Tissue Inhibitor of Metalloproteinases-3 Moderates the Proinflammatory Status of Macrophages

Sean E. Gill¹, Sina A. Gharib¹, Eli M. Bench¹, Samuel W. Sussman¹, Roy T. Wang¹, Cliff Rims¹, Timothy P. Birkland¹, Ying Wang¹, Anne M. Manicone¹, John K. McGuire¹, and William C. Parks¹

¹Center for Lung Biology, University of Washington, Seattle, Washington

Tissue inhibitor of metalloproteinases-3 (TIMP-3) has emerged as a key mediator of inflammation. Recently, we reported that the resolution of inflammation is impaired in *Timp3*^{-/-} mice after bleomycin-induced lung injury. Here, we demonstrate that after LPS instillation (another model of acute lung injury), *Timp3*^{-/-} mice demonstrate enhanced and persistent neutrophilia, increased numbers of infiltrated macrophages, and delayed weight gain, compared with wild-type (WT) mice. Because macrophages possess broad immune functions and can differentiate into cells that either stimulate inflammation (M1 macrophages) or are immunosuppressive (M2 macrophages), we examined whether TIMP-3 influences macrophage polarization. Comparisons of the global gene expression of unstimulated or LPS-stimulated bone marrow-derived macrophages (BMDMs) from WT and *Timp3*^{-/-} mice revealed that *Timp3*^{-/-} BMDMs exhibited an increased expression of genes associated with proinflammatory (M1) macrophages, including *Il6*, *Il12*, *Nos2*, and *Ccl2*. Microarray analyses also revealed a baseline difference in gene expression between WT and *Timp3*^{-/-} BMDMs, suggesting altered macrophage differentiation. Furthermore, the treatment of *Timp3*^{-/-} BMDMs with recombinant TIMP-3 rescued this altered gene expression. We also examined macrophage function, and found that *Timp3*^{-/-} M1 cells exhibit significantly more neutrophil chemotactic activity and significantly less soluble Fas ligand-induced caspase-3/7 activity, a marker of apoptosis, compared with WT M1 cells. Macrophage differentiation into immunosuppressive M2 cells is mediated by exposure to IL-4/IL-13, and we found that *Timp3*^{-/-} M2 macrophages demonstrated a lower expression of genes associated with an anti-inflammatory phenotype, compared with WT M2 cells. Collectively, these findings indicate that TIMP-3 functions to moderate the differentiation of macrophages into proinflammatory (M1) cells.

Keywords: resolution of inflammation; macrophage; metalloproteinase; lung injury

Several immunosuppressive mechanisms have evolved to ensure that acute, generally beneficial inflammation does not progress to chronic, destructive inflammation. Beyond reversal of the initiating event (e.g., bacterial clearance and wound closure), active host processes, such as the release of the anti-inflammatory

CLINICAL RELEVANCE

Our study addresses the role of tissue inhibitor of metalloproteinases (TIMP)-3 in regulating macrophage polarization after lung injury through the use of transgenic mice lacking TIMP-3. Ultimately, we have identified a key role for TIMP-3 in regulating macrophage differentiation, and subsequently, the ability to undergo proinflammatory polarization.

cytokines IL-4, IL-10, and IL-13, among other processes (1), actively repress the proinflammatory properties of various cell types. Macrophages, through their ability to clear neutrophils and release anti-inflammatory cytokines, are thought to be important in the resolution of inflammation (2).

Generally speaking, macrophages are key effectors promoting the shift from a proinflammatory to an anti-inflammatory environment, and can be broadly classified into two groups: classically activated (M1) and alternatively activated (M2) macrophages, which can be further subdivided into specialized M2 groups (3). M1 macrophages are induced by proinflammatory Th1 cytokines, such as IFN- γ , and are characterized by the production of proinflammatory factors such as IL-6, inducible nitric oxide synthase (iNOS), TNF- α , and monocyte chemoattractant protein (MCP)-1/CCL-2. The M2 phenotype is induced by the Th2 cytokines IL-4 and IL-13, and is characterized by the production of anti-inflammatory molecules such as IL-10 and transforming growth factor (TGF)- β 1 (2-6). Although key cytokine agonists of macrophage polarity have been identified, the functional heterogeneity of these effector leukocytes can be further honed by both tissue environmental factors (e.g., the extracellular matrix) and other cell autonomous mediators.

Acute lung injury (ALI), which can progress to acute respiratory distress syndrome, is characterized by profound inflammation and tissue injury, often resulting from severe pulmonary infection or trauma (7). In 25 to 40% of ALI cases, failure to resolve inflammation after injury occurs, leads to chronic inflammation and persistent pulmonary dysfunction. The role of macrophages in lung injury is diverse, and is shaped by the mode of injury. For example, a reduction of macrophages during ventilator-induced, hyperoxia-induced, or endotoxin-induced lung injury often leads to less severe inflammation, suggesting that macrophages are key propagators of the inflammatory response (8-11). In lung infection, however, the absence of macrophages often leads to enhanced inflammation and decreased survival, demonstrating a role for macrophages in clearing infection, as well as moderating the inflammatory response (12-14). As these studies suggest, macrophages are key contributors to both the promotion and resolution of lung inflammation. Thus, an accumulation of the appropriate population (M1 versus M2) of macrophages in the lung must be tightly regulated.

Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an inhibitor of both the matrix metalloproteinases (MMPs) and the

(Received in original form September 23, 2012 and in final form May 26, 2013)

This work was supported by National Institutes of Health grants HL082658, HL089455, HL098067, HL093022, and DK089507 (W.C.P.). S.E.G. is supported by a Parker B. Francis Fellowship.

