Experimental Pneumocystis lung infection promotes M2a alveolar macrophage-derived MMP12 production

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Nelson MP, Christmann BS, Dunaway CW, Morris A, Steele C. Experimental Pneumocystis lung infection promotes M2a alveolar macrophage-derived MMP12 production. Am J Physiol Lung Cell Mol Physiol 303: L469–L475, 2012. First published July 6, 2012; doi:10.1152/ajplung.00158.2012.—Among several bacterial and viral pathogens, the atypical fungal organism Pneumocystis jirovecii has been implicated as a contributor to the pathogenesis of chronic obstructive pulmonary disease (COPD). In a previous study, we reported that Pneumocystis-colonized HIV-positive subjects had worse obstruction of airways and higher sputum levels of macrophage elastase/matrix metalloproteinase 12 (MMP12), a protease strongly associated with the development of COPD. Here, we examined parameters of Pneumocystis-induced MMP12 in the lungs of mice and its role in the lung immune response to murine Pneumocystis. Initial studies demonstrated that P. murina exposure induced Mmp12 mRNA expression in whole lungs and alveolar macrophages (AMs), which was dependent on the presence of CD4+ T cells as well as signal transducer and activator of transcription 6. Mmp12 mRNA expression was upregulated in AMs by interleukin (IL)-4 treatment, but downregulated by interferon (IFN)-γ. Collectively, our data indicate that Mmp12 induction is a component of the P. murina-induced M2 response and thus provides insight into the link between Pneumocystis colonization/colonization and lung exacerbations in COPD.

MATERIALS AND METHODS

Mice. Male C57BL/6 or Balb/c mice, 6–8 wk of age, were purchased from Jackson Laboratories (Bar Harbor, ME). Mmp12−/−, signal transducer and activator of transcription (Stat) 4−, and Stat6-deficient mice were purchased from Jackson Laborato-
ries. Hck/Fgr/Lyn<sup>−/−</sup> mice (Snc TKO mice) originally developed by Dr. Clifford Lowell, University of California at San Francisco (23), were provided by Dr. Shaoqiang Li, University of Massachusetts. All animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to United States Public Health Service Office of Laboratory Animal Welfare policies after review and approval by the UAB Institutional Animal Care and Use Committee.

*P. murina* inoculation and lung burden quantification. A preparation of *P. murina* was prepared as previously described (16). Briefly, C.B-17 SCID mice previously inoculated with *P. murina* were injected with a lethal dose of ketamine/xylazine, and the lungs were aseptically removed and frozen at −80°C in 1 ml PBS. Frozen lungs were homogenized through a 70-μm filter and pelleted at 300 g for 10 min at 4°C. The pellet was resuspended in 1 ml of PBS, and a 1:10 dilution was stained with modified Giemsa stain (Diff-Quik). The number of *P. murina* cysts was quantified microscopically, and the concentration was adjusted to 2 × 10<sup>6</sup> cysts/ml. For in vivo challenge, mice were anesthetized with isoflurane and administered 2 × 10<sup>5</sup> cysts in a volume of 0.1 ml via intratracheal inoculation. Some preparations were also adjusted to 2 × 10<sup>5</sup> cysts/ml, and 50-μl aliquots were placed in tubes containing 200 μl of 90% PBS supplemented with 10% dimethyl sulfoxide and stored at −80°C. Employing this storage method, stable *P. murina* viability, as determined by quantitative real-time PCR, can be maintained for greater than one year (38). In some experiments, mice were depleted of CD4 T cells via intraperitoneal injections of GK 1.5 monoclonal antibody as described (22). For lung fungal burden analysis, total RNA was isolated from the right lung of infected mice by a single-step method using TRIzol reagent (Invitrogen Life Technologies) per the manufacturer’s instructions. Thereafter, RNA was transcribed to cDNA, and real-time PCR for *Pneumocystis* rRNA was performed as described previously (39, 41).

