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PAI-1 (Plasminogen Activator Inhibitor-1) Expression Renders Alternatively Activated Human Macrophages Proteolytically Quiescent

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Objective—Macrophages are versatile immune cells capable of polarizing into functional subsets depending on environmental stimulation. In atherosclerotic lesions, proinflammatory polarized macrophages are associated with symptomatic plaques, whereas Th2 (T-helper cell type 2) cytokine–polarized macrophages are inversely related with disease progression. To establish a functional cause for these observations, we analyzed extracellular matrix degradation phenotypes in polarized macrophages.

Approach and Results—We provide evidence that proinflammatory polarized macrophages rely on membrane-bound proteases including MMP-14 (matrix metalloproteinase-14) and the serine protease uPA (urokinase plasminogen activator) together with its receptor uPAR for extracellular matrix degradation. In contrast, Th2 cytokine alternatively primed macrophages do not show different proteolytic activity in comparison to unpolarized macrophages and lack increased localization of MMP-14 and uPA receptor to the cell membrane. Nonetheless, they express the highest amount of the serine protease uPA. However, uPA activity is blocked by similarly increased expression of its inhibitor PAI-1 (plasminogen activator inhibitor 1). When inhibiting PAI-1 or when analyzing macrophages deficient in PAI-1, Th2 cytokine–polarized macrophages display the same matrix degradation capability as proinflammatory-primed macrophages. Within atherosclerotic lesions, macrophages positive for the alternative activation marker CD206 express high levels of PAI-1. In addition, to test changed tissue remodeling capacities of alternatively activated macrophages, we used a bleomycin lung injury model in mice reconstituted with PAI-1$^{-/-}$ bone marrow. These results supported an enhanced remodeling phenotype displayed by increased fibrosis and elevated MMP activity in the lung after PAI-1 loss.

Conclusions—We were able to demonstrate matrix degradation dependent on membrane-bound proteases in proinflammatory stimulated macrophages and a forced proteolytical quiescence in alternatively polarized macrophages by the expression of PAI-1.

Visual Overview—An online visual overview is available for this article. (***Arterioscler Thromb Vasc Biol*. 2017;37: 1913-1922. DOI: 10.1161/ATVBAHA.117.309383.)**

Key Words: arteriosclerosis ■ macrophages ■ matrix metalloproteinases ■ serine proteases ■ serpins

Macrophages are versatile cells capable of adapting to different environmental stimuli to perform required tasks. Polarization of macrophages is induced by specific cytokines and dependent on tissue and context. Macrophage polarization responses can influence inflammatory reactions in opposite ways.[1] Polarization involving IFN (interferon)-γ with a TLR (Toll-like receptor) agonist such as LPS (lipopolysaccharide) is termed classical activation[2] and usually associated with a proinflammatory phenotype characterized by the secretion of TNF (tumor necrosis factor)-α, IL (interleukin)-1, IL-6, and surface receptors including the T-cell costimulatory receptors CD80 and CD86.[2–4] In these polarization conditions, macrophages are considered to be potent effector cells that kill intracellular microorganisms and tumor cells.[5] In addition, proinflammatory macrophages are present in the early phase of tissue injury and get replaced by a wound healing macrophage subset termed alternatively activated macrophages after the early inflammatory phase subsides.[6] These macrophages are characterized by

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Nonstandard Abbreviations and Acronyms	
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MMP	matrix metalloproteinase
PAI-1	plasminogen activator inhibitor 1
TIMP	tissue inhibitor of MMP
TLR	Toll-like receptor
TNF	tumor necrosis factor
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor

increased expression of anti-inflammatory IL-10 and increased expression of scavenger receptors. Besides scavenging debris, promoting angiogenesis, tissue remodeling, and repair, alternatively activated macrophages are able to fine-tune inflammatory responses and adaptive Th2 (T-helper cell type 2) immunity.[7] The classic activation pattern for alternative activation includes the Th2 cytokines IL-4 and IL-13. To stimulate macrophage polarization in vitro, a proinflammatory macrophage phenotype is achieved by stimulating with LPS and IFN-γ, whereas alternative activation is achieved by stimulation with IL-4 and IL-13. After the proposed nomenclature by Murray et al,[8] LPS-treated and IFN-γ–treated macrophages are termed M(LPS+IFN), and alternatively activated macrophages are termed M(IL-4+IL-13) throughout this article. Classification of macrophages to M(LPS+IFN) and M(IL-4+IL-13) polarized conditions is an oversimplification considering the dynamic nature of macrophage plasticity, but allows a reductionist approach of defining specific functions for macrophages under controlled conditions.

Polarized macrophage subsets are well established to have a prominent role in tissue remodeling and wound repair.[6] Depending on the context, macrophage polarization can be beneficial or detrimental. In atherosclerotic lesions, proinflammatory macrophages dominate the rupture-prone shoulder regions of the plaque over alternatively activated macrophages, whereas the fibrous caps of lesions showed no significant differences between subsets.[9] This is further supported by the association of Th1-associated cytokines with symptomatic plaques.[10] Alternatively activated macrophages are positively connoted with wound healing and tissue repair.[6] However, overactivation of a wound healing phenotype can have detrimental consequences as in pulmonary fibrosis.[11]

Tissue degradation requires the expression and activation of MMPs (matrix metalloproteinases). MMPs are mediators of change and physical adaption, whether developmentally regulated, environmentally induced, or disease associated.[12] These enzymes can degrade structural components of the extracellular matrix allowing space for cells to migrate or proliferate. In addition, MMP cleavage of proteins can produce fragments with various biological activities.[13] Even though macrophages have been already prominently associated with modulated MMP expression, little functional data are available for polarization conditions.

MMPs are secreted in a pro-MMP form that needs to be cleaved to be activated, hence allowing a tight and spatial control of MMP activation patterns. Membrane-bound MMPs can be cleaved and activated within the trans-Golgi network by furins.[14] Some soluble MMPs can then be cleaved by those membrane-bound and activated MMPs as demonstrated for the activation of secreted MMP-2 by membrane-bound MMP-14.[15] In addition, serine proteases have been reported to activate several MMPs. Among them, particularly plasmin has been associated with in vivo activation of MMPs.[16] Another serine protease demonstrated to activate MMPs is uPA (urokinase plasminogen activator), either indirectly via cleavage of plasminogen to plasmin or directly.[17] Interestingly, uPA is under tight control of its inhibitor PAI-1 (plasminogen activator inhibitor 1).[18] In addition, the catalytic domain of MMPs can be targeted by the inhibitory TIMPs (tissue inhibitors of MMPs).[19] These different activation systems allow for a wide range of control options to ensure proper activation and deactivation of tissue remodeling.