Correspondence and requests for reprints should be addressed to Sean E. Gill, Ph.D., now at the Victoria Research Laboratories, Centre for Critical Illness Research, 800 Commissioners Road East, A6-134, London, ON, N6A 4G5 Canada. E-mail: sgill8@uwo.ca

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 5, pp 768-777, Nov 2013

Copyright © 2013 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2012-0377OC on June 6, 2013

Internet address: www.atsjournals.org

related ADAM (a disintegrin and metalloproteinase) family of extracellular enzymes (15–17). Through the inhibition of these enzymes, particularly ADAM-17, TIMP-3 plays a key role in regulating inflammation after injury (18–20). TIMP-3 is expressed by multiple cell types throughout many tissues, including macrophages, and via its ability to silence various metalloproteinases, it regulates the shedding of cytokines and their receptors from the cell surface of macrophages (20).

TIMP-3 also affects macrophage function in atherosclerosis, suggesting that this inhibitor may regulate macrophage polarization in response to the environment (21, 22). Recently, we demonstrated that the absence of TIMP-3 resulted in increased neutrophil accumulation in the lungs after bleomycin-induced lung injury, ultimately leading to an impaired resolution of inflammation (23). In this study, we confirm the role of TIMP-3 in regulating inflammation after lung injury, and identify TIMP-3 as a key moderator of macrophage proinflammatory (M1) polarization.

MATERIALS AND METHODS

For detailed methods, please see the online supplement.

Mouse Model and Treatments

The Institutional Animal Care and Use Committee at the University of Washington approved all animal use procedures. Eight-week-old wild-type (WT; C57Bl/6J) and *Timp3*^{-/-} mice were anesthetized, intubated, and exposed to LPS (3.5 mg/kg). Mice were weighed daily and killed at the indicated times, their lungs were harvested, and bronchoalveolar lavage (BAL) was isolated as described elsewhere (23).

Mouse Bone Marrow–Derived Macrophages

Bone marrow was isolated from WT and *Timp3*^{-/-} mice and cultured for 6 days, as described elsewhere (23–26). In a subset of experiments, recombinant His-tagged TIMP-3 (rTIMP-3-His) was added at 10 µg/ml to the culture medium.

Microarray Experiments and Data Analysis

Microarray experiments. Bone marrow–derived macrophages (BMDMs) were maintained in medium (M0 macrophages) or exposed to 100 ng/ml LPS (M1 macrophages). Total RNA was isolated 24 hours later (RNeasy Mini-Kit; Qiagen, Inc., Valencia, CA), labeled, and hybridized to the Mouse Ref-8 version 2.0 Expression Bead Chip Kit (Illumina, Inc., San Diego, CA). Background adjustment and quantile normalization were performed using Bead Studio software (Illumina, Inc.).

Correspondence analysis. We performed multidimensional scaling of whole-genome transcriptional profiles of the 16 samples, using correspondence analysis (27).

Differential gene expression and functional analysis. Normalized microarray data that met the detection threshold were used to identify differentially expressed genes, using a Bayesian implementation of the parametric *t* test (28), adjusted for multiple comparisons using a *Q*-value cutoff of less than 0.05 (29). We applied pairwise comparisons using these tests to identify significant gene-expression changes between genotypes (i.e., *Timp3*^{-/-} M0 versus WT M0 and *Timp3*^{-/-} M1 versus WT M1). Differentially expressed genes (*Q*-value < 0.05) underwent functional analysis, using the Database for Annotation, Visualization, and Integrated Discovery (30). A permutation-based false-discovery analysis was used to correct for multiple hypothesis testing, with a significance cutoff of less than 0.05.

Cluster analysis. Two-dimensional hierarchical clustering was performed, using normalized gene-expression values based on the Pearson correlation metric (31).

Transcription factor analysis. A computational transcription factor (TF) analysis of differentially expressed genes in *Timp3*^{-/-} M1 versus WT M1 BMDMs was performed, using Expander software (32). The enrichment of TFs was determined using a Bonferroni-adjusted *P*-value cutoff of less than 0.01.

Figure 1. Inflammation is enhanced in mice lacking tissue inhibitor of metalloproteinases-3 (*Timp3*^{-/-}) compared with wild-type (WT) mice after LPS-induced lung injury. (A) An examination of the bronchoalveolar lavage (BAL) fluid of WT and *Timp3*^{-/-} mice at 2, 4, and 6 days after LPS instillation revealed that total neutrophils were significantly increased at all time points examined, and that total macrophages were significantly increased at 2 days after LPS instillation in *Timp3*^{-/-} mice compared with WT mice (**P* < 0.05, unpaired *t* test between genotypes at each time point; *n* = 3–12). (B) Weight loss in WT and *Timp3*^{-/-} mice after the instillation of LPS was similar between genotypes. However, weight gain was delayed by 1 day in *Timp3*^{-/-} mice compared with WT mice (*n* = 3–9). All graphs include error bars (± SEM).

Network analysis. Differentially expressed genes were also used as seeds to create a gene product network derived from interaction data-bases, including Ingenuity and STRING (33, 34).

Quantitative RT-PCR

Total RNA from BMDMs was isolated and analyzed as described elsewhere (23). Samples from *Timp3*^{-/-} BMDMs were normalized to those from WT BMDMs.

Flow Cytometry

WT BMDMs, cultured with or without rTIMP-3-His for 24 hours, were incubated with Fc receptor block (BD Pharmingen, San Diego, CA) and stained with Penta-His-Alexa 488 (Qiagen, Inc.), according to the manufacturer's protocol.

Neutrophil Chemotaxis Assay

WT neutrophil chemotaxis toward conditioned media from unstimulated or LPS-stimulated WT and *Timp3*^{-/-} BMDMs was measured using a microchemotaxis assay, as described elsewhere (23, 24, 35).

