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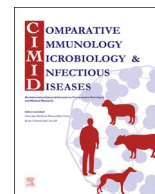
Clarithromycin suppresses induction of monocyte chemoattractant protein-1 and matrix metalloproteinase-9 and improves pathological changes in the lungs and heart of mice infected with influenza A virus

Etsuhisa Takahashi, Irene L. Indalao, Takako Sawabuchi, Keiko Mizuno, Satoko Sakai, Takashi Kimoto, Hyejin Kim, Hiroshi Kido*

Division of Enzyme Chemistry, Institute for Enzyme Research, Tokushima University, Kuramoto-cho 3-18-15, Tokushima 770-8503, Japan

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ABSTRACT

The influenza A virus (IAV)–cytokine–trypsin/matrix metalloproteinase-9 (MMP-9) cycle is one of the important mechanisms of multiple organ failure in severe influenza. Clarithromycin, a macrolide antibiotic, has immune modulatory and anti-inflammatory effects. We analyzed the effects of clarithromycin on the induction of chemokines, cytokines, MMP-9, trypsin, vascular hyper-permeability and inflammatory aggravation in mice with IAV infection. IAV/Puerto Rico/8/34(H1N1) infection increased the levels of monocyte chemoattractant protein-1 (MCP-1) and cytokines in serum, and MMP-9 and trypsin in serum and/or the lungs and heart. Clarithromycin significantly suppressed the induction of serum MCP-1 and MMP-9 and vascular hyperpermeability in these organs in the early phase of infection, but did not suppress the induction of trypsin, IL-6 or IFN-γ. Histopathological examination showed that clarithromycin tended to reduce inflammatory cell accumulation in the lungs and heart. These results suggest that clarithromycin suppresses infection-related inflammation and reduces vascular hyperpermeability by suppressing the induction of MCP-1 and MMP-9.

1. Introduction

Influenza A virus (IAV) is the most common infectious pathogen in humans and causes significant morbidity and mortality, particularly in infants and the elderly population [1,2]. Multiple organ failure (MOF) with vascular hyperpermeability is reported in the progressive stage of seasonal influenza virus pneumonia, particularly in patients with underlying risk factors [3] and is also common in infection with the highly pathogenic avian influenza viruses [4]. We reported previously that the "influenza–cytokine–trypsin/matrix metalloprotease-9 (MMP-9)" cycle is one of the key pathogenic mechanisms that interact with IAV multiplication, vascular hyperpermeability and MOF in severe influenza [5–8].

Marked increases in the levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (i.e., cytokine storm) affect cell adhesion, permeability, apoptosis, mitochondrial energy metabolism and reactive oxygen species, and can potentially result in vascular dysfunction, hyperpermeability and MOF [9,10]. These cytokines upregulate cellular trypsin [5–8] and MMP-9 through the activation of nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1) [11,12]. Induced and secreted trypsin potentiates viral entry and replication in various organs and vascular endothelial cells, because IAV has no viral hemagglutinin processing protease in its genes and post-transcriptional hemagglutinin cleavage by cellular trypsin-type proteases is indispensable for viral membrane fusion activity, virus entry into cells and multiple replication

cycles [7,13–15]. In addition, secreted trypsin induces cytokine release via proteinase-activated receptor (PAR)-2 [16] and also plays a role in blood-brain barrier (BBB) destruction by increasing intracellular calcium concentrations and loss of tight-junction protein, zonula occludens-1, via PAR-2 [5]. Furthermore, the secreted trypsin efficiently converts proMMPs to active MMPs [18,19]. Among the activated MMPs, upregulation of MMP-9 promotes endothelial hyperpermeability and enhances inflammatory cell migration through the degradation of type IV collagen in the vascular basement membrane [17,20].

There is growing evidence that macrolides with a 14-member ring, such as erythromycin and clarithromycin, are bifunctional drugs; they have an anti-inflammatory [21–24] and immune-modulatory properties [25–28], in addition to their antimicrobial effects. We reported previously that clarithromycin enhances secretory IgA production and its neutralizing activities through the induction of IgA class switching recombination and upregulation of B-cell-activating factor of the tumor necrosis factor family molecule in mucosal dendritic cells as an adjuvant or immunomodulatory compound in IAV-infected mice [28]. Thus, oral administration of clarithromycin in pediatric patients with influenza enhanced anti-viral mucus and systemic immunity and reduced the re-infection rate in the subsequent year [27]. While previous studies described the immunomodulatory effects of clarithromycin [26–28], the anti-inflammatory effects of clarithromycin on vascular hyperpermeability in the pathogenesis of MOF in severe influenza are not fully understood.