The purpose of this study was to identify proteolytic capacities of polarized macrophage subsets. We found a strong proteolytic activity in M(LPS+IFN) polarized macrophages, whereas M(IL-4+IL-13) polarization did not change the matrix degrading capacity compared with unpolarized macrophages. Whereas M(LPS+IFN) macrophages use a combination of cell surface activators including MMP-14 and the serine protease uPA, proteolytic activity of M(IL-4+IL-13) macrophages is inhibited by the uPA inhibitor PAI-1. By removing PAI-1 from M(IL-4+IL-13) macrophages, proteolytic activity increased to levels comparable with M(LPS+IFN) macrophages.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Proteolytic Capacity of Macrophage Subsets

Macrophage polarization changes the phenotypic behavior and exerts certain functional changes in macrophages. Polarization to a proinflammatory and an alternative phenotype has been widely recognized in different pathological settings. For a simplistic approach, macrophages can be polarized in vitro toward M(LPS+IFN) and M(IL-4+IL-13) phenotypes using specific cytokines and identified with distinct characteristic markers and activation-dependent downstream cytokines. We polarized human macrophages toward M(LPS+IFN) and M(IL-4+IL-13) and determined the polarization phenotype. In short, both M(LPS+IFN) and M(IL-4+IL-13) demonstrated expected polarization patterns including increased expression of CD80 and CD86 in M(LPS+IFN) macrophages and high levels of CD206 and IL-10 in M(IL-4+IL-13) macrophages (Figure I in the online-only Data Supplement). The role of polarized macrophage subsets in tissue degradation and proteolysis is to date controversially discussed. To analyze the effect of different macrophage subsets on matrix degradation, we used a fluorescence-based assay to determine the lysis of gelatin during polarization. Our results indicated a robust proteolytic capacity of human M(LPS+IFN) polarized macrophages, whereas M(IL-4+IL-13) macrophages showed only a limited degradation potential similar to unpolarized macrophages (Figure 1A). Matrix degradation can usually be associated with changes in MMP and TIMP expression profiles.[20] We applied an antibody

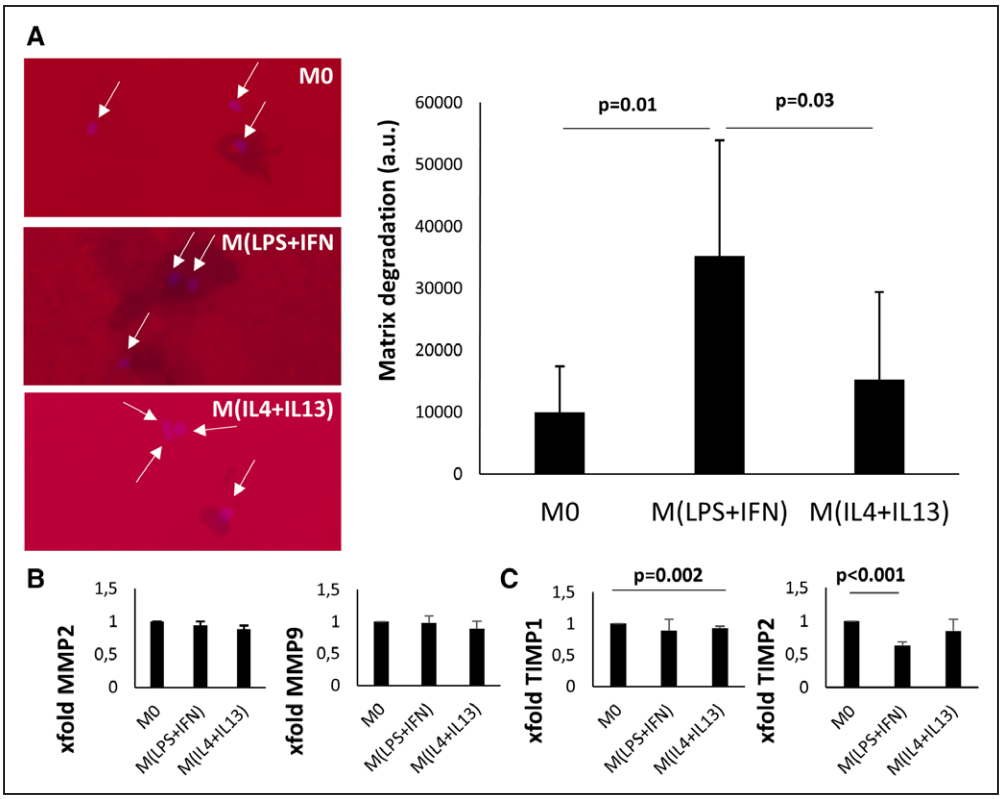

Figure 1. Proteolytic capability of polarized macrophages. **A**, Macrophages were seeded onto indocarbocyanine-gelatin–coated glass slides and polarized. Matrix degradation was evaluated by measuring the degraded black areas. DAPI stained nuclei are marked by white arrows on representative pictures. Area of degradation was determined as described under Materials and Methods and is given as arbitrary units (a.u.). Data represent mean±SD of 5 independent donors. **B**, Total matrix metalloproteinase 2 (MMP-2) and MMP-9 levels were measured in supernatants of polarized macrophages using an antibody array. Signal intensity was quantified by ImageJ. Data are given as x-fold change vs unpolarized M0 macrophages and represent mean±SD of 4 independent donors. **C**, Total TIMP1 (tissue inhibitors of MMP-1) and TIMP2 levels were measured in supernatants of polarized macrophages using an antibody array. Signal intensity was quantified by ImageJ. Data are given as x-fold change vs unpolarized M0 macrophages and represent mean±SD of 4 independent donors. IFN indicates interferon; IL, interleukin; and LPS, lipopolysaccharide.