BMDM Apoptosis Assay

BMDMs were stimulated with PBS, staurosporine (2 μ M), or soluble Fas ligand (sFasL; 500 pg/ml). BMDM apoptosis was determined using a caspase-3/7 activity assay, according to the manufacturer's protocol (Cell Technology, Mountain View, CA).

RESULTS

TIMP-3 Regulates Inflammation after Lung Injury

We reported that neutrophil accumulation persists in the lungs of bleomycin-injured *Timp3*^{-/-} mice, indicating that TIMP-3 functions in the resolution of inflammation (23). To assess whether TIMP-3 serves a similar function in other models of lung inflammation, we challenged WT and *Timp3*^{-/-} mice with instilled LPS. Similar to bleomycin-induced injury, *Timp3*^{-/-} mice demonstrated significantly increased neutrophil accumulation compared with WT mice at all times after the instillation of LPS (2, 4, and 6 days after instillation; Figure 1A), particularly at 6 days, when neutrophilia was resolving in WT mice but persisted (> 2-fold) in *Timp3*^{-/-} mice. Mice lacking TIMP-3 also exhibited significantly increased macrophage accumulation on Day 2 after LPS, compared with WT mice (Figure 1A). Furthermore, whereas WT mice began to gain weight at 3 days after LPS, *Timp3*^{-/-} mice continued to lose weight until Day 4 (Figure 1B). Complementary to our previous findings (23), these data demonstrate that TIMP-3 functions to moderate inflammation after lung injury.

Macrophage Polarization Is Altered in *Timp3*^{-/-} Macrophages

We focused on TIMP-3 functioning in macrophages for two reasons. First, TIMP-3 does not appear to exert a direct effect on neutrophil function (23). Thus, the increased neutrophil accumulation observed in *Timp3*^{-/-} mice after lung injury would likely be attributable to the action of an immunoeffector cell population. Second, depending on their state of activation, macrophages are capable of promoting or suppressing inflammation.

Although murine macrophages are known to express *Timp3*, its relative expression among functionally distinct macrophage subpopulations has not been determined (20). Thus, we compared *Timp3* mRNA levels in unstimulated (M0), LPS-stimulated (M1), and IL-4/IL-13-stimulated (M2) macrophages at 24 and 48 hours. Whereas *Timp3* was expressed at relatively

low levels in M0 and M2 macrophages at both times (threshold cycle [C_T] = 30–31), a fourfold increase in *Timp3* expression was evident in M1 macrophages at 24 hours after stimulation. This increase returned to baseline levels by 48 hours (Figure 2A).

The expression data indicate that TIMP-3 appears to be expressed preferentially, and hence may function in M1-polarized macrophages. To assess this idea, we used microarray analyses with RNA from WT and *Timp3*^{-/-} BMDMs, treated without or with LPS (M0 and M1, respectively) for 24 hours, to examine the role of TIMP-3 in regulating proinflammatory macrophage polarization. Correspondence analyses of WT and *Timp3*^{-/-} BMDMs revealed a distinct clustering of samples based on both exposure (LPS) and genotype, indicating that genotype was responsible for a significant amount of the variance in overall gene expression (Figure 2B).

We further explored genotype-dependent transcriptional perturbations by identifying many differentially expressed genes between WT and *Timp3*^{-/-} BMDMs at baseline (404 unique genes) and after LPS stimulation (148 unique genes; Figure 3 and Tables E1 and E2 in the online supplement). A subset of genes identified by microarray analyses as differentially expressed between WT and *Timp3*^{-/-} BMDMs was confirmed by quantitative

Figure 2. Absence of TIMP-3 leads to altered gene expression in bone marrow-derived macrophages (BMDMs). (A) An examination of *Timp3* expression by quantitative PCR in BMDMs stimulated with LPS or IL-4/IL-13 revealed that *Timp3* expression was significantly increased in LPS-stimulated BMDMs at 24 hours after stimulation (* $P < 0.05$, versus 24 hours of unstimulated BMDMs, one-way ANOVA with the Tukey *post hoc* test; $n = 3$). All graphs include error bars ($\pm$ SEM). (B) Correspondence analysis of WT and *Timp3*^{-/-} BMDMs treated with and without LPS (M1 and M0, respectively) demonstrated a distinct clustering of samples, based first on exposure and second on genotype, implying that genotype was responsible for a significant amount of the variation ($n = 4$).

Figure 3. Bone marrow-derived macrophages (BMDMs) from wild-type (WT) and tissue inhibitor of metalloproteinases-3 null (*Timp3*^{-/-}) mice are significantly different at baseline. (A) Heat maps depict genes with significantly different expression levels between WT and *Timp3*^{-/-} BMDMs that were unstimulated (M0, 404 genes) or lipopolysaccharide (LPS)-stimulated (M1, 148 genes; *n* = 4). (B) Functional analyses highlighted a number of processes that were significantly enriched in *Timp3*^{-/-} BMDMs compared with WT BMDMs, including many groups associated with the promotion of inflammation (*n* = 4).

RT-PCR (Figure E1). Furthermore, within our microarray experiments, *Il6* did not meet the detection threshold for transcripts, and *Ccl2* was detected but not significantly different between genotypes. Analysis according to quantitative RT-PCR, however, revealed that the expression of *Il6* and *Ccl2* was significantly increased in *Timp3*^{-/-} BMDMs compared with WT BMDMs after LPS stimulation (Figure E1). We also found that *Timp3* was detected by microarray analysis in the macrophages of *Timp3*^{-/-} mice. This finding is attributable to the design of the *Timp3* deletion, which may allow for some transcription to occur, but importantly, does not allow for the translation of a functional protein (38). Functional analyses of these differentially expressed genes revealed a number of processes that were significantly enriched in both unstimulated (M0) and LPS-stimulated (M1) *Timp3*^{-/-} BMDMs compared with WT BMDMs (Figure 3). Many of these functional groups were associated with the promotion of inflammation, and included processes associated with immunity, the response to wounding, leukocyte chemotaxis, and apoptosis (Figure 3).