Vascular permeability is controlled by a "barrier" comprised of the micro-vascular wall, which includes the endothelial glycocalyx, endothelium, basement membrane, and all accessory cells wrapped around the outer surface of the vessel [29]. Though each of these components contributes to vascular wall permeability, we focused on disruption of the basement membrane and up-regulation of MMP-9, since MMP-9 is one of the important factors in the cycle of "influenza–cytokine–trypsin/MMP-9" described above.

The present study was conducted to determine the anti-inflammatory effects of clarithromycin on the induction of MMP-9, cytokines and chemokines in serum, and MMP-9, trypsin, vascular hyperpermeability and pathological changes in the lungs and heart of mice infected with IAV.

2. Materials and methods

2.1. Animals and virus infection

Four-week-old Balb/c female mice just after weanling were purchased from Japan SLC (Shizuoka, Japan) and used in all experiments. Influenza A/Puerto Rico/8/34(H1N1) (PR8) was kindly provided by The Research Foundation for Microbial Diseases of Osaka University (Kagawa, Japan). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996), and the study was approved by the Tokushima University Animal Research Ethics Committee (#T27-37).

Under ketamine and xylazine anesthesia, we administrated intranasally 25 plaque-forming units (pfu) of PR8 in 15 μl of saline, which induces cytokine storm but does not cause death up to post-infection day 9 (PID-9). Saline alone was instilled intranasally in the control mice. Immediately after infection, each group of animals were treated orally with a solution of clarithromycin at 150 μg/head in 100 μl methyl cellulose (MC) or MC as the vehicle every 12 h for 9 days.

2.2. Histological analysis

The isolated perfused lungs and heart of each group of animals (n = 3) were fixed with 10% buffered formalin, pH 7.2, and then embedded in paraffin. The paraffin blocks were cross-sectioned at 5 μm thickness using microtome (Leica Instruments, Nusslosh, Germany). The sections were subjected to hematoxylin-eosin staining (H&E

staining). Microscopic images of the cranial lobe of the right lung and heart were acquired with an all-in-one Fluorescence Microscope (BZ-X710; Keyence Corporation, Osaka, Japan). Evans' blue-treated tissue sections were also analyzed by a Fluorescence Microscope BZ-X710. The hypercellular areas with infiltrating inflammatory cells (shown in red) and the hypocellular areas (shown in green) in three different areas of the lung and heart of each animal were quantified by BZ-X analyzer software (Keyence Corporation), as described previously [31,32], and presented as percentages of the hypercellular area in the lung and heart section areas.

2.3. Evans' blue treatment

To examine changes in vascular permeability in the lungs and heart during the early phase of IAV infection, each non-infected and infected mouse (n = 3) received intraperitoneal injection of 0.2% Evans' blue in 100 μl of phosphate-buffered saline (PBS) on post-infection days (PIDs) 3, 6 and 9, as described previously [30]. One hour after the injection, the entire body was perfused through the left ventricle with 30 ml of saline, and the heart and lungs were excised. The tissues were homogenized with 500 μl of saline, and then centrifuged at $6000 \times g$ for 20 min at 4 °C to collect the supernatant. The concentration of Evans' blue dye in the supernatant was measured at a wavelength of 590 nm with a SPECTRA max PLUS (Molecular Devices, Sunnyvale, CA).

2.4. Western immunoblotting

The lungs and heart of each group of animals treated with clarithromycin or MC (n = 5) were homogenized with extraction buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP 40, 0.5% deoxycholate, 0.4 mM EDTA, and 0.5 mM sodium orthovanadate, and centrifuged at $12,000 \times g$ for 20 min at 4 °C. The protein concentration of the supernatant was measured by a BCA assay kit (Thermo Scientific, Rockford, IL), according to the protocol provided by the manufacturer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10–20% gradient gels (Multigel II mini, COSMO BIO, Tokyo, Japan) under reducing conditions. Proteins separated by SDS-PAGE were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and incubated overnight with anti-MMP-9 or anti-β-actin antibodies at 4 °C. After washing, the membranes were incubated with peroxidase-conjugated anti-mouse (Invitrogen, Carlsbad, CA) or −rabbit (Sigma, St. Louis, MO) IgG antibodies for one hour at room temperature, and proteins were detected using enhanced chemiluminescence detection reagent (GE Healthcare Biosciences, Uppsala, Sweden).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Blood samples collected from the mouse heart of each group of animals treated with clarithromycin or MC (n = 5) were centrifuged at $2000 \times g$ for 10 min at 4 °C and the serum was used for measurement of MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), IL-6, interferon-γ (IFN-γ), and TNF-α by ELISA kits (R&D Systems, Minneapolis, MN), using the method recommended by the manufacturer. The isolated lungs and heart were minced and homogenized with 30 vols of PBS, and then centrifuged at $12,000 \times g$ for 20 min at 4 °C to collect the supernatant for measurement of MMP-9 and trypsin levels. Trypsin levels were measured with mouse Trypsin ELISA kit (MyBioSource, San Diego, CA) according to the protocol provided by the manufacturer.

