a broad-spectrum tetracycline antibiotic whose mode of action is to prevent access of acyl transfer-RNA to the acceptor site on the mRNA-30S ribosomal subunit complex, thus inhibiting the elongation process of bacterial protein synthesis. It possesses bacteriostatic, antiprotozoal, and antihelmintic effects (Smith and Cook 2004; Batty et al. 2007). Doxycycline also acts as a nonspecific MMP inhibitor, and its roles in MMP-2 and MMP-9 inhibition have been extensively studied. Some proposed mechanisms of MMP inhibition by doxycycline include downregulating MMP gene expression, chelating to zinc at the catalytic site, inhibiting pro-MMP activation, or scavenging of ROS (Curci et al. 2000; Cena et al. 2010; Chang et al. 2010). Ng et al. (2012) showed that oral administration of a low dose of doxycycline not only reduces inflammation following influenza virus infection in mice but also leads to significant reduction of host lung injury by minimizing the destruction of pulmonary epithelium and endothelium, and by decreasing leakage of proteinaceous material into the airways. Influenza-induced host lung injury is effectively improved by lower doses of doxycycline. However, higher doses of the drug substantially reduce inflammation to render viral clearance inefficient, thus resulting in high virus load, direct cytopathic effects on the host cells, and eventually aggravating pulmonary damage. It is thus vital to use an optimal (but not excessive) dosage of doxycycline to mitigate inflammation and gelatinase activities in influenza virus infection to alleviate acute lung injury.

23.4 Study Objectives

The first objective of this study was to analyze the in vitro production of MMP-2 and MMP-9 gelatinases following influenza H3N2 virus infection of neutrophils. The main rationale of this aim was predicated on previous in vivo studies showing that mouse-adapted influenza A/Aichi/2/1968(H3N2) passage 10 (P10) virus infection elicits an exaggerated inflammatory response in the lungs with extensive infiltration of macrophages and neutrophils (which are sources of gelatinases) that contribute to pulmonary damage (Narasaraju et al. 2009). Subsequent studies examining severe influenza pneumonitis in mice revealed excessive neutrophilic infiltration, NETs, alveolar damage, heightened viral load, and ARDS-like pathology (Narasaraju et al. 2011). This objective was to focus on the production of MMP-2 and MMP-9 in neutrophils differentiated from the MPRO murine pro-myelocytic cell line, following infection with mouse-adapted influenza H3N2 virus.

The second objective was to investigate the effects of doxycycline on expression of MMP-2 and MMP-9 at transcriptional and translational levels in neutrophils in the context of influenza virus infection. Since excessive neutrophil and MMP activities are implicated in the pathogenesis of ALI and ARDS (Narasaraju et al. 2011), doxycycline may serve as a potential therapeutic to ameliorate host damage caused by neutrophil gelatinases during severe pulmonary influenza infection.

23.5 Materials and Methods

Mouse lung-adapted influenza A/Aichi/2/1968(H3N2) virus was prepared as described previously (Narasaraju et al. 2009; Ivan et al. 2012). One batch of BALB/c mice was infected with mouse lung-adapted passage 14 (P14) H3N2 virus, and their lung homogenates harvested to generate passage 15 (P15) virus. P15 virus was then used for infecting a larger batch of mice to generate passage 16 (P16) virus. Automated cycle sequencing of the P16 virus hemagglutinin (HA) and nonstructural 1 (NS1) genes amplified by reverse transcription-polymerase chain reaction (RT-PCR) confirmed the presence of mutations previously identified in P10 virus (i.e., G218E in HA and D125G in NS1). Virus plaque assay using MDCK cells was performed for viral quantification of P16-infected lung homogenates which were used for infection of MPRO cells.

MPRO cell culture and differentiation into neutrophils were carried out as described previously (Ivan et al. 2013). Total cell count and differential cell count were determined using trypan blue exclusion and Giemsa staining, respectively. MPRO cells were treated with 10 μM ATRA to induce differentiation into neutrophils. Figure 23.1 shows that MPRO cell differentiation peaked at day 6, during which the majority of differentiated cells acquired neutrophil-like morphologic features such as multilobed or segmented nucleus with granulated cytoplasm (Fig. 23.2).