Network analysis based on known gene-product interactions was performed on genes expressed differentially between WT and *Timp3*^{-/-} LPS-stimulated BMDMs, including those identified by quantitative RT-PCR. This analysis revealed that LPS-treated macrophages from *Timp3*^{-/-} mice exhibited an increased expression of proinflammatory or M1-associated genes (specifically, *Nos2*, *Il6*, *Cd40*, *Tnfa*, *Ccl2*, *Ccl3*, *Ccl4*, and *Cxcl10*), compared with LPS-treated WT macrophages (Figure 4). Furthermore, computational TF analyses of these differentially expressed genes revealed that a significant number possessed interferon-stimulated response elements (ISREs; Figure 4). Interferon response factor-1, a TF known to bind ISREs, was one of these up-regulated genes (Figure 4).

To confirm that the differential gene expression observed in *Timp3*^{-/-} macrophages was attributable to a lack of TIMP-3, WT and *Timp3*^{-/-} BMDMs were treated with rTIMP-3-His during macrophage differentiation. Using flow cytometry, we were able to detect that rTIMP-3-His had bound to the surface of WT macrophages (Figure 5A). Furthermore, the treatment of *Timp3*^{-/-}

Figure 4. Polarization of macrophages lacking tissue inhibitor of metalloproteinases-3 (*Timp3*^{-/-}) is skewed toward a proinflammatory phenotype. The gene expression of WT and *Timp3*^{-/-} BMDMs stimulated with LPS (100 ng/ml) for 24 hours was analyzed by microarrays (Illumina Mouse Ref-8 Bead Chip; Illumina, Inc., San Diego, CA). A network analysis, based on known gene-product interactions, was performed on genes differentially expressed between WT and *Timp3*^{-/-} BMDMs. This analysis revealed an up-regulation of genes (in red) associated with proinflammatory (M1) macrophages in *Timp3*^{-/-} BMDMs (*n* = 4). The interferon-stimulated response element (ISRE) binding site was highly overrepresented among these differentially expressed genes, and is a known target of the up-regulated transcription factor interferon response factor (IRF)-1.

BMDMs with rTIMP-3-His returned gene expression to levels similar to those observed in WT macrophages (Figure 5B). However, this rescue of gene expression by rTIMP-3-His only occurred when BMDMs were cultured with rTIMP-3-His during differentiation. The addition of rTIMP-3-His to differentiated WT and *Timp3*^{-/-} BMDMs during stimulation with LPS exerted a varied effect on gene expression in both WT and *Timp3*^{-/-} BMDMs (Figure E2). We also observed that the treatment of WT macrophages with rTIMP-3 led to a significant reduction in the expression of *Nos2*, compared with WT macrophages treated with PBS alone (Figure 5B).

We next examined the expression of a subset of genes at different time points after stimulation. Analyses of *Il6* and *Nos2* expression by WT and *Timp3*^{-/-} BMDMs at 8 and 24 hours after LPS revealed that in some cases, the differential expression between genotypes appeared to be dependent on time (Figure 5C). These data, together with our rTIMP-3-His rescue data, suggest that TIMP-3 may be involved in macrophage differentiation. Analyses of *Timp3* expression in bone marrow cells throughout macrophage differentiation revealed that the highest level of *Timp3* expression occurred on Days 0 and 1 in undifferentiated bone marrow cells (*C_T* = 28–29), and decreased thereafter (Figure 5D).

We also examined the expression of genes associated with alternatively activated (M2) macrophages after 48 hours of stimulation with IL-4/IL-13. The expression of these genes, including *Arg1*, *Fizz1*, *Mrc1*, and *Ccl2*, was decreased in *Timp3*^{-/-} BMDMs compared with WT BMDMs, suggesting that the ability of *Timp3*^{-/-} macrophages to respond to IL-4/IL-13 and polarize toward an M2 phenotype was blunted (Figure 5E). Collectively, these data support a role for TIMP-3 in the regulation of macrophage differentiation, and ultimately in the moderation of the proinflammatory polarization of macrophages. Compared with WT macrophages, *Timp3*^{-/-} macrophages demonstrate an increased expression of genes associated with proinflammatory (M1) macrophages after LPS stimulation, and

a decreased expression of genes associated with the anti-inflammatory/reparative (M2) macrophages after IL-4/IL-13 stimulation.

TIMP-3 Regulates Macrophage Function

Our functional analysis of genes differentially expressed between WT and *Timp3*^{-/-} BMDMs revealed specific biological processes that were enriched, including genes associated with endocytosis, leukocyte chemotaxis, and apoptosis (Figure 3). To confirm these findings, we examined the ability of macrophages to induce neutrophil chemotaxis, as well as undergo apoptosis, in response to multiple stimuli (Figure 6). For these experiments, we used conditioned media collected from WT and *Timp3*^{-/-} BMDMs that were unstimulated (data not shown) or stimulated with LPS to induce WT neutrophil chemotaxis. *Timp3*^{-/-} BMDMs induced a 30% increase in neutrophil chemotaxis, compared with WT BMDMs (Figure 6A).

Analyses of caspase-3/7 activity in unstimulated (M0) and LPS-stimulated (M1) WT and *Timp3*^{-/-} BMDMs after incubation with either staurosporine or sFasL were used to examine macrophage apoptosis. LPS-stimulated WT macrophages were found to be resistant to staurosporine-induced apoptosis. However, stimulation with sFasL led to a striking increase in apoptosis among WT M1 macrophages, compared with M0 macrophages. Caspase-3/7 activity increased 4.4-fold over PBS control samples in M1 macrophages, versus 2.9-fold in M0 macrophages (Figure 6C). *Timp3*^{-/-} BMDMs appear to be resistant to apoptosis compared with WT BMDMs under all conditions, with significantly less caspase-3/7 activity in M0 macrophages when stimulated with staurosporine, as well as in M1 macrophages when stimulated with sFasL (Figure 6B).