array capable of detecting multiple MMPs and TIMPs. Of the analyzed proteins, only MMP-8 was not detectable in the supernatant of macrophages (Figure IIA in the online-only Data Supplement). Surprisingly, most of the MMPs were not differentially produced from polarized macrophage subsets after 48 hours of polarization (Figure IIB in the online-only Data Supplement). The 2 main MMPs for degrading gelatin substrates are MMP-2 and MMP-9, but polarization did not change total protein expression of these MMPs (Figure 1B). In contrast, only MMP-10 was significantly upregulated after polarization under both M(LPS+IFN) and M(IL-4+IL-13) polarizing conditions (Figure IIB in the online-only Data Supplement). However, upregulation was statistically not significantly different between different polarization conditions. Another way of shifting the proteolytic balance of MMPs is via downregulation of TIMPs. Of the 3 analyzed TIMPs, TIMP1 was only slightly, albeit significantly downregulated in M(IL-4+IL-13) macrophages, whereas TIMP2 was downregulated in M(LPS+IFN) polarizing conditions (Figure 1C).

Membrane-Bound MMP-14 Is Increased in M(LPS+IFN) Macrophages

MMPs are usually produced as a pro-form that needs further activation. For membrane-bound MMPs, this activation usually occurs in the trans-Golgi via a furin-mediated mechanism. Soluble MMPs can be activated via several mechanisms including cleavage by membrane-bound and previously activated MMP-14 or by serine proteases.[14] A key feature for macrophages during matrix degradation is the ability to form filopodia and localize MMPs to these cell protrusions.[21] We found an increase of filopodia in human M(LPS+IFN) polarized macrophages over M0 and M(IL-4+IL-13) polarized macrophages (Figure 2A). Filopodia, especially in endothelial cells, were associated with not only tissue degradation but also migration.[22] To determine if increased matrix degradation might be related to increased cell mobility and hence a larger area covered, we analyzed the migratory speed of macrophage subsets in a transwell assay. M(LPS+IFN) and M(IL-4+IL-13) macrophages showed similar and faster migration patterns compared with M0 macrophages. We therefore suggest that matrix degradation is independent from migration capacity of macrophages (Figure IIIA in the online-only Data Supplement). Filopodia were stained for MMP-14, which can locally activate MMPs at the cellular surface. Whereas M0 and M(IL-4+IL-13) macrophages showed prominent staining mainly around the nucleus, M(LPS+IFN) filopodia stained positive for MMP-14 (Figure 2B). This increased localization of MMP-14 to filopodia is in no relation to total MMP-14

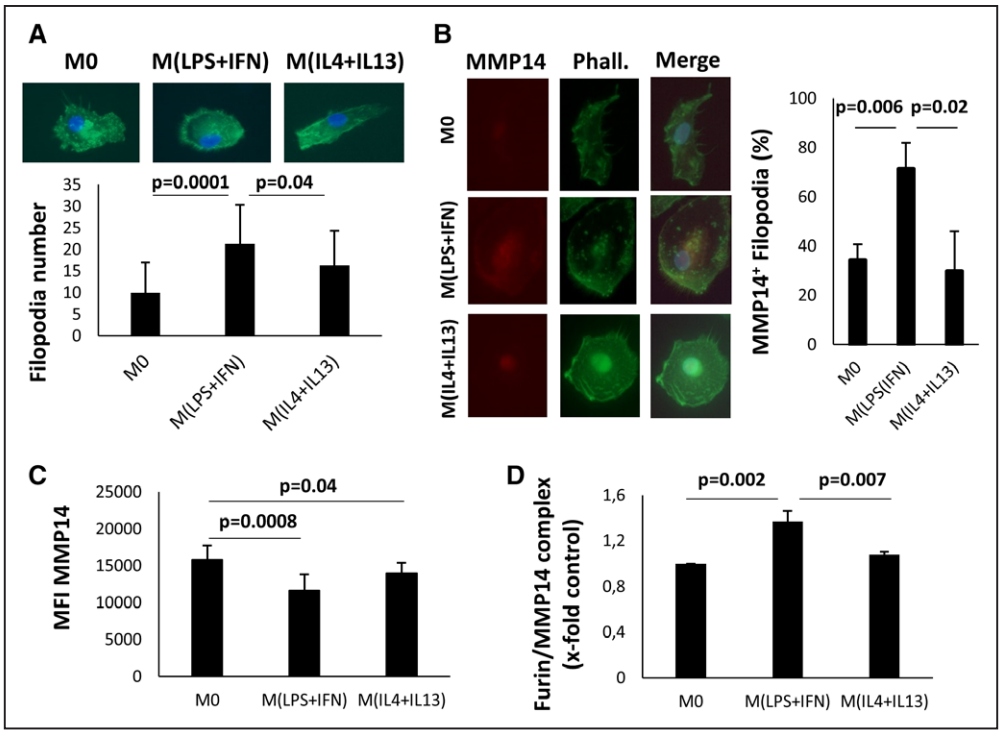

Figure 2. Matrix metalloproteinase 14 (MMP-14) localization in polarized macrophages. **A**, Total filopodia number was evaluated in polarized macrophages by manual counting of phalloidin stained filopodia. Pictures represent filopodia staining under different polarization conditions. Data represent mean number of filopodia±SD of 7 independent donors. At least 20 individual cells per donor and polarization condition were counted. **B**, MMP-14 localization was evaluated on filopodia. Representative pictures demonstrate the merged staining including phalloidin (green), DAPI (blue), and MMP-14 (red). The red MMP-14 channel is displayed to the left of the merged pictures. Total number of filopodia and total number of MMP14⁺ filopodia were evaluated. Data represent average percent of filopodia positive for MMP-14±SD of 3 independent donors. At least 20 individual cells per donor and polarization condition were counted. **C**, Total MMP-14 protein content was evaluated in polarized macrophages using flow cytometry. Data are given in mean fluorescence intensity (MFI) of MMP-14 and represent mean±SD of 3 independent donors. **D**, Furin/MMP-14 complexes in cell lysates were determined as stated under Materials and Methods. Data are given as x-fold change vs unpolarized M0 macrophages and represent mean signal intensity±SD of 3 independent donors. IFN indicates interferon; IL, interleukin; and LPS, lipopolysaccharide.