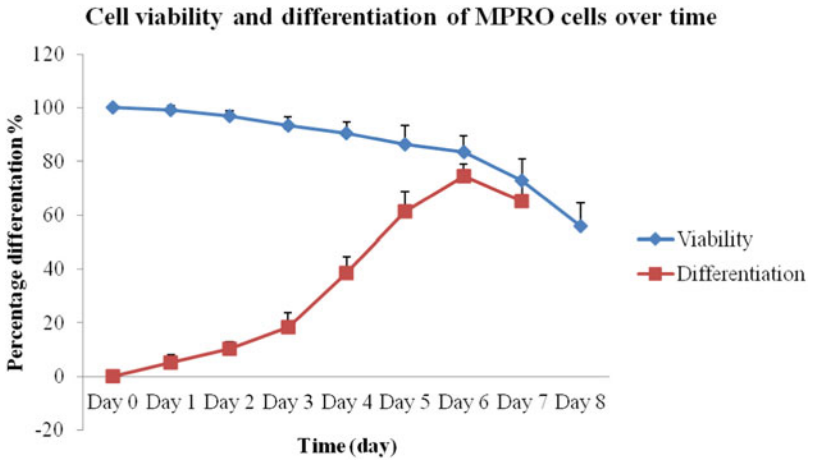

Fig. 23.1 Comparison of percentage of MPRO cell viability and cell differentiation over time. Cells were subjected to trypan blue staining for cell viability, and to Giemsa staining for neutrophil differentiation, and counted by microscopy. The graphs display the mean and standard deviation for each time-point ($n = 12$ each). Day 0 indicates time of addition of all-trans retinoic acid (ATRA). Neutrophil differentiation peaked at day 6, while cell viability decreased progressively over time

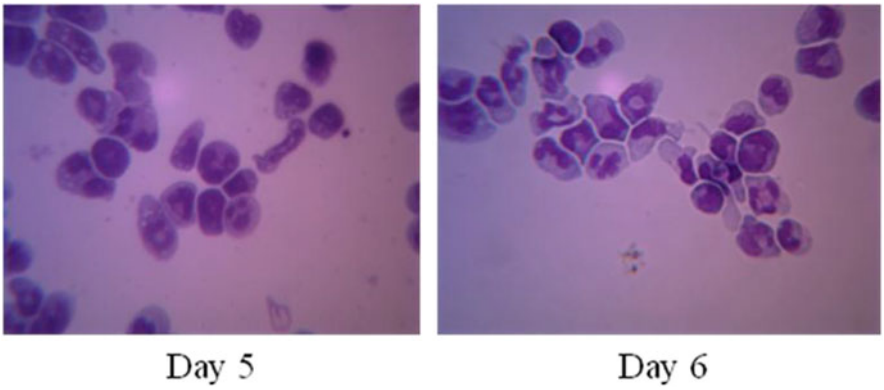

Fig. 23.2 Giemsa staining of differentiating MPRO cells at days 5 and 6 after the addition of ATRA. Representative images to exemplify MPRO cells differentiating and acquiring neutrophil-like morphologic characteristics such as segmented nucleus and granulated cytoplasm on day 5 and especially on day 6. Differentiated MPRO cells at day 6 following ATRA treatment were used for influenza virus infection

MPRO cells at day 6 following ATRA treatment were used for virus infection at multiplicity of infection (MOI) of 0.1. Three million cells were seeded in each well of 24-well plates. The four experimental groups were: control uninfected and untreated cells; infected but untreated cells; uninfected cells treated with 50 μM doxycycline (DOX); infected cells treated with 50 μM doxycycline. For the infected

and DOX-treated group, neutrophils were incubated at 37 °C for 1 h before addition of virus. Cells were incubated at various time-points of 1, 7, and 10 h postinfection before harvesting samples for analyses.

Cell pellets were subjected to RNA extraction followed by reverse transcription and real-time quantitative PCR using SYBR Green marker to analyze mRNA levels of MMP-2 and MMP-9, as described previously (Ng et al. 2012).

Culture supernatants were harvested and subjected to Western blot analyses to evaluate expression levels of MMP-2 and MMP-9 proteins; and to gelatinase zymography to assess gelatinase activity, as described previously (Ng et al. 2012).