To explore the role of TIMP-3 further in mediating macrophage apoptosis, we treated WT M0 and M1 BMDMs with rTIMP-3-His during either differentiation or polarization, and then induced apoptosis through treatment with sFasL (Figures

Figure 5. Tissue inhibitor of metalloproteinases (TIMP)-3 regulates macrophage polarization after stimulation with LPS or IL-4/IL-13. (A) Recombinant TIMP-3 (rTIMP-3) was detected on the cell surface of WT BMDMs by flow cytometric analysis ($n = 4$). (B) An analysis by quantitative PCR of gene expression in LPS-stimulated WT and *Timp3*^{-/-} BMDMs in the absence (Ctl) or presence of rTIMP-3 demonstrated that treatment with rTIMP-3 returned gene expression in *Timp3*^{-/-} BMDMs to levels similar to those observed in the WT BMDMs (* $P < 0.05$, versus WT Ctl; # $P < 0.05$, versus *Timp3*^{-/-} Ctl; two-way ANOVA with Bonferroni *post hoc* test; $n = 4-8$). Data are expressed as percent WT LPS-stimulated Ctl. (C) An experiment regarding *Il6* and *Nos2* expression by quantitative PCR in WT and *Timp3*^{-/-} BMDMs at 8 and 24 hours after stimulation with LPS revealed that the differential expression between genotypes is time-dependent for certain genes. (* $P < 0.05$, versus WT at each time point, two-way ANOVA with Bonferroni *post hoc* test; $n = 4$). (D) *Timp3* gene expression, as determined by quantitative PCR, was highest in undifferentiated bone marrow cells, and decreased throughout macrophage differentiation. (E) An examination by quantitative PCR of genes associated with anti-inflammatory (M2) macrophage polarization in WT and *Timp3*^{-/-} BMDMs stimulated with IL-4/IL-13 for 48 hours revealed that *Arg1*, *Mrc1*, and *Ccl2* expression was significantly decreased in *Timp3*^{-/-} BMDMs, compared with WT BMDMs (* $P < 0.05$, unpaired *t* test; $n = 6$). All graphs include error bars ($\pm$ SEM).

E3A and E3B, respectively). Treatment with rTIMP-3-His during differentiation exerted little effect on the ability of the macrophage to respond to apoptotic stimuli. However, the addition

of rTIMP-3 during polarization resulted in significantly more caspase-3/7 activity in M1-polarized macrophages when stimulated with sFasL (Figure E3B).

Figure 6. Lack of tissue inhibitor of metalloproteinase (TIMP)-3 alters macrophage function. (A) Conditioned media from LPS-stimulated *Timp3*^{-/-} BMDMs elicited significantly more neutrophil chemotaxis than conditioned media from LPS-stimulated WT BMDMs (**P* < 0.05, unpaired *t* test; *n* = 4). (B) An analysis of caspase-3/7 activity in unstimulated (M0) and LPS-stimulated (M1) WT and *Timp3*^{-/-} BMDMs after 2, 4, and 8 hours treatment with staurosporine or soluble Fas ligand (sFasL) revealed that *Timp3*^{-/-} BMDMs demonstrate significantly less caspase-3/7 activity. This difference was most apparent in M1 BMDMs after 2 and 4 hours of treatment with sFasL (**P* < 0.05, unpaired *t* test between genotypes at each time point; *n* = 3–6). All graphs include error bars ($\pm$ SEM).

Together, these data suggest that *Timp3*^{-/-} macrophages are functionally different from WT macrophages. Furthermore, the chemotaxis data suggest that the *Timp3*^{-/-} macrophages may, at least in part, be responsible for the increased accumulation of neutrophils in *Timp3*^{-/-} mice after lung injury, and this resistance to apoptosis may be responsible for the accumulation of macrophages in the lungs of *Timp3*^{-/-} mice after lung infection.

DISCUSSION

In this study, we identify TIMP-3 as a mediator of macrophage polarization and function (Figure 7). In the absence of TIMP-3, macrophage differentiation is altered, resulting in macrophages that are skewed toward a more proinflammatory polarization, with an increased expression of genes associated with classically activated (M1) macrophages and a decreased expression of genes associated with alternatively activated (M2) macrophages after stimulation with LPS or IL-4/IL-13, respectively. Furthermore, we demonstrate that in addition to macrophage polarization, macrophage function is also altered in *Timp3*^{-/-} macrophages. A lack of TIMP-3 leads to increased recruitment of neutrophils, and survival. We also provide evidence that the control of macrophage function may be one of the mechanisms used by TIMP-3 to moderate inflammation after lung infection.

The role of TIMP-3 in cells of the myeloid lineage has been previously examined in a few focused studies. In bone marrow, *Timp3* is predominantly expressed by stromal cells, with much lower expression in hematopoietic stem cells (HSCs). However, TIMP-3 plays a role in regulating HSC proliferation and differentiation (36, 37). TIMP-3, independent of its ability to inhibit metalloproteinases, promotes HSC proliferation, and the absence of TIMP-3 leads to impaired recovery from myelosuppression (37). In addition, the overexpression of human TIMP-3 in mouse HSCs leads to increased myelopoiesis and subsequently increased CD11b⁺ myeloid cells (36). Although we did not observe differences in the overall composition of white blood cell populations,

our experiments provide support for these earlier findings, because we observed differences between unstimulated WT and *Timp3*^{-/-} BMDMs at baseline (Figures 2 and 3). Moreover, we found that only rTIMP-3-His, added during macrophage differentiation, rescued gene expression in *Timp3*^{-/-} BMDMs, and that *Timp3* expression decreased throughout macrophage differentiation (Figure 5). These data suggest that *Timp3*^{-/-} BMDMs already possess a different capacity to respond to stimuli, and that TIMP-3 plays a role in regulating macrophage differentiation and function.