**Alveolar macrophage isolation and culture.** Naïve mice were anesthetized with intraperitoneal ketamine/xylazine and killed by exsanguination. Thereafter, lungs were lavaged through an intra-tracheal catheter with prewarmed (37°C) calcium- and magnesium-free PBS supplemented with 0.6 mM EDTA. A total of 10 ml were used in each mouse in 0.5-ml increments with a 30-s dwell time. The lavage fluids were pooled and centrifuged at 600 g for 10 min, and the cells were collected for the coculture assay. Cell preparations were generally >98% enriched for alveolar macrophages (AMs). Macrophages (1 × 10<sup>5</sup> in 100 μl) were left alone or treated with 10 ng/ml of interleukin (IL)-1 or interferon (IFN)-γ (both from eBioscience) for 4 h (RNA) or 24 h (cell lysate).

**Real-time PCR analysis for Mmp12 expression in AMs and lung tissue.** Mice were infected with *P. murina* for 7 or 14 days followed by either lung lavage or lung tissue collection. Lung lavage cells were adhered to plastic for 1 h at 37°C in 5% CO<sub>2</sub> (to enrich for AMs) followed by the removal of nonadherent cells. Total RNA was isolated from enriched AMs or the right lung of infected mice by a single-step method using TRIzol reagent as per the manufacturer’s instructions. Thereafter, RNA was transcribed to cDNA (iScript cDNA synthesis kit; Bio-Rad), and real-time PCR for *Mmp12* (Mm00500554_m1; Applied Biosystems) was performed (iQ Supermix; Bio-Rad). Western blot analyses, the two-tailed paired Student’s t-test and the two-tailed Mann-Whitney U-test when not normally distributed. In real-time PCR and Western blot analyses, the two-tailed paired Student’s t-test was employed. Significance was accepted at a value of *P* < 0.05.

**RESULTS**

*P. murina* lung exposure results in the induction of Mmp12. In our previous study, we observed that *P. jirovecii* colonization was associated with worse airway obstruction in HIV-infected subjects and correlated with increased levels of Mmp12 in sputum (25). In an effort to better understand this correlation, we took a “bedside to bench” approach and questioned whether lung exposure to *Pneumocystis* (*P. murina*) induced *Mmp12* mRNA expression in a murine model. For this, C57BL/6J mice (Jackson) were challenged with *P. murina* for 7 and 14 days, and whole lungs or bronchoalveolar lavage cells were collected. Results in Fig. 1A show that exposure to *P. murina* for 7 days resulted in no significant induction of *Mmp12* expression in whole lungs and a minimal increase in lung lavage cells. In contrast, by 14 days postinfection, *Mmp12* mRNA expression was increased by >5-fold in whole lungs and by >38-fold in lung lavage cells compared with that isolated from uninfected mice. In a separate experimental design, C57BL/6Ncr mice (NCI) were challenged with *P. murina* for 14 and 28 days, and whole lungs were collected. *P. murina* infection in this B6 strain induced significantly higher *Mmp12* expression at 14 days postinfection that remained elevated through 28 days postinfection (Fig. 1B). Thus, experimental infection with *P. murina* results in significant, yet time-dependent induction of *Mmp12* expression in the lung.

**Active Mmp12 is preferentially induced in M2a AMs.** We have recently reported that macrophage activation during *P. murina* lung infection in resistant mice favors alternative macrophage activation (28). Although Mmp12 expression may be associated with alternative macrophage activation (14), data also support a role for IFN-γ in the induction of *Mmp12* (10, 19). CD4 T cells produce IL-4 and IFN-γ in the lung and draining lymph nodes during *P. murina* lung infection with IL-4 predominating through the first 3 wk of infection (34). Therefore, to specifically determine the pattern of Mmp12 induction in M1 vs. M2a macrophage activation states, we treated AMs from naïve mice with IL-4 or IFN-γ and determined *Mmp12* mRNA and Mmp12 protein levels. Results in Fig. 2A demonstrate that IL-4 was a potent inducer of *Mmp12* mRNA expression, whereas IFN-γ surprisingly downregulated *Mmp12* mRNA expression. Examination of whole cell lysates for Mmp12 protein

**Statistical analysis.** Data were analyzed using GraphPad Prism statistical software (GraphPad Software, San Diego, CA). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student’s t-test and the two-tailed Mann-Whitney U-test when not normally distributed. In real-time PCR and Western blot analyses, the two-tailed paired Student’s t-test was employed. Significance was accepted at a value of *P* < 0.05.