protein, as M0 macrophages have the highest MMP-14 overall content (Figure 2C). MMP-14 is activated from the pro-MMP to the active form by furin in the trans-Golgi network.[23] Of note, furin protein amount was not changed under polarizing conditions (Figure IIIB in the online-only Data Supplement). We assessed the amount of furin-bound MMP-14 in cellular lysates of macrophage subsets. M(LPS+IFN) polarized macrophages had increased intracellular MMP-14/furin complexes indicating increased MMP-14 activation (Figure 2D).

uPA/uPA Receptor Availability in Polarized Macrophages

In addition to matrix-bound MMPs, certain cell receptors can contribute to localized activation of matrix degradation. The serine protease uPA has been associated with activation of MMPs.[16] Localized to its receptor uPAR (uPA receptor), uPA can activate MMPs at cell filopodia and hence govern a directed matrix degradation.[24] We identified human M(LPS+IFN) macrophages as having significantly higher uPAR receptor surface density levels compared with M0 and M(IL-4+IL-13) polarized human macrophages (Figure 3A). Furthermore, M(LPS+IFN) macrophages showed increased localization of uPAR to cellular protrusions (Figure 3B). This increase of cellular uPAR was accompanied by increased uPA localization to the membrane in M(LPS+IFN) macrophages (Figure 3C). To evaluate the importance of uPAR-dependent gelatin degradation, we added a uPA antibody to the gelatin degradation assay. Inhibition of uPA did not change the proteolytic capacity of M0 and M(IL-4+IL-13) macrophages. However, after uPA blockade, M(LPS+IFN) macrophages had reduced proteolytic capacities indicating at least a partial role for uPA-dependent activation of matrix degradation (Figure 3D).

uPA/PAI-1 expression in Polarized Macrophages

uPA is a serine protease usually secreted from cells. Macrophages have been described to express uPA.[25] To identify whether a macrophage polarizing condition would increase uPA protein levels, we used an ELISA to quantify uPA levels in supernatants of human M0, M(LPS+IFN), and M(IL-4+IL-13) macrophages. Surprisingly, uPA levels were highest in M(IL-4+IL-13) macrophages and lowest in M(LPS+IFN) macrophages (Figure 4A). However, when assessing uPA activity, we found only a statistically relevant significance between uPA activity in M0 and M(LPS+IFN) macrophages, where M(LPS+IFN) macrophages showed significantly more activity in cleaving a uPA substrate (Figure 4B). uPA can be inhibited by PAI-1. Determining the levels of PAI-1 in cell culture supernatants of human M0, M(LPS+IFN), and M(IL-4+IL-13) macrophages revealed the by far highest levels of PAI-1 to be associated with M(IL-4+IL-13) macrophages (Figure 4C). Intracellular PAI-1 levels