Statistical analyses. Results were expressed as mean value ± standard deviation. Statistical analyses and comparisons of samples were performed using Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

23.6 Results and Discussion

In this study, it was hypothesized that influenza H3N2 virus could induce the production and gelatinase activity of MMP-2 and MMP-9 in neutrophils in vitro. Another hypothesis was that doxycycline could inhibit the expression and activity of MMP-2 and MMP-9 at protein and transcriptional levels.

23.6.1 Mouse-Adapted Influenza H3N2 P16 Virus Infection of MPRO Neutrophils Enhances MMP-2 and MMP-9 Protein Expression, Gelatinase Activity, and MMP-9 Transcription

Western blot analyses showed that mouse-adapted influenza A/H3N2 P16 virus infection of MPRO neutrophils was indeed able to induce and elevate protein expression of both MMP-2 and MMP-9 at all time-points. However, this degree of enhanced expression of MMP-2 and MMP-9 over time was somewhat different.

Furthermore, gelatinase zymography also revealed that influenza H3N2 P16 virus could also induce an overall significant increase in both MMP-2 and MMP-9 gelatinolytic activities.

Real-time qRT-PCR also demonstrated that H3N2 P16 virus could significantly induce MMP-9 mRNA expression, which exhibited a time-dependent increase in transcriptional response to influenza virus infection.

The above findings are summarized in Tables 23.1 and 23.2.

Table 23.1 Comparison of percentage change in MMP-9 expression by Western blotting, gelatinase zymography, and real-time quantitative RT-PCR in test versus control neutrophils at different time-points

	Time-points	Infected versus uninfected	Uninfected control	Infected
		No DOX	DOX versus No DOX	DOX versus No DOX
Western blot (WB)	1 h	↑ 87%*	↓ 60%*	↓ 20%*
	7 h	↑ 26%*	↓ 60%*	↓ 23%*
	10 h	↑ 64%*	↓ 23%*	↓ 48%*
Gelatinase zymography (GZ)	1 h	↑ 30%*	↓ 80%*	↓ 40%*
	7 h	↑ 150%*	NS	↓ 60%*
	10 h	↑ 80%*	↓ 10%*	↓ 50%*
Real-time quantitative RT-PCR	1 h	↑ 30%*	↓ 10%	↓ 8%
	7 h	↑ 70%*	↓ 20%*	↓ 20%*
	10 h	↑ 100%*	↓ 40%*	↓ 40%*

First, comparison of influenza virus-infected neutrophils versus uninfected control neutrophils without doxycycline (DOX) treatment (at 1, 7, and 10 h postinfection). Second, comparison of uninfected control neutrophils treated with 50 μM DOX versus uninfected control neutrophils without DOX treatment. Third, comparison of infected neutrophils treated with 50 μM DOX versus infected neutrophils without DOX treatment

*Denotes statistically significant difference of $P < 0.05$ as determined by two-sample, two-tailed test with equal variances. *NS* no significant difference

Table 23.2 Comparison of percentage change in MMP-2 expression by Western blotting and gelatinase zymography in test versus control neutrophils at different time-points

	Time-points	Infected versus uninfected	Uninfected control	Infected
		No DOX	DOX versus No DOX	DOX versus No DOX
Western blot (WB)	1 h	↑ 30%*	↓ 40%*	↓ 66%*
	7 h	↑ 100%*	↓ 20%*	↓ 100%*
	10 h	↑ 150%*	↓ 14%*	↓ 25%*
Gelatinase zymography (GZ)	1 h	↑ 30%*	↓ 20%*	↓ 20%*
	7 h	↑ 150%*	NS	↓ 20%*
	10 h	↑ 60%*	↓ 30%*	↓ 20%*

First, comparison of influenza virus-infected neutrophils versus uninfected control neutrophils without doxycycline (DOX) treatment (at 1, 7, and 10 h postinfection). Second, comparison of uninfected control neutrophils treated with 50 μM DOX versus uninfected control neutrophils without DOX treatment. Third, comparison of infected neutrophils treated with 50 μM DOX versus infected neutrophils without DOX treatment

*Denotes statistically significant difference of $P < 0.05$ as determined by two-sample, two-tailed test with equal variances. *NS* no significant difference

23.6.2 *Doxycycline Treatment Inhibits MMP-2 and MMP-9 Protein Expression, Gelatinase Activity, and MMP-9 Gene Expression in Neutrophils Infected With Influenza H3N2 P16 Virus*