**MMP12 AND *PNEUMOCYSTIS* LUNG INFECTION**

L470

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levels revealed that AMs cultured overnight in medium alone produced high levels of the 45-kDa NH₂-terminal active form of Mmp12, low levels of the 54-kDa proenzyme form, and no detectable levels of the 22-kDa processed active form (Fig. 2B). In contrast, although IL-4 pretreatment resulted in no change in the 45-kDa form, significant production of both the 54- and 22-kDa forms was observed (Fig. 2C). IFN-γ pretreatment had no effect on the levels of the 45-kDa form and did not induce either the 54- or 22-kDa forms. Quantitative analysis indicated that IL-4 pretreatment results in a more than eightfold and sevenfold increase or 22-kDa forms. Quantitative analysis indicated that IL-4 pretreatment resulted in no change in the 45-kDa form and did not induce either the 54- or 22-kDa forms. Quantitative analysis indicated that IL-4 pretreatment results in a more than eightfold and sevenfold increase in the 54- and 22-kDa forms, respectively (Fig. 2C). Thus, the induction of fully active Mmp12 during P. murina lung infection is a parameter of M2a macrophage activation.

Differential expression of Mmp12 in P. murina-infected Src-deficient vs. CD4-depleted mice. We have previously reported that mice deficient in the myeloid Src family kinases Hck, Fgr, and Lyn (Src TKO mice) demonstrated an increase in alternatively activated macrophages (28), which we have demonstrated to have an enhanced capacity to kill P. murina (28, 29). Because Src TKO mice are more resistant to P. murina lung infection (29), we questioned whether this observation correlated with Mmp12 expression. Results in Fig. 3A show that Src TKO mice had increased Mmp12 expression in the lungs 7 days after P. murina lung challenge, a time point at which M2 signature genes are increased (28). It is important to note that Src TKO mice had enhanced Mmp12 expression by day 7, which is a week earlier than wild-type control mice express significant Mmp12 in the lungs (Fig. 1). Depletion of CD4 T cells in experimental animal models leads to an inability to clear P. murina from the lungs (1). Because Mmp12 expression was elevated in mice with augmented P. murina clearance [Src TKO (29)], we next determined whether Mmp12 expression

Fig. 1. Pneumocystis murina lung exposure results in the induction of matrix metalloproteinase (Mmp) 12. A: C57BL/6J mice (Jackson) were administered 2 × 10⁵ Pneumocystis cysts via intratracheal inoculation. Seven or fourteen days postinoculation, lung cells were isolated by bronchoalveolar lavage, or whole lungs were extracted. Total RNA was isolated and transcribed to cDNA, and quantitative real-time PCR was performed for Mmp12. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (Gapdh), and fold changes between naïve (set at 1) and infected mice were determined using the 2⁻ⁿ(DCt) method. Cumulative data from two independent studies with an n = 4–5 mice per group/per study are shown. B: C57BL/6Ncr mice (NCI) were similarly inoculated with P. murina, and whole lungs were collected at 14 and 28 days postinoculation. Mmp12 levels were quantified as in A. P value of <0.05 (*) and <0.001 (****) (paired 2-tailed Student’s t-test).