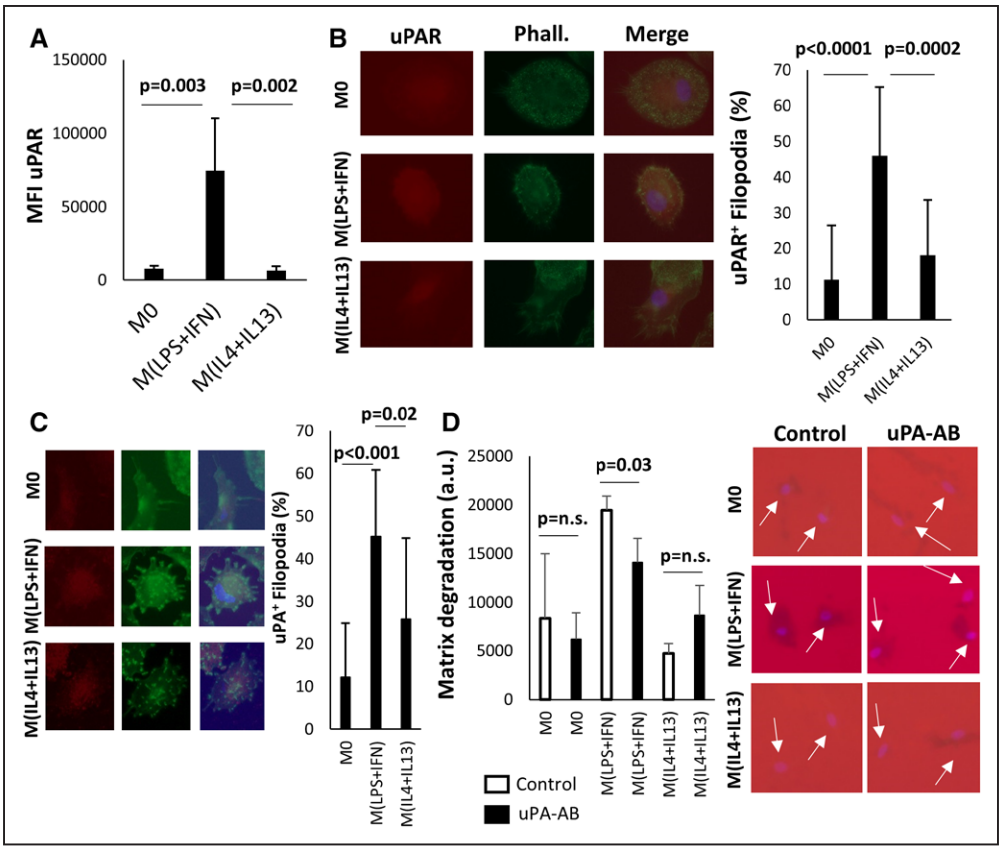

Figure 3. uPAR (urokinase plasminogen activator receptor) localization in polarized macrophages. **A**, Total uPAR protein content was evaluated in polarized macrophages using flow cytometry. Data are given in mean fluorescence intensity (MFI) of uPAR and represent mean±SD of 5 independent donors. **B**, uPAR localization was evaluated on filopodia. Representative pictures demonstrate the merged staining including phalloidin (green), DAPI (blue), and uPAR (red). The red uPAR channel is displayed to the left of the merged pictures. Total number of filopodia and total number of uPAR⁺ filopodia were evaluated. Data are given in percent of filopodia positive for uPAR and represent mean±SD of 4 independent donors. At least 20 individual cells per donor and polarization condition were counted. **C**, uPA localization was evaluated on filopodia. Representative pictures demonstrate the merged staining including phalloidin (green), DAPI (blue), and uPA (red). The red uPA channel is displayed to the **left**. Total number of filopodia and total number of uPA⁺ filopodia were evaluated. Data are given in percent of filopodia positive for uPA and represent mean±SD of 4 independent donors. At least 20 individual cells per donor and polarization condition were counted. **D**, Macrophages were seeded onto indocarbocyanine-gelatin–coated glass slides and polarized. Matrix degradation was evaluated by measuring the degraded black areas under control conditions or in the presence of a uPA-inhibiting antibody (uPA-AB). DAPI stained nuclei are marked by white arrows on representative pictures. Area of degradation was determined as described under Materials and Methods and is given as arbitrary units (a.u.). Data represent mean±SD of 3 independent donors. IFN indicates interferon; IL, interleukin; and LPS, lipopolysaccharide.

were also increased in M(IL-4+IL-13) macrophages, however, not statistically significant (Figure IVA in the online-only Data Supplement). Induction of PAI-1 via IL-4 and IL-13 is dependent on STAT6 (signal transducer and activator of transcription 6) signaling, as pharmacological inhibition of STAT6 abrogated the induction of PAI-1 by M(IL-4+IL-13) polarization (Figure IVB in the online-only Data Supplement). Although PAI-1 can inhibit uPA extracellularly, it can also inhibit furin and subsequent MMP activation in the trans-Golgi intracellularly. To determine if PAI-1 would be involved in intracellular furin inhibition, we performed a furin/PAI-1 complex ELISA. We did not observe a difference in furin/PAI-1 complexes in any subtype suggesting a solely extracellular role for PAI-1 (Figure IVC in the online-only Data Supplement). PAI-1 active antigen was still slightly but significantly increased in M(IL-4+IL-13) macrophages compared with unpolarized macrophages supporting the complete uPA activity blockade observed (Figure 4D). To determine the proteolytic potential of uPA produced by the respective macrophages, we used a PAI-1 blocking monoclonal antibody MA-MP6H6 described previously in the gelatin degrading assay.[26] Under PAI-1 blockade, M(IL-4+IL-13) macrophages had similar matrix degrading capacities to M(LPS+IFN) polarized macrophages (Figure 4E). Results were confirmed in M(IL-4+IL-13) macrophages using an siRNA-mediated PAI-1 knockdown (Figure IVD in the online-only Data Supplement). In contrast, adding PAI-1 protein during polarization of M(LPS+IFN) macrophages led to reduced matrix degradation (Figure IVE in the online-only Data Supplement). To analyze whether polarized macrophages of PAI-1 knockout mice would reproduce our blocking antibody results in human macrophages, we isolated and polarized mouse macrophages. Polarized macrophages from PAI-1–deficient mice did not display a different polarization profile concerning M(LPS+IFN) induced TNF-α and IL-1 levels and M(IL-4+IL-13) induced arginase 1 compared with wild-type polarized macrophages (Figure V in the online-only Data Supplement). Results obtained with macrophages from PAI-1–deficient mice confirmed our initial finding that M(IL-4+IL-13) macrophages regain gelatin degradation