Doxycycline treatment of uninfected and virus-infected MPRO neutrophils resulted in inhibition of expression of both MMP-2 and MMP-9 proteins, although to varying extents at different time-points (Figs. 23.3 and 23.4). Doxycycline also suppressed MMP-9 mRNA levels, with greater inhibition of gene expression the longer the incubation with doxycycline. Doxycycline treatment also decreased the gelatinolytic activity of both MMP-2 and MMP-9, with the latter exhibiting a more marked and time-dependent reduction (Tables 23.1 and 23.2). These findings indicate that the

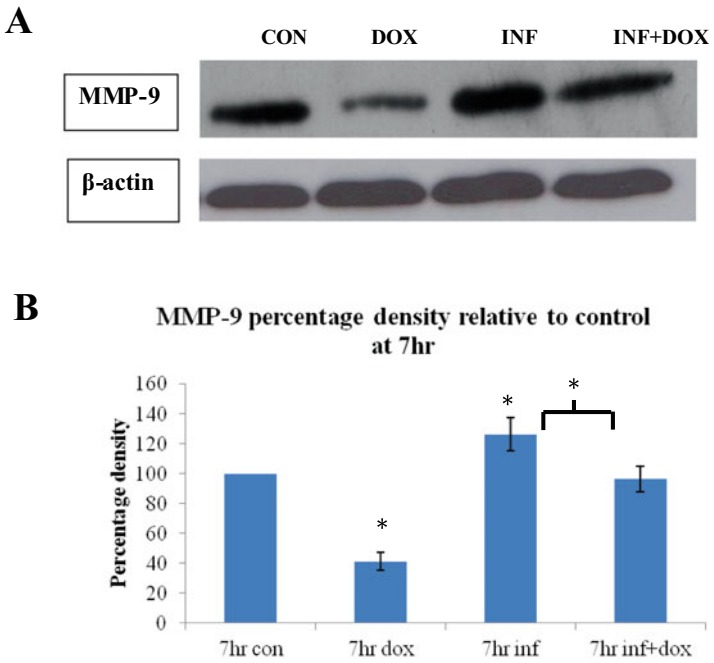

Fig. 23.3 Western blot analyses depicting MMP-9 protein expression in cell supernatant samples at 7-h time-point. (**a**) Representative immunoblots of supernatant samples from control and infected neutrophils at 7 h postinfection, showing both MMP-9 and housekeeping β-actin expression. There were four experimental groups ($n = 4$ per group). CON: uninfected and untreated control neutrophils. DOX: uninfected neutrophils with doxycycline (DOX) treatment. INF: influenza infection without DOX treatment. INF + DOX: influenza infection with DOX treatment. (**b**) Densitometric analyses of each MMP-9 protein band normalized against β-actin, and then expressed as a percentage of the band density relative to the control at 7 h (which was designated as 100%). *Denotes the statistically significant difference of $P < 0.05$ as determined by two-sample, two-tailed test with equal variances