2.6. Statistical analysis

The results are expressed as mean ± standard deviation (SD). Differences between groups were examined using Student's t-test. P
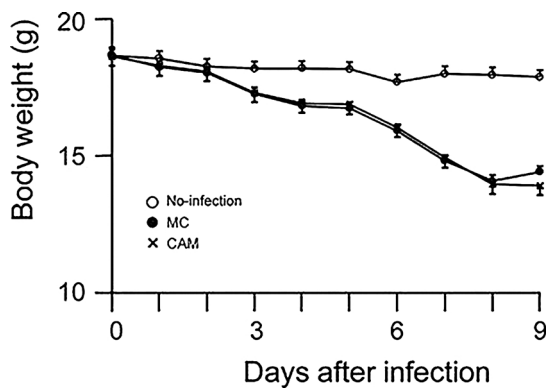
Fig. 1. Effects of clarithromycin (CAM) on changes in body weight of mice infected with IAV PR8. Changes in body weight of infected mice treated with CAM, vehicle MC and non-infected mice ($n = 5$). Data are mean $\pm$ SD.

values < 0.05 were considered significant. All statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA).

3. Results

3.1. Effects of clarithromycin on cytokines and chemokines in serum of mice infected with PR8

Since the cytokine family in the "influenza–cytokine–trypsin/MMP-9" cycle is largely involved in the pathological changes and vascular hyperpermeability in severe IAV infection, we measured the levels of various cytokines and chemokines in the serum of infected mice.

Immediately after viral infection with nasal instillation of PR8, each mouse was treated orally with clarithromycin or the vehicle twice daily for 9 days. Changes in body weight of these mice were monitored till the end of the study. While all infected animals survived to PID-9, the body weight decreased to 75.3% and 74.7% of no infection control in MC- and clarithromycin-treated mice, respectively (Fig. 1), indicating that clarithromycin by itself has no significant toxicity during the experimental period.

Under the experimental conditions described above, the levels of MCP-1 and IL-6 in serum started to increase at PID-3 and then gradually decreased until PID-9, while those of IFN-γ transiently increased at PID-6 (Fig. 2). Influenza A virus infection-induced increase in MCP-1, which guides the migration and infiltration of monocytes/macrophages to inflammation sites, was significantly suppressed by clarithromycin at PID-6, and also tended to be suppressed, though not significantly, until PID-9. However, clarithromycin had no significant effect on the up-regulation of IL-6 and IFN-γ in serum of mice after IAV infection. The serum levels of the other cytokines and chemokines, such as TNF-α and MIP-1α, in mice infected with IAV were below the detection limits throughout the experiment (data not shown).

3.2. Clarithromycin reduces spread of inflammation and inflammatory cell accumulation in the lungs and heart of IAV-infected mice

Histopathological evaluation of the lungs of MC-treated infected mice by H&E staining showed the presence of inflammatory cells at PID-3, and further accumulation with time after infection, not only in the bronchi and alveolar walls but also in arterial and venous areas, resulting in abnormal thickening, hence, the strong staining with hematoxylin (Fig. 3a, upper panel). Damaged alveolar structure was detected at PIDs 6 and 9. However, mild distribution and limited accumulation of inflammatory cells was noted in the lungs of infected mice treated with clarithromycin. Histological quantification of the percentage of the red-colored hypercellular area in the lungs showed suppression of inflammatory cell accumulation in clarithromycin-treated mice, compared with MC-treated mice at PIDs 6 and 9, although the
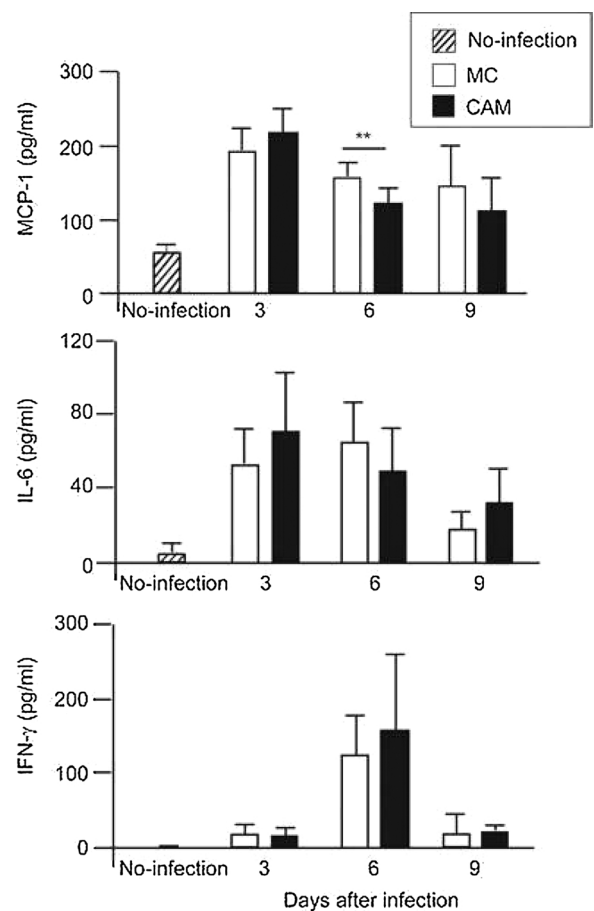