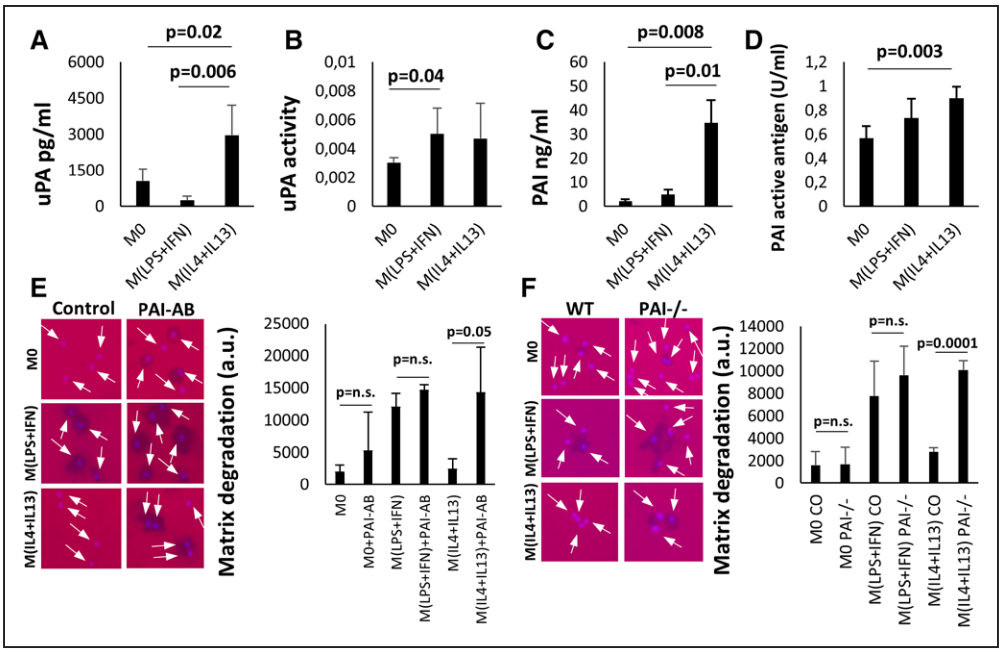

Figure 4. uPA (urokinase plasminogen activator) and PAI-1 (plasminogen activator inhibitor 1) expression in polarized macrophages. **A**, uPA protein was evaluated in the supernatant of polarized macrophages using a specific ELISA and is given in pg/mL. Values represent mean±SD of 5 independent donors. **B**, uPA activity was evaluated as stated in Materials and Methods and is given in IU/mL. Values represent mean±SD of 5 independent donors. **C**, PAI-1 protein was evaluated in the supernatant of polarized macrophages using a specific ELISA and is given in ng/mL. Values represent mean±SD of 5 independent donors. **D**, PAI-1 active antigen in the supernatant of polarized macrophages was evaluated using a specific ELISA as stated in Materials and Methods and is given as IU/mL. Values represent mean±SD of 5 independent donors. **E**, Macrophages were seeded onto indocarbocyanine-gelatin–coated glass slides and polarized. Matrix degradation was evaluated by measuring the degraded black areas under control conditions or in the presence of a PAI-1–inhibiting antibody (PAI-AB). DAPI stained nuclei are marked by white arrows on representative pictures. Area of degradation was determined as described under Materials and Methods and is given as arbitrary units (a.u.). Data represent mean±SD of 5 independent donors. **F**, Macrophages from wild-type (control [CO]) or PAI-1$^{-/-}$ (PAI$^{-/-}$) mice were seeded onto indocarbocyanine-gelatin–coated glass slides and polarized. Matrix degradation was evaluated by measuring the degraded black areas. DAPI stained nuclei are marked by white arrows on representative pictures. Area of degradation was determined as described under Materials and Methods and is given as arbitrary units (a.u.). Data represent mean±SD of 3 mice. IFN indicates interferon; IL, interleukin; LPS, lipopolysaccharide; and n.s., non-significant.

capacities on loss of PAI-1 and exert similar degradation capacities as M(LPS+IFN) macrophages (Figure 4F).

In Vivo Expression and Epigenetic Changes of PAI-1 in Macrophages

The long-term effects of macrophage polarization are currently under debate, and polarization could only be a transient and reversible effect of certain conditions.[27] We analyzed tissue sections of 18 human atherosclerotic plaques to determine whether the observed PAI-1 expression can be found in a pathological setting as well. Our results indicated a strong correlation of the M(IL-4+IL-13) marker CD206 with PAI-1 protein levels supporting our initial hypothesis of a PAI-1 producing M(IL-4+IL-13) macrophage subset (Figure 5A). To further analyze the influence of an initial M(IL-4+IL-13) polarization on future PAI-1 expression, we restimulated polarized macrophages with IL-1β to simulate an inflammatory milieu. PAI-1 only remained inducible by proinflammatory stimulation in M(IL-4+IL-13) macrophages (Figure 5B). Induction of PAI-1 in M(IL-4+IL-13) macrophages was dependent on NF-kB (nuclear factor-kB) signaling, as the NF-kB inhibitor dimethyl fumarate was able to abolish IL-1β–induced PAI-1 induction (Figure VIA in the online-only Data Supplement). However, nuclear levels of p65, a key NF-kB component, after IL-1β stimulation were elevated in all macrophages including M0, M(LPS+IFN), and M(IL-4+IL-13) macrophages (Figure VIB in the online-only Data Supplement). Epigenetic changes in a promoter region have the potential to change the expression of genes.[28] To analyze if M(IL-4+IL-13) polarization might have effects on DNA substructure organization, we determined the accessibility of the PAI-1 promoter in polarized macrophages. Primers for the PAI-1 promoter were designed according to the previously published PAI-1 promoter sequence.[29] After polarization, the PAI-1 promoter was on average 4-fold more accessible in M(IL-4+IL-13) polarized macrophages compared with M0 and M(LPS+IFN) polarization conditions (Figure 5C).

In Vivo Deletion of PAI-1 Increases Fibrosis and MMP Activity in Lung Tissue

Alternatively activated macrophages are associated with a tissue remodeling phenotype. To determine the consequences of a PAI-1 loss in macrophages in a setting of tissue remodeling, we used a bleomycin-induced lung injury model after reconstituting mice with either wild-type bone marrow or bone marrow from PAI-1$^{-/-}$ mice. This model is characterized by an initial infiltration of proinflammatory macrophages followed by a phase of tissue remodeling subsequently leading to fibrosis.[30] After bleomycin treatment, there is a significant weight loss of treated animals compared with untreated ones. However, there was no difference between mice receiving either wild-type bone marrow

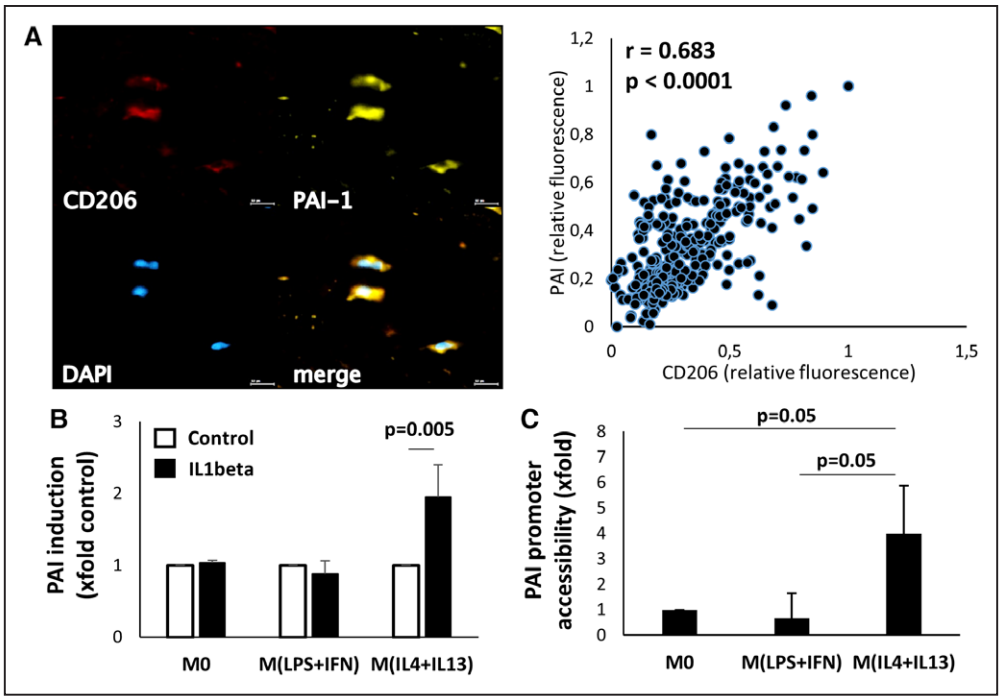

Figure 5. In vivo expression and epigenetic changes of PAI-1 (plasminogen activator inhibitor 1) in macrophages. **A**, Atherosclerotic plaques of 18 patients were stained for PAI-1 and the alternative activation marker CD206 ex vivo as described under Materials and Methods and a total of 333 macrophages were analyzed. Macrophages with a high fluorescence intensity of CD206 (red) displayed also high levels of PAI-1 (yellow). A representative picture given to the left shows 3 stained macrophages, whereby the macrophage with the highest CD206 signal (red) also displays the highest PAI-1 (yellow) fluorescence. Statistical analysis revealed a significant correlation with a Pearson Correlation Factor r=0.683, P<0.0001. **B**, Polarized macrophages were stimulated with IL (interleukin)-1β, and PAI-1 protein was determined after 24 h by ELISA. Data are given as x-fold change vs unpolarized M0 macrophages and represent mean±SD of five independent donors. **C**, Openness of the PAI-1 promoter was evaluated using nuclease digestion as indicated under Materials and Methods. Data are given as x-fold change in promoter accessibility vs unpolarized M0 macrophages and represent mean±SD of 3 independent donors. IFN indicates interferon; IL, interleukin; and LPS, lipopolysaccharide.