Fig. 2. Active Mmp12 is preferentially induced in M2 alveolar macrophages. A: alveolar macrophages were isolated from 6- to 8-wk-old, male C57BL/6 via bronchoalveolar lavage and cultured in medium alone or in the presence of interleukin (IL)-4 or interferon (IFN)-γ (10 ng/ml) for 4 h. Thereafter, total RNA was isolated and transcribed to cDNA, and quantitative real-time PCR was performed for Mmp12. Gene expression was normalized to Gapdh, and fold changes between untreated (set at 1) and infected mice were determined using the 2⁻ⁿ(DCt) method. B: alveolar macrophages were isolated from 6- to 8-wk-old, male C57BL/6 mice and cultured in medium alone or in the presence of IL-4 or IFN-γ (10 ng/ml) for 24 h. Thereafter, cell lysates were extracted, subjected to SDS-PAGE and polyvinylidene difluoride (PVDF) transfer, and immunoblotted with anti-murine Mmp12 IgG. Positive bands were identified using an enhanced chemiluminescence (ECL) Western blot detection kit and subsequently analyzed using Quantity One 1-D Analysis Software (Bio-Rad). A representative image of Mmp12 levels in unstimulated vs. IL-4- or IFN-γ-stimulated alveolar macrophages is shown (two lanes included IL-4-treated cells). Un, untreated. C: cumulative data from four independent studies employing Quantity One 1-D software to determine the densitometry values for the different Mmp12 isoforms and their β-actin controls. Data are expressed as fold change of Mmp12 to actin after normalizing unstimulated samples to a value of 1. *P value of <0.05 (paired 2-tailed Student’s t-test).
was modulated in mice with defective *P. murina* clearance (i.e., CD4-depleted mice). Results in Fig. 3B show that whole lung *Mmp12* expression is reduced significantly in *P. murina*-exposed mice subjected to depletion of CD4+ T cells. CD4 depletion also resulted in significantly lower expression of other M2 markers, such as RELM-α (Fig. 3C) and CCL17 (Fig. 3D). Thus, *Mmp12* induction in the lungs during *P. murina* infection directly correlates to the magnitude of the M2 response, which is dependent on CD4+ T cells.

Both STAT4 and STAT6 are required for optimal *Mmp12* induction during *P. murina* lung infection. Our data support a role for IL-4 signaling and M2a AMs in the generation of lung *Mmp12* expression after *P. murina* exposure. Moreover, CD4 T cells are required for maintaining M2 polarization and *Mmp12* expression during *P. murina* lung infection (Fig. 3).

Recent studies have implicated a role for IL-17A in the induction of *Mmp12* expression in the lungs during cigarette smoke exposure (5, 33). IL-17A production by CD4 T cells requires IL-23-mediated activation of the transcription factors STAT3 and STAT4 (21, 40). Because IL-4 signals through STAT6 (15), we determined the relative contribution of STAT4 and STAT6 in *Mmp12* expression during *P. murina* lung infection. Results in Fig. 4 show that mice deficient in STAT4 had a moderate, yet significant, reduction in lung *Mmp12* expression 14 days post-*P. murina* challenge. However, *Mmp12* expression 14 days post-*P. murina* challenge was more dependent on STAT6 signaling, since STAT6-deficient mice demonstrated a more marked reduction (∼2.3-fold). Thus, *Mmp12* expression during *P. murina* lung infection requires STAT6, and to a lesser extent STAT4.
MMP12 does not contribute to lung host defense against *P. murina*. Recent studies have reported that MMP12 expressed in macrophage phagolysosomes has potent antimicrobial activity (12). Moreover, data presented in Fig. 4 suggest that the ability to clear *P. murina* from the lungs correlates with the level of Mmp12 induction, indicating the possibility that MMP12 may function in a host defense capacity. To address this, we challenged *Mmp12−/−* mice with *P. murina* and assessed the burden after 7 and 14 days. Results in Fig. 5A show that, despite the ability of *P. murina* to induce Mmp12 expression in the lungs, MMP12 is not required for clearance of *P. murina* from the lungs. Moreover, MMP12 deficiency had no effect on the lung inflammatory response to *P. murina* in that IL-1, granulocyte-macrophage colony-stimulating factor (G-CSF), CCL2, and CXCL1 were not modulated in *Mmp12−/−* mice at 7 (Fig. 5B) and 14 (Fig. 5C) days postinfection. Thus, although Mmp12 expression is induced readily in M2a AMs during *P. murina* lung infection, MMP12 plays no role in immune-mediated clearance of *P. murina*.