Fig. 2. Effects of clarithromycin (CAM) on induction of chemokines and cytokines in serum of mice after PR8 infection. MCP-1 (top), IL-6 (middle) and IFN-γ (bottom) levels in serum determined by ELISA at PID-3, −6 and −9 of infected mice treated with CAM, vehicle MC and non-infected mice ($n = 5$). Data are mean $\pm$ SD. **$P < 0.01$ versus MC (by Student's t-test). No-infection, no infection control at day 0.

differences between the two were not statistically significant (Fig. 3a, lower panel).

A similar effect for clarithromycin was noted in the heart (Fig. 3b). In the MC-treated mice, infiltrated inflammatory cells appeared initially in the subepicardium at PID-3, followed by extensive infiltration across the interstitium and perivascular areas deep into the myocardium, accompanied by progressive destruction of the extracellular matrix after IAV infection. Treatment with clarithromycin tended to reduce the area and extent of inflammatory cell accumulation in the heart of IAV-infected mice, relative to MC treatment, during IAV infection with a significant difference at PID-3 (Fig. 3b, lower left panel).

3.3. Clarithromycin attenuates vascular hyperpermeability in the lungs and heart of IAV-infected mice

Extravasation of Evans' blue examined by fluorescence microscopy showed increased vascular permeability in the lung (Fig. 4a) and coronary arteries (Fig. 4b) at PID-3, with gradual worsening with time after infection until PID-9. Treatment with clarithromycin tended to suppress Evans' blue extravasation in the lungs and heart. Quantitative analysis of Evans' blue dye levels in the supernatants of heart and lung homogenates showed significant suppression of IAV infection-induced Evans' blue extravasation at PID-6 in the lungs and at PID-3 and −6 in the heart (Fig. 4c). These results showed that clarithromycin induced suppression of vascular hyperpermeability in the early phase of IAV infection (at PID-3 and/or −6, but not at PID-9) in the lungs and heart.
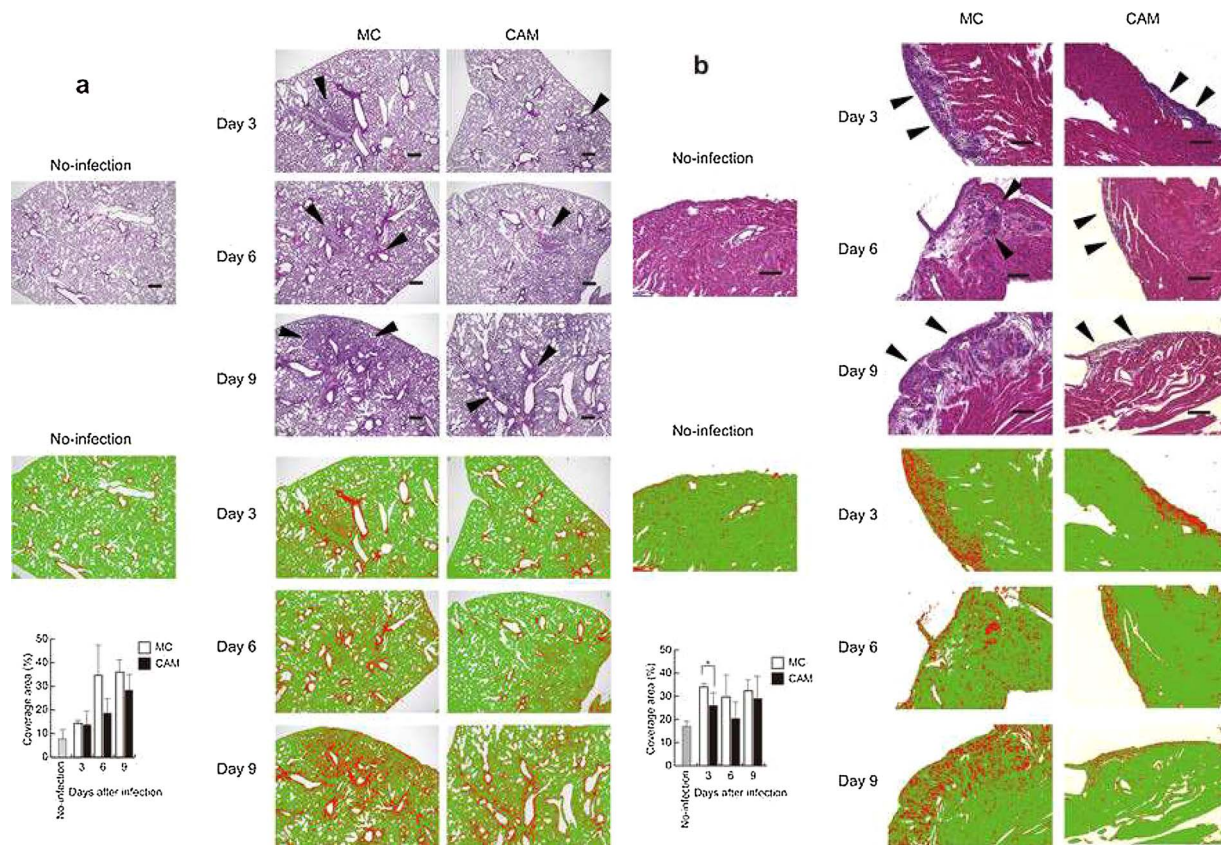
Fig. 3. Effects of clarithromycin (CAM) on changes in inflammatory cell accumulation in the lungs and heart of mice infected with PR8. Sections from the lungs (a, top panels) and heart (b, top panels) were stained with hematoxylin-eosin (H&E), and evaluated for tissue inflammation and inflammatory cell accumulation. The hypercellular areas with inflammatory cell accumulation shown in red and the hypocellular areas shown in green were quantified using BZ-X analyzer software (bottom right panels). Photographs are representative sections of the lung and heart of mice of each group ($n = 3$). *Arrowheads*: loci of inflammatory cell accumulation. Scale bars, 300 μm (a), 100 μm (b). The percentage of the hypercellular area in the three different areas of the lung and heart sections was analyzed using BZ-X analyzer software (a and b, bottom left panels). Data are mean ± SD. *$P < 0.05$ versus MC (by Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Clarithromycin suppresses increase in MMP-9 levels in serum and tissue extracts of IAV-infected mice