or PAI$^{-/-}$ bone marrow (data not shown). Macrophages in recipient mice of wild-type bone marrow stained positive for PAI-1, whereas macrophages of PAI-1$^{-/-}$ reconstituted bone marrow mice stained negative for PAI-1 (Figure 6A). When analyzing morphological parameters of the lung, we found a reduction of remaining alveoli space in mice receiving PAI-1$^{-/-}$ bone marrow compared with mice receiving wild-type bone marrow indicating a higher degree of tissue fibrosis (Figure 6B). In addition, MMP activity measured in lung tissue was increased in PAI-1$^{-/-}$ bone marrow recipients, supporting our previous findings (Figure 6C).

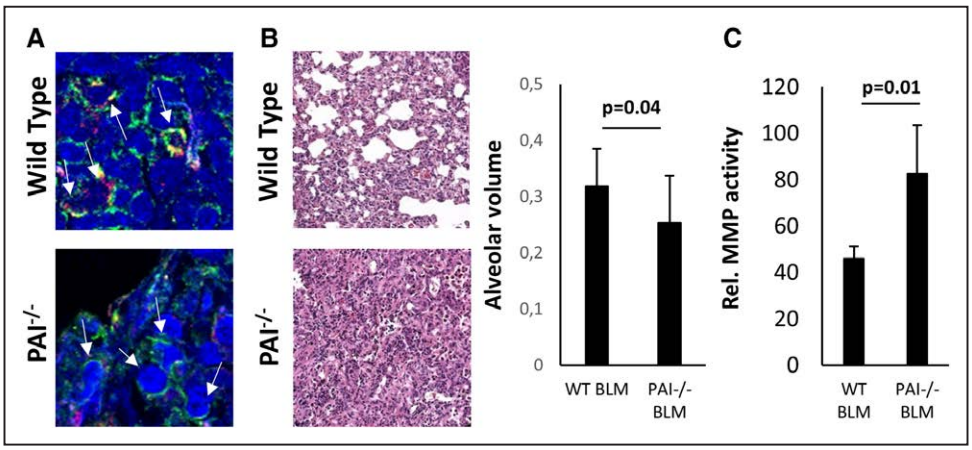

Figure 6. Bleomycin lung injury model. **A**, Macrophages were stained in mouse lung tissue paraffin sections using F4/80 (green) and costained for expression of PAI-1 (red). Macrophages in wild-type bone marrow reconstituted lungs stained positive for F4/80 and PAI-1 (yellow overlay), whereas PAI-1$^{-/-}$ bone marrow reconstituted mice did not show staining for PAI-1 in macrophages. **B**, Fibrosis was evaluated in paraffin sections of mouse lung tissue using hematoxilin and eosin staining. To determine degree of fibrosis, the remaining unstained alveolar volume was determined. Data are given as remaining alveolar volume of the total lung section in mm^2 ±SD from 12 mice per group. **C**, Matrix metalloproteinase (MMP) activity was measured in lung tissue from mice reconstituted either with wild-type bone marrow or PAI-1$^{-/-}$ bone marrow before bleomycin-induced injury. Data are given as relative MMP activity per total protein extract±SD of 4 lung tissue samples per group. MMP activity was calculated as a ratio to total protein extracted from the respective tissue.

Overall, bleomycin-induced lung fibrosis was increased together with increased MMP activity in mice negative for PAI-1 in macrophages.

Discussion

Macrophages are important contributors in extracellular matrix remodeling.[6] Whereas tissue remodeling is positive in wound healing and repair, it can be detrimental in diseases such as atherosclerosis or rheumatoid arthritis.[31–33] Macrophage function is tightly regulated by the tissue environment leading to differentially polarized macrophages. This polarization can be, in a simplified approach, mimicked in vitro allowing to study specific polarization conditions.[2] Previously, inflammatory polarized macrophages were associated with increased tissue degradation, although a clear mechanism for increased proteolytic activity was missing.[34] In this article, we demonstrate for the first time that the serine protease inhibitor PAI-1 is crucial for different proteolytic capacities in differently polarized macrophages. Our data indicate that because of robust and STAT6-dependent expression of PAI-1 in alternatively activated macrophages, these cells lose the capability to degrade extracellular matrix. By removing PAI-1 or by its inhibition, M(IL-4+IL-13) macrophages show similar extracellular matrix degradation as proinflammatory macrophages. We suggest that PAI-1 is a quiescence factor for alternatively activated macrophages, controlling excessive remodeling behavior of this polarization condition.

Previously, proinflammatory macrophages were demonstrated to have a different expression profile of MMPs and TIMPs compared with alternatively activated macrophages.[35,36] However, these data relied on quantification of MMPs solely by mRNA and did not include functional assays. In our current study, we demonstrate increased proteolytic activity in M(LPS+IFN) macrophages in a matrix degrading assay. However, protein data did not show massive changes in MMP and TIMP expression levels indicating different activation pathways of tissue degradation rather than sole increase of MMP protein levels or reduction of TIMP protein levels.