**DISCUSSION**

COPD patients colonized with *P. jirovecii* are reported to have higher proinflammatory cytokine levels than noncolonized patients (3). In experimental models, *Pneumocystis* infection in mice exposed to cigarette smoke results in airspace enlargement and increased inflammation (6), whereas simian/human immunodeficiency virus-infected/Pneumocystis-colonized primates developed progressive obstructive pulmonary disease characterized by increased emphysematous tissue (35). Collectively, clinical and experimental data indicate that *Pneumocystis* is associated with higher inflammation and decreases in lung function that contribute to the physiopathology and progression of COPD. In addition, we have previously reported that *P. jirovecii* colonization in HIV-positive COPD patients is associated with worse airway obstruction and correlates with increased levels of MMP12 in sputum (25). In the current report, we show that the induction of MMP12 expression in the lungs is part of the normal immune response to experimental *Pneumocystis* infection.

In our initial analysis, we observed a relative delay in the induction of *Mmp12* expression in the lungs after *P. murina* challenge. By 1 wk postchallenge, there was no significant change in the expression of *Mmp12*. However, by 2 wk postchallenge, we observed robust upregulation of *Mmp12* mRNA expression in both whole lungs and bronchoalveolar lavage cells in which the largest cellular constituent was AMs (unpublished data). *Mmp12* mRNA expression was enriched in bronchoalveolar lavage cells compared with whole lungs, sug-

![Graph](http://ajplung.physiology.org/)

**Fig. 4.** Both signal transducer and activator of transcription (STAT) 4 and STAT6 are required for optimal *Mmp12* induction during *P. murina* lung infection. BALB/c-, *Stat4−/−*, and *Stat6−/−* deficient mice were administered 2 × 10^5 *Pneumocystis* cysts via intratracheal inoculation. Fourteen days postinoculation, whole lungs were extracted, total RNA was isolated and transcribed to cDNA, and quantitative real-time PCR was performed for *Mmp12*. Gene expression was normalized to *Gapdh*, and fold changes between BALB/c (set at 1) and infected *Stat4−/−* and *Stat6−/−* deficient mice were determined using the 2^−ΔΔCt method. Cumulative data from two independent studies with an n = 4–5 mice per group per study are shown. P value of <0.05 (*) and 0.001 (***)(paired 2-tailed Student’s t-test).

**Fig. 5.** MMP12 does not contribute to lung host defense against *P. murina*. A: C57BL/6 (WT) and *Mmp12−/−* mice were administered 2 × 10^5 *Pneumocystis* cysts via intratracheal inoculation. Seven or fourteen days postinoculation, lungs were collected, and *Pneumocystis* burden was determined by real-time PCR for *Pneumocystis* rRNA copy number. Representative data from one of two independent studies with an n = 5 mice per group per time point are shown. Data are expressed as mean *Pneumocystis* rRNA copy number. KO, knockout. B and C: mice were infected as described. At 7 (B) or 14 (C) days postchallenge, lungs were collected, and clarified supernatants from lung homogenates were analyzed for granulocyte macrophage colony-stimulating factor (G-CSF), CXCL1, CCL2, and IL-1β levels by Bio-Plex. Representative data from one of two independent studies with an n = 5 mice per group per time point are shown. Data are expressed as mean pg/ml + SE.
gesting that structural cells such as epithelial cells or fibroblasts were likely not a significant source of *Mmp12*. Moreover, *Mmp12* expression was not transient, since 4 wk post-*P. murina* challenge, *Mmp12* mRNA levels remained elevated. We have recently reported that alternative macrophage activation is a component of the lung immune response to *P. murina* in normal mice and is enhanced in mice that demonstrate increased resistance to *P. murina* (28). Because alternative (M2a) vs. classical (M1) macrophage activation is driven by IL-4 and IFN-γ, respectively, we determined whether these mediators modulated *Mmp12* expression. Indeed, *Mmp12* expression was favored by M2 AMs, since IL-4 treatment significantly upregulated *Mmp12* expression and MMP12 protein production. It was interesting to note that IL-4 treatment specifically induced more total, i.e., unprocessed (54 kDa), MMP12 protein levels than IFN-γ. Consequently, only fully processed, active MMP12 (22 kDa) was observed in IL-4-treated AMs.