Influenza A virus infection-induced up-regulation of MMP-9 is one of the key factors involved in increased vascular permeability [5–9]. Accordingly, we measured the effects of clarithromycin on MMP-9 up-regulation in serum and tissues during the 9-day course of IAV infection. Clarithromycin significantly suppressed the marked increase in serum MMP-9 levels at PID-3, as monitored by ELISA, and also tended to suppress the level, though not significantly, at PID-6 and −9 (Fig. 5a). In addition, western immunoblotting showed that clarithromycin significantly suppressed the activated MMP-9 levels in the heart and lungs of IAV-infected-mice at PID-6 (Fig. 5b).

3.5. Clarithromycin has no effect on trypsin levels in the lungs and heart, and TIMP-1 levels in serum

Trypsin plays an important role in IAV multiplication *in vivo* [7,8,13] and also efficiently converts proMMPs to active MMPs [6,19,21]. In the MC-treated mice, lung trypsin levels increased gradually after infection and reached peak levels during PID-3 to −6 then decreased at PID-9 (Fig. 6a). Clarithromycin had no significant effect on lung trypsin levels. Furthermore, clarithromycin did not change the up-regulated trypsin levels in the heart (data not shown). Next, we analyzed the effect of clarithromycin on levels of MMP-9 inhibitor, TIMP-1, in serum. TIMP-1 levels increased markedly and continuously after IAV infection through PID-9 while clarithromycin had no effect on the levels (Fig. 6b).

4. Discussion

Multiple organ failure with vascular hyperpermeability is the progressive stage of influenza virus pneumonia [4,33]. We reported previously that severe IAV infection was associated with increased intracellular calcium levels and loss of tight junction constituent, zonula occludens-1, through the upregulation of cytokines and trypsin [5] and also with glycogen synthase kinase-3β-mediated β-catenin degradation in adherens junctions [34]. In addition to the impairment of tight junction and adherens junction, IAV infection induces degradation of extracellular matrix by MMPs and trypsin, thus further increasing vascular hyperpermeability. Taken together, these findings add support to the "influenza–cytokine–trypsin/MMP-9" hypothesis, and that it could be one of the key mechanisms of vascular hyperpermeability and MOF in severe influenza [5,7]. In the present study, we investigated the anti-inflammatory effects of clarithromycin on MMP-9 upregulation associated with IAV infection, which may play a role in endothelial hyperpermeability and inflammatory cell migration.