Macrophages can sense their environment by membrane protrusions or filopodia. By localizing membrane-bound proteins to certain cellular areas, a spatial organization can be acquired. We showed increased filopodia formation in M(LPS+IFN) macrophages. These filopodia were also increasingly occupied by MMP-14, a membrane-bound MMP. MMP-14 is activated in the trans-Golgi by furin and exerts its proteolytic activity as soon as it anchors in the cell membrane.[34] Besides its role in collagen proteolysis, MMP-14 was reported to further activate soluble MMPs including MMP-2 and MMP-9.[35,36] Even though we found slightly reduced overall MMP-14 protein levels in M(LPS+IFN) polarized macrophages compared with M0 and M(IL-4+IL-13) macrophages, furin/MMP-14 complexes were increased indicating more availability of active MMP-14 in M(LPS+IFN) macrophages compared with other polarization conditions. Another membrane anchored MMP activation system is the uPA/uPAR axis.[24] Activation via uPA can occur either directly or indirectly via plasmin-mediated MMP activation.[37] Again, by localizing uPAR to filopodia, cells can direct the proteolytic activity to a certain location. Similar to MMP-14, we could show that uPAR is increasingly localized to M(LPS+IFN) macrophage filopodia. This increased localization is accompanied by increased protein concentration at the cellular membrane, rendering uPAR a possible M(LPS+IFN) macrophage marker protein. Together with increased receptor localization, the ligand uPA can also be found at higher levels on the filopodia of proinflammatory activated macrophages. Using a uPA inhibitory antibody, we were able to show a partial reduction of matrix degradation capacity in M(LPS+IFN) macrophages with no changes in proteolytic activity for other polarization conditions. In addition, adding PAI-1 protein to M(LPS+IFN) polarization had similar effects on matrix degradation capacities. Our data suggest the importance of membrane-bound protease activity for tissue degradation for M(LPS+IFN) polarized macrophages that is lacking completely in M(IL-4+IL-13) polarized macrophages.

uPA is a secreted protein that can exert its proteolytic function either bound to uPAR or in solution.[24] uPA levels detected in the conditioned media were highest in M(IL-4+IL-13) macrophages, whereas M(LPS+IFN) macrophages showed the lowest production. However, this increased uPA production did not lead to increased proteolytic capacity of M(IL-4+IL-13) macrophages as overall uPA activity present in conditioned media was similar in M(LPS+IFN) and M(IL-4+IL-13) macrophages. The lack of uPA activity in M(IL-4+IL-13) macrophages can be explained by the robust amount of simultaneously produced PAI-1, which renders uPA inactive. Indeed, by inhibiting PAI-1 either via an antibody, siRNA or genetically, we could restore the tissue degradation capacity of M(IL-4+IL-13) macrophages to a similar level as observed in M(LPS+IFN) macrophages. We were able to confirm the association of PAI-1 with alternatively activated macrophages in human atherosclerotic plaques in vivo as levels of the alternative activation marker CD206 correlated significantly with levels of PAI-1 expression in plaque macrophages.

Macrophage polarization is a transient process allowing the macrophage to respond to a changed environment.[27] Previous reports showed a possible beneficial effect of prior M(IL-4+IL-13) polarization ex vivo followed by transfusion in a murine model of pancreatic and renal injury.[38,39] Interestingly, a recent report suggested a transient repolarization of M(IL-4) macrophages to proinflammatory macrophages. However, M(LPS+IFN) macrophages were no longer repolarizable to M(IL-4) macrophages because of a mitochondrial dysfunction.[40] In contrast, alternatively polarized macrophages were demonstrated to have altered reprogramming capabilities because of increased p50 availability.[5] We observed in vitro that only macrophages polarized previously toward the M(IL-4+IL-13) lineage were capable of upregulating PAI-1 expression after a proinflammatory stimulus. This upregulation was dependent on the transcription factor NF-kB. However, even though translocation of NF-kB to the nucleus after IL-1β stimulation was observed regardless of previous polarization, induction of PAI-1 was limited to macrophages previously polarized with IL-4 and IL-13. We found that the promoter region for PAI-1 in M(IL-4+IL-13) polarized macrophages showed a loosened chromatin structure as analyzed by promoter accessibility. Hence, we suggest that a previously alternatively activated macrophage retains its capacity to upregulate PAI-1 expression via changes to the PAI-1 promoter region.

Alternative activation of macrophages is largely associated with tissue remodeling. Furthermore, alternative activation of macrophages in atherosclerotic plaques is associated with plaque regression and smaller plaque size.[41,42] In addition, tumor-associated macrophages were able to promote cancer cell invasion via MMP modulation.[43] To determine a role of PAI-1 under remodeling conditions, we used a murine bleomycin-induced lung injury model characterized by a fibrotic phase including IL-4–mediated macrophage activation.[30] We observed that lungs from mice reconstituted with PAI-1$^{-/-}$ bone marrow showed increasing signs of tissue fibrosis and had increased levels of MMP activation. This observation is in line with wound healing phenotypes observed in PAI-1 knockout mice, where loss of PAI-1 leads to increased wound healing.[44] Therefore, we suggest that PAI-1 expression in vivo can modulate the invasiveness and capacity of macrophages and reduce the overall potential of the cell toward tissue degradation.

In conclusion, we suggest that polarization differentially affects the proteolytic activity of macrophages. M(LPS+IFN) polarization leads to increased proteolytic activity mainly via membrane-bound proteins including MMP-14 and uPAR/uPA localized to filopodia. In contrast, proteolytic activity in M(IL-4+IL-13) polarized macrophages is controlled by increased PAI-1 expression. In addition, M(IL-4+IL-13) polarized macrophages retain the capacity to upregulate PAI-1 and thus inhibit proteolytic activity dependent on uPA also in a proinflammatory setting via changes in the PAI-1 promoter structure. Our data indicate a complex and variable regulation of proteolytic activity in macrophage subsets.

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Disclosures

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Highlights

- M(LPS [lipopolysaccharide]+IFN [interferon]) macrophages use membrane-bound matrix metalloproteinases and uPA (urokinase plasminogen activator)/uPAR (urokinase plasminogen activator receptor) for matrix degradation.
- M(IL [interleukin]-4+IL-13) macrophages have a forced proteolytical quiescence because of PAI-1 (plasminogen activator inhibitor 1) expression.
- PAI-1 expression can be confirmed in macrophages from atherosclerotic tissue and is epigenetically controlled.
- PAI-1 loss leads to increased fibrosis and matrix metalloproteinase activation in a mouse model in vivo.