These same studies also found that *Timp3* expression in mature leukocytes is low (36), a finding our data support (average C_T in BMDMs = 31.3). However, macrophages likely synthesize only the amount of TIMP-3 (or any protein) to affect specific processes, and we did observe an increase in *Timp3* expression in LPS-stimulated BMDMs over both unstimulated and IL-4/IL-13-stimulated BMDMs (Figure 2A). Furthermore, our data demonstrating that the addition of rTIMP-3-His during polarization (and hence its addition to differentiated BMDMs) leads to increased caspase-3/7 activity (Figure E3) suggest that this increased *Timp3* expression in M1-polarized BMDMs may be responsible for the enhanced response to sFasL. Together, these data suggest that even low *Timp3* expression by mature macrophages plays a role in the differentiated macrophage. Furthermore, the timing of the addition of rTIMP-3-His appears to be an important factor in determining how macrophage function is mediated by TIMP-3. Specifically, the addition of rTIMP-3-His during differentiation modifies gene expression, but exerts no effect on the response to apoptotic stimuli, whereas the addition of rTIMP-3-His during polarization exerts a variable effect on gene expression, but enhances the response to apoptotic stimuli. This suggests that endogenous levels of *Timp3* expression are finely tuned to keep its activity limited to the desired targets, and emphasizes the importance of the careful interpretation of data obtained from the addition of exogenous TIMP-3, because of possible off-target effects and unpredictable findings. Studies

Figure 7. Tissue inhibitor of metalloproteinase (TIMP)-3 promotes the resolution of inflammation through the modulation of macrophage polarization. The expression of *Timp3* during macrophage differentiation and by proinflammatory (M1) macrophages allows for the moderation of M1 polarization and a shift toward anti-inflammatory (M2) polarization. In the absence of TIMP-3 (*Timp3*^{-/-} mice), macrophage polarization is skewed toward a proinflammatory phenotype (M1), leading to continued neutrophil accumulation after lung injury and failed resolution of inflammation.

are ongoing to further understand the role of TIMP-3 in macrophage function.

TIMP-3 has been shown by others to play a functional role in macrophages (20, 22, 36). Smookler and colleagues found that TIMP-3 regulates the shedding of TNF- α and the TNF- α receptor (TNFR) from the cell surface of macrophages *in vitro* and *in vivo* after stimulation with LPS (20). Moreover, TIMP-3 was found to inhibit ADAM-17 activity specifically in human monocytes after stimulation with LPS, thereby restricting TNF- α shedding (39). TNF- α activates pathways regulated by the interferon-stimulated response element, and thus the increased shedding of TNF- α from BMDMs could provide a potential mechanism driving proinflammatory macrophage polarization.

Although we did detect significant increases in the TNF- α shed from *Timp3*^{-/-} compared with WT BMDMs, in line with the findings of Smookler and colleagues (20), these differences were only minimal in LPS-stimulated (M1) macrophages (an approximately 12% increase). In unstimulated (M0) macrophages, TNF- α levels were almost below the limit of detection (< 10 pg/ml). Thus, although one likely function for macrophage-derived TIMP-3 involves the regulation of ADAM-17 activity, we conclude that the enhanced shedding of TNF- α was unlikely to be responsible for the altered macrophage polarization we observed.

Other groups have demonstrated that TIMP-3 affects atherosclerosis, likely through regulating macrophage function (21, 22). The overexpression of TIMP-3, specifically in macrophages, leads to smaller, more stable atherosclerotic plaques that contain fewer inflammatory cells (specifically, T cells and macrophages) (22). These findings suggest that increasing *Timp3* expression in macrophages protects against atherosclerotic plaque formation, and potentially promotes an anti-inflammatory phenotype in macrophages. Conversely, other studies have identified a subset of foamy macrophages with low *Timp3* expression and high *Mmp14* expression that are highly invasive and that demonstrate increased proliferation and apoptosis, which further supports a role for TIMP-3 in the regulation of inflammation in atherosclerosis. Our data that *Timp3*^{-/-} macrophages were skewed toward proinflammatory polarization and promoted increased neutrophil chemotaxis support the findings that TIMP-3 in macrophages is associated with decreased inflammation in atherosclerotic plaques. Together, these studies and our data suggest that macrophage-derived TIMP-3 serves to moderate inflammation, and thereby allow for anti-inflammatory (M2) macrophage polarization.

Macrophages have the ability to both promote and inhibit inflammation, both of which are required to allow for tissue repair after injury and infection (2–4). This dual role for macrophages is especially true in the lung, where macrophages are required to clear bacteria and promote inflammation after lung infection, but are also required to promote the resolution of inflammation and fibrosis after injury (8–14). Thus, mechanisms regulating macrophage function in the lung, especially those that mediate the ability of the macrophage to promote the resolution of inflammation, must be determined. One possible mechanism involves the clearance of proinflammatory macrophages via apoptosis. Although multiple members of the TNF superfamily, including TNF- α and sFasL, are known to induce macrophage apoptosis, in the setting of lung injury, different subsets of macrophages appear susceptible to different stimuli (40, 41). For example, recruited macrophages, but not resident macrophages, appear to be highly susceptible to sFasL-mediated apoptosis after the instillation of LPS or viral infection (40).