It is not uncommon in Japan to prescribe clarithromycin combined with antiviral neuraminidase inhibitors to prevent complications and aggravation of the flu symptoms. In this regard, we reported previously that oral administration of clarithromycin enhances airway mucosal immunity through the induction of IgA class switching recombination [28] and significantly reduces the re-infection rate in the subsequent year in pediatric patients with influenza treated with anti-viral neuraminidase inhibitors [27]. In addition to the remarkable immunomodulatory effects of clarithromycin, we evaluated here the anti-inflammatory properties of clarithromycin in the lungs and heart of IAV-infected mice.
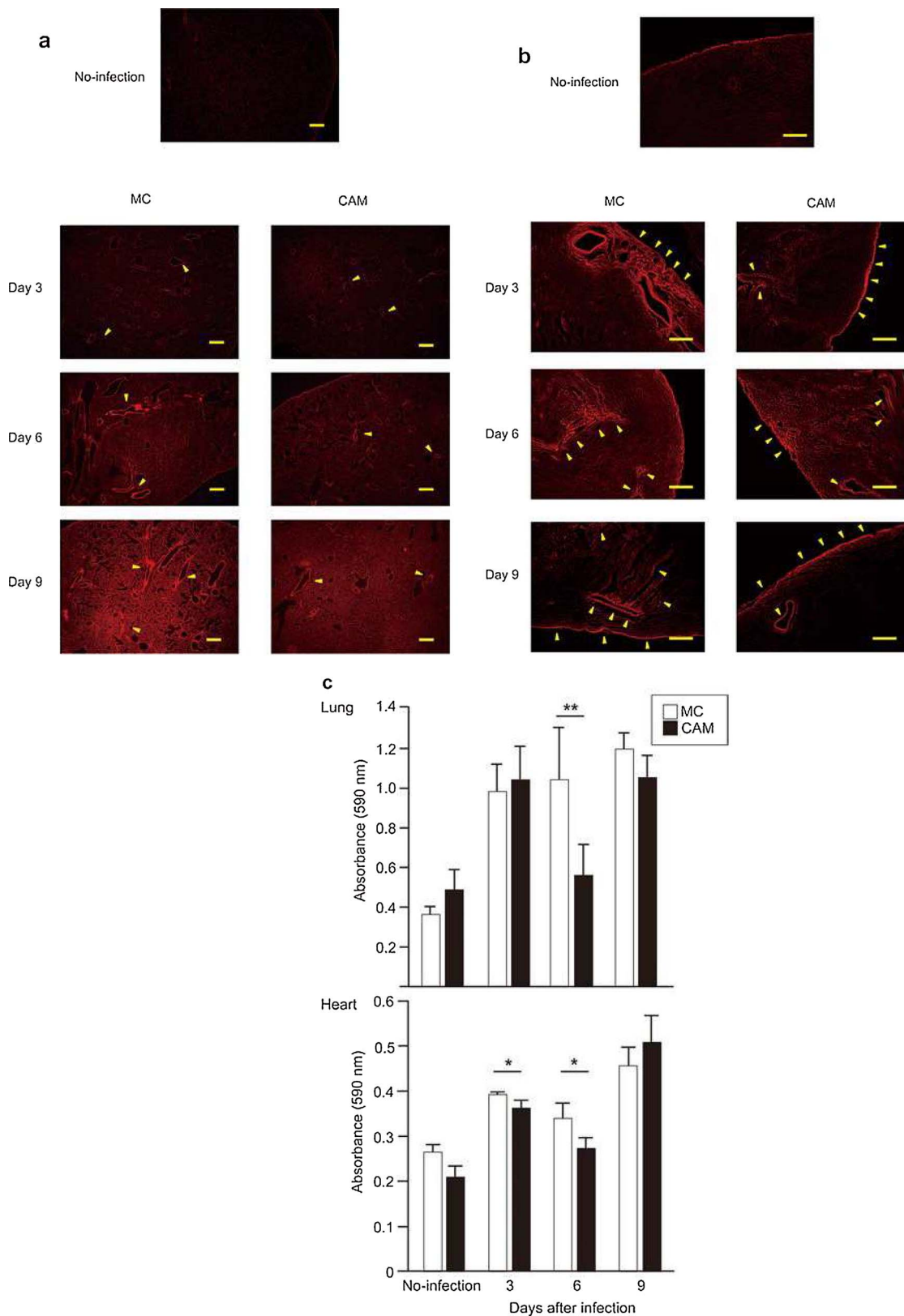
Fig. 4. Clarithromycin (CAM) suppresses Evans' blue extravasation in the lungs and heart of mice infected with PR8. Evans' blue extravasation in lung (a) and heart (b) sections was analyzed by fluorescence microscopy. *Arrowheads*: sites of Evans' blue extravasation. Scale bars, 300 μm (a), 100 μm (b). Photographs are representative sections of the lung and heart of mice of each group ($n = 3$). (c) Evans' blue