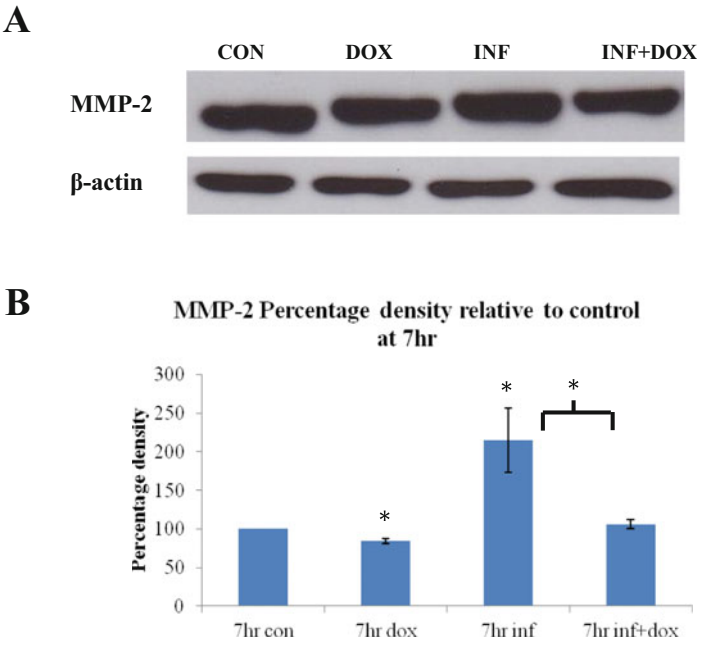

Fig. 23.4 Western blot analyses depicting MMP-2 protein expression in cell supernatant samples at 7-h time-point. (**a**) Representative immunoblots of supernatant samples from control and infected neutrophils at 7 h postinfection, showing both MMP-2 and housekeeping β-actin expression. There were four experimental groups ($n = 4$ per group). CON: uninfected and untreated control neutrophils. DOX: uninfected neutrophils with doxycycline (DOX) treatment. INF: influenza infection without DOX treatment. INF + DOX: influenza infection with DOX treatment. (**b**) Densitometric analyses of each MMP-2 protein band normalized against β-actin, and then expressed as a percentage of the band density relative to the control at 7 h (which was designated as 100%). *Denotes the statistically significant difference of $P < 0.05$ as determined by two-sample, two-tailed test with equal variances

expression of MMP-2 and MMP-9 in influenza virus-infected neutrophils could be affected by doxycycline via different mechanisms. Such differences between MMP-2 and MMP-9 may include their constitutive expression, mRNA and protein stability and half-life, negative feedback loops among others (Ben-Yosef et al. 2005).

23.6.3 Future Perspectives and Repurposing Doxycycline for Other Infections

This study focused on neutrophils, and further investigations are warranted on other relevant tissues and cell types. For example, in endothelial cells, doxycycline can affect MMP-9 production but not MMP-2 production (Hanemaaijer et al. 1998). The

effects of doxycycline on other MMPs in influenza virus-infected neutrophils should also be investigated, such as MMP-7, MMP-8, MMP-13, MMP-19, MMP-25, and MMP-27. Interestingly, MMP-25 is also an activator of pro-MMP-2, and may potentially be involved in the mechanism underpinning doxycycline's effects on gelatinase expression in infected neutrophils.

This study only analyzed the impact of doxycycline treatment on MMP-2 and MMP-9 inhibition, but there are likely to be other known and unknown molecular mechanisms of doxycycline. Further analyses of doxycycline treatment of infected versus uninfected neutrophils by harnessing transcriptomics and proteomics may elucidate additional genes, pathways, and networks that mediate the underlying molecular mechanisms. It would also be interesting to explore whether combination therapy with doxycycline together with antiviral agents such as oseltamivir can confer synergistic effects to ameliorate influenza pathogenesis.

Doxycycline therapy has also been considered and explored for the management of other infectious diseases, including COVID-19 (Narendrakumar et al. 2021)—one study found circulating MMP-9 as an early biomarker of respiratory failure in COVID-19 (Ueland et al. 2020). Doxycycline can inhibit feline coronavirus replication in vitro (Dunowska and Ghosh 2021), and can act synergistically with remdesivir antiviral to significantly reduce murine coronavirus replication in macrophages (Tan et al. 2021). A randomized controlled trial exploring doxycycline (versus placebo) when added to standard antituberculous therapy for pulmonary tuberculosis can ameliorate immunopathology and disease parameters in doxycycline-treated patients (Miow et al. 2021). Much remains to be explored to harness the repurposing of doxycycline in the management of MMP-related pathological disorders and other microbial infections (Liu and Khalil 2017).

23.7 Summary

In conclusion, MMP-2 and MMP-9 production and gelatinase activity were induced by infection of differentiated neutrophils with mouse-adapted influenza A/Aichi/2/1968(H3N2) virus. Furthermore, doxycycline treatment was able to abrogate this increase in MMP-2 and MMP-9 expression and gelatinase activity. Given that excessive neutrophil infiltration and uncontrolled neutrophil gelatinase activity in pulmonary tissues play critical roles in the pathogenesis of ALI and ARDS, this study provides insights into the mechanism of excessive MMP-2 and MMP-9 production and activity in neutrophils during influenza virus infection. Thus, harnessing doxycycline as an MMP inhibitor represents a potential therapeutic strategy to mitigate influenza-associated pulmonary injury by targeting MMPs, gelatinase production, and activity in neutrophils. Doxycycline therapy (alone or in combination with other agents) has also been considered and explored for the management of other infectious diseases such as coronavirus infections and tuberculosis.

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