In our study, we demonstrate that the absence of TIMP-3 leads to altered macrophage function, and most notably, increased resistance to apoptotic stimuli (Figure 6C). Furthermore, the addition of exogenous TIMP-3 to WT macrophages leads to an enhanced response to apoptotic stimuli (Figure E3). TIMP-3 has long been associated with apoptosis, and is generally thought to be proapoptotic. The overexpression of TIMP-3 in a number of different cell lines leads to the stabilization of cell-surface “death receptors” (Fas and TNFR), increased caspase activation, and cell death (42–47). Although our finding that *Timp3*^{-/-} BMDMs were resistant to sFasL-induced apoptosis supports these previous studies, we have been unable to demonstrate increased Fas shedding from *Timp3*^{-/-} cells, as might be predicted (data not shown). These negative data suggest that increased resistance to apoptosis was not attributable to enhanced Fas shedding, but instead was potentially attributable to the uninhibited shedding of another death receptor, such as TNFR-1, which is a relevant

target in Fas-induced apoptosis in the liver (47). Our findings, however, that TIMP-3 moderates the macrophage response to apoptotic stimuli suggest that TIMP-3 is a crucial factor in determining whether proinflammatory, recruited macrophages are cleared appropriately after lung injury. The prolonged survival of these proinflammatory macrophages would be detrimental and would likely lead to enhanced neutrophilia and an impaired resolution of inflammation, similar to what we observed in *Timp3*^{-/-} mice after bleomycin-induced or LPS-induced lung injury.

Thus, our study, along with previous studies, provides evidence that TIMP-3 plays a critical role in regulating macrophage differentiation and, consequently, macrophage polarization and function during injury (Figure 7). A lack of TIMP-3 leads to a more proinflammatory, apoptosis-resistant macrophage, and subsequently, increased neutrophil accumulation in the lung after injury or larger, less stable plaques in atherosclerosis. The overexpression of TIMP-3, however, results in a more anti-inflammatory macrophage, which potentially promotes the resolution of inflammation. Although the inhibition of ADAM-17 is likely one of the mechanisms used by TIMP-3 to regulate macrophage function, it is unlikely the sole mechanism. Ongoing studies will address this question, and will further examine the role of TIMP-3 in regulating the resolution of inflammation after lung injury.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Charles Frevert, Brian Johnson, and Cara Appel for technical and analytic assistance.

References

- Nathan C, Ding A. Nonresolving inflammation. *Cell* 2010;140:871–882.
- Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* 2011;11:723–737.
- Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J Immunol* 2008;181:3733–3739.
- Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3:23–35.
- Ma J, Chen T, Mandelin J, Ceponis A, Miller NE, Hukkanen M, Ma GF, Kontinen YT. Regulation of macrophage activation. *Cell Mol Life Sci* 2003;60:2334–2346.
- Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity* 2005;23:344–346.
- Wheeler AP, Bernard GR. Acute lung injury and the acute respiratory distress syndrome: a clinical review. *Lancet* 2007;369:1553–1564.
- Berg JT, Lee ST, Thepen T, Lee CY, Tsan MF. Depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphate. *J Appl Physiol* 1993;74:2812–2819.
- Berg JT, White JE, Tsan MF. Response of alveolar macrophage-depleted rats to hyperoxia. *Exp Lung Res* 1995;21:175–185.
- Eyal FG, Hamm CR, Parker JC. Reduction in alveolar macrophages attenuates acute ventilator induced lung injury in rats. *Intensive Care Med* 2007;33:1212–1218.
- Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA. Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L1191–L1198.
- Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R III, Standiford TJ. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumoniae*: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect Immun* 1997;65:1139–1146.
- Hashimoto S, Pittet JF, Hong K, Folkesson H, Bagby G, Kobzik L, Frevert C, Watanabe K, Tsurufuji S, Wiener-Kronish J. Depletion of alveolar macrophages decreases neutrophil chemotaxis to *Pseudomonas* airspace infections. *Am J Physiol* 1996;270:L819–L828.
- Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, Sawa T. Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect Immun* 1998;66:3164–3169.
- Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J Cell Sci* 2002;115:3719–3727.
- Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim Biophys Acta* 2000;1477:267–283.
- Wei S, Kashiwagi M, Kota S, Xie Z, Nagase H, Brew K. Reactive site mutations in tissue inhibitor of metalloproteinase-3 disrupt inhibition of matrix metalloproteinases but not tumor necrosis factor- α -converting enzyme. *J Biol Chem* 2005;280:32877–32882.
- Black RA. TIMP3 checks inflammation. *Nat Genet* 2004;36:934–935.
- Mohammed FF, Smookler DS, Taylor SE, Fingleton B, Kassiri Z, Sanchez OH, English JL, Matrisian LM, Au B, Yeh WC, et al. Abnormal TNF activity in *Timp3*^{-/-} mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet* 2004;36:969–977.
- Smookler DS, Mohammed FF, Kassiri Z, Duncan GS, Mak TW, Khokha R. Tissue inhibitor of metalloproteinase 3 regulates TNF-dependent systemic inflammation. *J Immunol* 2006;176:721–725.
- Johnson JL, Sala-Newby GB, Ismail Y, Aguilera CM, Newby AC. Low tissue inhibitor of metalloproteinases 3 and high matrix metalloproteinase 14 levels define a subpopulation of highly invasive foam-cell macrophages. *Arterioscler Thromb Vasc Biol* 2008;28:1647–1653.
- Casagrande V, Menghini R, Menini S, Marino A, Marchetti V, Cavalera M, Fabrizi M, Hribal ML, Pugliese G, Gentileschi P, et al. Overexpression of tissue inhibitor of metalloproteinase 3 in macrophages reduces atherosclerosis in low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* 2012;32:74–81.
- Gill SE, Huizar I, Bench EM, Sussman SW, Wang Y, Khokha R, Parks WC. Tissue inhibitor of metalloproteinases 3 regulates resolution of inflammation following acute lung injury. *Am J Pathol* 2010;176:64–73.
- Tanino Y, Coombe DR, Gill SE, Kett WC, Kajikawa O, Proudfoot AE, Wells TN, Parks WC, Wight TN, Martin TR, et al. Kinetics of chemokine-glycosaminoglycan interactions control neutrophil migration into the airspaces of the lungs. *J Immunol* 2010;184:2677–2685.
- Johnston LK, Rims CR, Gill SE, McGuire JK, Manicone AM. Pulmonary macrophage subpopulations in the induction and resolution of acute lung injury. *Am J Respir Cell Mol Biol* 2012;47:417–426.
- Manicone AM, Birkland TP, Lin M, Betsuyaku T, van Rooijen N, Lohi J, Keski-Oja J, Wang Y, Skerrett SJ, Parks WC. Epilysin (MMP-28) restrains early macrophage recruitment in *Pseudomonas aeruginosa* pneumonia. *J Immunol* 2009;182:3866–3876.
- Fellenberg K, Hauser NC, Brors B, Neutzner A, Hoheisel JD, Vingron M. Correspondence analysis applied to microarray data. *Proc Natl Acad Sci USA* 2001;98:10781–10786.
- Baldi P, Long AD. A Bayesian framework for the analysis of microarray expression data: regularized *t*-test and statistical inferences of gene changes. *Bioinformatics* 2001;17:509–519.
- Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA* 2003;100:9440–9445.
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003;4:3.
- Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, et al. TM4: a free, open-source system for microarray data management and analysis. *Biotechniques* 2003;34:374–378.
- Ulitsky I, Maron-Katz A, Shavit S, Sagir D, Linhart C, Elkon R, Tanay A, Sharan R, Shilo Y, Shamir R. Expander: from expression microarrays to networks and functions. *Nat Protoc* 2010;5:303–322.
- Calvano SE, Xiao W, Richards DR, Feliciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, et al. Inflammatory and Host Response to Injury Large Scale Collaboratory Research Program: a network-based analysis of systemic inflammation in humans. *Nature* 2005;437:1032–1037.
- Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A, Simonovic M, et al. STRING 8: a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res* 2009;37:D412–D416.