levels in the supernatants of the lungs and heart homogenates of mice of each group. Data are mean ± SD. No-infection, no infection control at day 0. $*P < 0.05$, $**P < 0.01$, versus MC (by Student's *t*-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
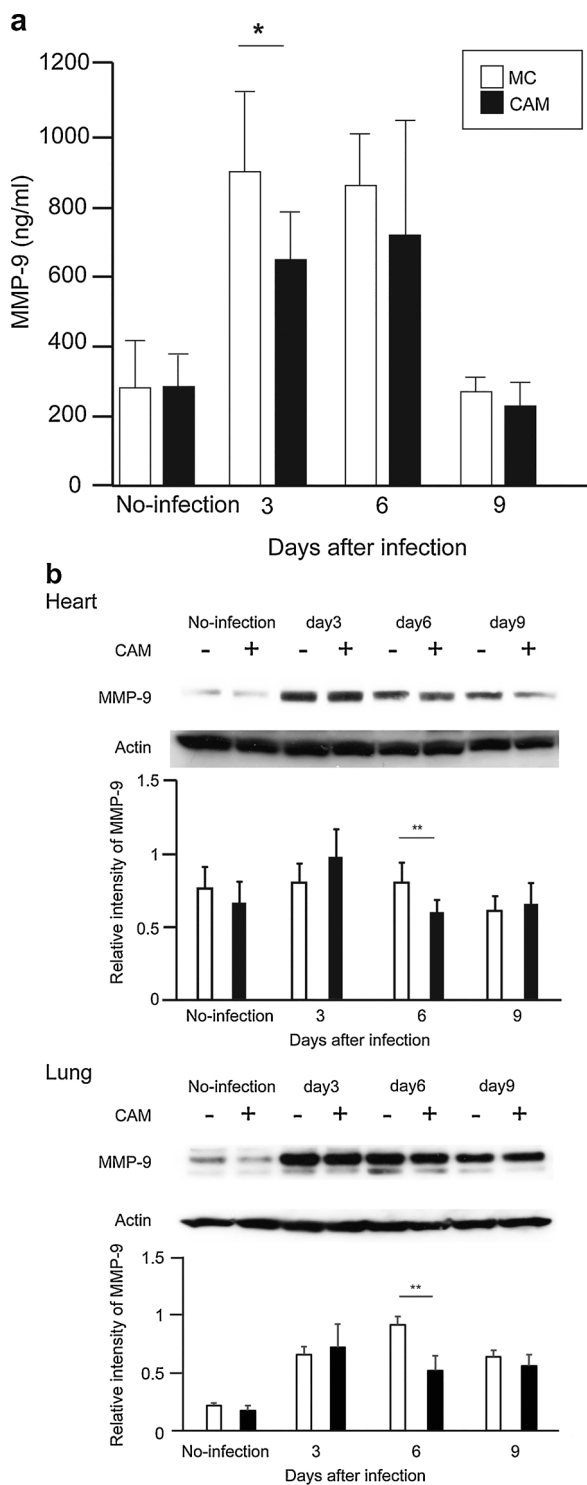
Fig. 5. Clarithromycin (CAM) suppresses induction of MMP-9 in serum, heart and lungs of mice infected with PR8. (a) MMP-9 levels in serum of infected mice treated with CAM (*solid bars*) and MC (*open bars*) at PID-3, −6 and −9 and non-infected control at day 0 (*n* = 5, each group) measured by ELISA. Data are mean ± SD. (b) MMP-9 expression levels in the lungs and heart of mice treated with CAM (+, *solid bars*) and MC (-, *open bars*) at PID-3, −6, and −9 and uninfected control (day 0) (*n* = 5, each group) were analyzed by western immunoblotting. 50 μg protein/lane. β-actin as an internal control. Image J software was used to quantify the band intensities. Top panels of western immunoblotting show representative results of five experiments. *$P < 0.05$, **$P < 0.01$, versus MC (by Student's *t*-test).
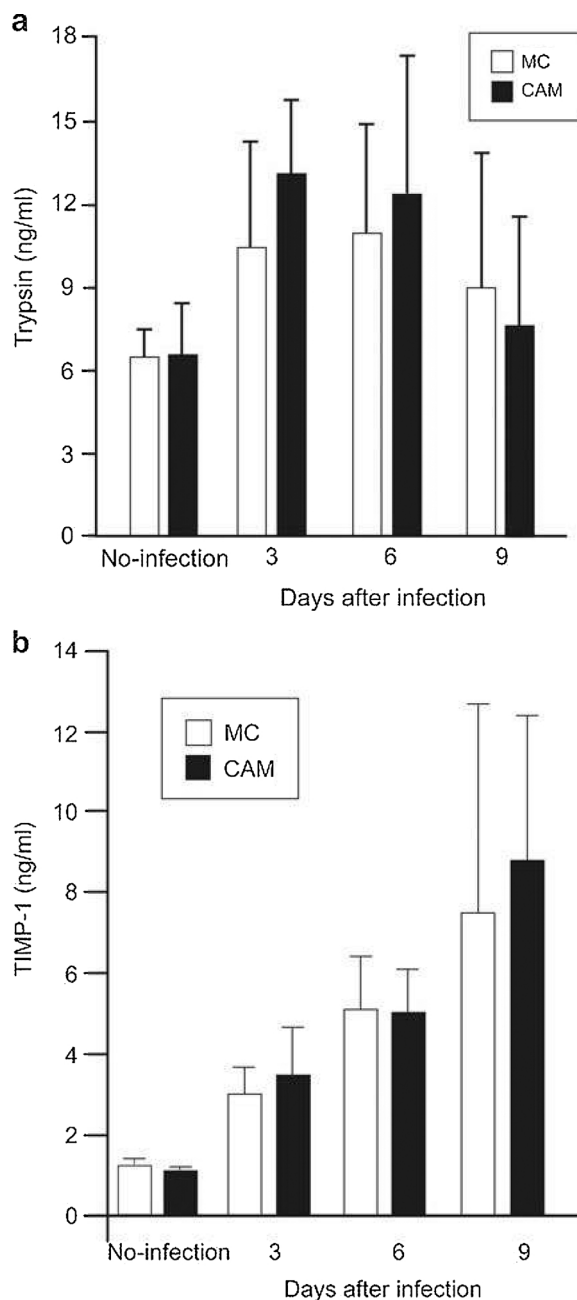