We next determined whether specific components of the immune system modulated *Mmp12* expression. We first examined mice deficient in the myeloid Src family tyrosine kinases Hck, Fgr, and Lyn, since we have previously reported that these mice had higher AM effector function against *P. murina* and were more resistant to infection (29). Moreover, we have shown that these mice demonstrate enhanced M2a macrophage polarization during *P. murina* lung infection (28). In line with this, we found that *Mmp12* expression was also elevated in these mice. We also observed a direct effect of CD4 T cell depletion on *Mmp12* expression, since elimination of this cell population resulted in significantly lower *Mmp12* expression. Of note, we further discovered that additional M2a macrophage activation markers, RELM-α and CCL17 specifically, were also reduced in CD4-depleted mice. These results indicate that CD4 T cells are required for maintaining M2a macrophage polarization during *P. murina* lung infection. Because we have shown that M2a AMs are potent effector cells against *P. murina* (28, 29), it is possible that one mechanism contributing to the development of fulminant *P. murina* lung infection during CD4 T cell depletion is the loss of M2a macrophage polarization. Furthermore, our data suggest that STAT6, and to a lesser extent STAT4, are required for optimal lung *Mmp12* expression during *P. murina* infection. Collectively, our data point to Src tyrosine kinases, CD4 T cells and STAT6, contributing to the magnitude of *Mmp12* expression in the lung during *P. murina* infection.

Our data suggested that the level of *Mmp12* expression correlated with the level of *P. murina* clearance from the lungs, thus bringing into question whether MMP12 possessed the ability to directly inhibit the growth of *P. murina*. Indeed, a 20-amino acid sequence in the carboxy-terminal domain of MMP12 has been shown to be a potent growth inhibitor of multiple Gram-positive and Gram-negative bacteria (12). The proenzyme form of MMP12 is secreted and subsequently activated in the phagolysosome of macrophages (12). Because we have shown that blocking phagocytosis inhibits AM-mediated killing of *P. murina* (38), we hypothesized that MMP12 was a mediator involved in the killing process. AMs from MMP12-deficient mice demonstrated no impairment in *P. murina* killing in vitro (data not shown), and we failed to observe an effect of *Mmp12* deficiency on clearance of *P. murina* from the lungs, even at time points in which *Mmp12* was highly expressed. There was also no effect on *Mmp12* deficiency on the lung inflammatory response to *P. murina*. Although we cannot exclude the possibility that immunosuppressed MMP12-deficient mice (CD4-depleted, steroid-treated, etc.) would demonstrate a clearance defect compared with immunosuppressed control mice, our data indicate that, in immunocompetent mice, MMP12 is not required for *P. murina* clearance.

In summary, we took a bedside-to-bench approach to better understand the association between *Pneumocystis* colonization and elevated MMP12 levels. Our data show that MMP12 is induced primarily in alternatively activated (M2a) AMs in a CD4-dependent, STAT6-dependent manner during *P. murina* lung infection. However, MMP12 is not required for the elimination of *P. murina* from the lungs. Collectively, these results suggest that a consequence of *P. murina* lung infection is the normal development of alternatively activated AMs, a component of which is MMP12 production. Acknowledging the pathogenic association of MMP12 with COPD, our data provide critical insight into why *Pneumocystis* colonization may be associated with this lung disease.

**REFERENCES**