35. Frevert CW, Wong VA, Goodman RB, Goodwin R, Martin TR. Rapid fluorescence-based measurement of neutrophil migration *in vitro*. *J Immunol Methods* 1998;213:41–52.
36. Shen Y, Winkler IG, Barbier V, Sims NA, Hendy J, Lévesque JP. Tissue inhibitor of metalloproteinase-3 (TIMP-3) regulates hematopoiesis and bone formation *in vivo*. *PLoS ONE* 2010;5.
37. Nakajima H, Ito M, Smookler DS, Shibata F, Fukuchi Y, Morikawa Y, Ikeda Y, Arai F, Suda T, Khokha R, *et al.* TIMP-3 recruits quiescent hematopoietic stem cells into active cell cycle and expands multipotent progenitor pool. *Blood* 2010;116:4474–4482.
38. Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, Mak TW, Khokha R. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest* 2001;108:817–829.
39. Brennan FM, Green P, Amjadi P, Robertshaw HJ, Alvarez-Iglesias M, Takata M. Interleukin-10 regulates TNF-alpha-converting enzyme (TACE/ADAM-17) involving a TIMP-3 dependent and independent mechanism. *Eur J Immunol* 2008;38:1106–1117.
40. Janssen WJ, Barthel L, Muldrow A, Oberley-Deegan RE, Kearns MT, Jakubzick C, Henson PM. Fas determines differential fates of resident and recruited macrophages during resolution of acute lung injury. *Am J Respir Crit Care Med* 2011;184:547–560.
41. Ortiz LA, Moroz K, Liu JY, Hoyle GW, Hammond T, Hamilton RF, Holian A, Banks W, Brody AR, Friedman M. Alveolar macrophage apoptosis and TNF-alpha, but not p53, expression correlate with murine response to bleomycin. *Am J Physiol* 1998;275:L1208–L1218.
42. Ahonen M, Baker AH, Kähäri VM. Adenovirus-mediated gene delivery of tissue inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. *Cancer Res* 1998;58:2310–2315.
43. Ahonen M, Poukkula M, Baker AH, Kashiwagi M, Nagase H, Eriksson JE, Kähäri VM. Tissue inhibitor of metalloproteinases-3 induces apoptosis in melanoma cells by stabilization of death receptors. *Oncogene* 2003;22:2121–2134.
44. Bond M, Murphy G, Bennett MR, Amour A, Knauper V, Newby AC, Baker AH. Localization of the death domain of tissue inhibitor of metalloproteinase-3 to the N terminus: metalloproteinase inhibition is associated with proapoptotic activity. *J Biol Chem* 2000;275:41358–41363.
45. Bond M, Murphy G, Bennett MR, Newby AC, Baker AH. Tissue inhibitor of metalloproteinase-3 induces a Fas-associated death domain-dependent Type II apoptotic pathway. *J Biol Chem* 2002;277:13787–13795.
46. Finan KM, Hodge G, Reynolds AM, Hodge S, Holmes MD, Baker AH, Reynolds PN. *In vitro* susceptibility to the pro-apoptotic effects of TIMP-3 gene delivery translates to greater *in vivo* efficacy versus gene delivery for TIMPs-1 or -2. *Lung Cancer* 2006;53:273–284.
47. Murthy A, Defamie V, Smookler DS, Di Grappa MA, Horiuchi K, Federici M, Sibilia M, Blobel CP, Khokha R. Ectodomain shedding of EGFR ligands and TNFR1 dictates hepatocyte apoptosis during fulminant hepatitis in mice. *J Clin Invest* 2010;120:2731–2744.