Fig. 6. Effects of clarithromycin (CAM) on lung trypsin levels and serum TIMP-1 in mice infected with PR8. (a) Trypsin levels were measured in the supernatants of lung homogenates of mice treated with CAM and vehicle MC at PID-3, −6, and −9 and uninfected control (*n* = 5, each group) by ELISA. Data are mean ± SD. (b) Serum TIMP-1 levels at PID-3, −6, and −9 and uninfected control measured by ELISA. Data are mean ± SD.

Our results showed that IAV infection rapidly induced MCP-1 in serum at PID-3 and the levels were sustained until PID-9 (Fig. 2), which is one of the key chemokines known to stimulate the migration and infiltration of monocytes/macrophages [35]. MCP-1 induction was associated with increased MMP-9 levels in the lungs and heart during PID-3 to −6, followed by gradual decrease in the levels (Fig. 5b). MMP-9 levels also increased in serum with a peak during PID-3 to −6, showing a pattern similar to that of MCP-1 (Fig. 2 and Fig. 5a). In contrast, TIMP-1 levels in serum after IAV-infection continuously increased during the 9-day experimental period (Fig. 6b). Oral administration of clarithromycin significantly suppressed the induction of MCP-1 at PID-6 in serum, and induction of MMP-9 in the lungs and heart at PID-6 and in serum at PID-3 (Fig. 2 and Fig. 5a). These results suggest that

clarithromycin suppressed MCP-1 induction, resulting in the inhibition of monocytes/macrophages accumulation and MMP-9 induction at inflammation sites in the lungs and heart. MMP-9 preferentially degrades type IV collagen in the basement membrane of blood vessels, resulting in vascular hyperpermeability [17–20]. Therefore, clarithromycin also significantly suppressed Evans' blue extravasation (Fig. 4C) in the lungs and heart at PID-3 and/or −6, similar to the time courses of MCP-1 and MMP-9 suppression.

The production of MMPs and MCP-1 is enhanced by transcriptional factors NF-κB and AP-1 [11,12,35]. Clarithromycin inhibit NF-κB and AP-1 binding to the promoters of target genes, resulting in suppression of the transcription [36]. Our findings of clarithromycin-induced suppression of MMP-9 and MCP-1 and accumulation of inflammatory cells in the infected loci may add support to the above findings.

In the present study, we used Balb/C mice with a Th2 biased immune response [37–39], in a series of study on immunomodulatory effects of clarithromycin [28]. Further studies in C57BL/6 mice with a more Th1 dominated immune response are required. In addition, our study indicates that clarithromycin treatment requires 3–6 days to reach a clear suppression effect on MMP-9 and MCP-1 and the finding was supported by the previous report in rat autoimmune myocarditis [40]. Further study on pre-administration of clarithromycin could better define its effects on IAV infection.

5. Conclusions

In addition to the marked immunomodulatory effects of clarithromycin reported previously [26–28], including enhancement of secretory IgA production, and its neutralizing activities and ability to reduce re-infection rate in pediatric patients, the present study showed that clarithromycin suppressed the inductions of MCP-1, MMP-9, inflammatory cell accumulation, and vascular hyperpermeability in the lungs and heart of mice infected with PR8. Although the anti-inflammatory effects of clarithromycin were transient; occurring mainly during PID-3 to −6 at the early phase of infection, our results may add support to the practice of clarithromycin use in the treatment of IAV progression.

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