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Thromboxane Receptor Signaling Is Required for Fibronectin-induced Matrix Metalloproteinase 9 Production by Human and Murine Macrophages and Is Attenuated by the Arhgef1 Molecule*

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Background: Arhgef1$^{-/-}$ macrophages exhibit exaggerated matrix metalloproteinase (MMP) 9 production when cultured on fibronectin.
Results: Thromboxane is produced by myeloid cells when cultured on fibronectin, and treatment with thromboxane receptor (TP) antagonist ablates MMP9 production.
Conclusion: TP signaling is required for MMP9 production by myeloid cells cultured on fibronectin.
Significance: TP antagonists may be therapeutic for reducing myeloid MMP9 production in inflammatory diseases.

During an inflammatory response, resident and newly recruited tissue macrophages adhere to extracellular matrix and cell-bound integrin ligands. This interaction induces the expression of pro-inflammatory mediators that include matrix metalloproteinases (MMPs). Arhgef1 is an intracellular signaling molecule expressed by myeloid cells that normally attenuates murine macrophage MMP production *in vivo* and *in vitro* after cell culture on the extracellular matrix protein, fibronectin. In this study, we have extended the characterization of this fibronectin-induced Arhgef1-regulated signaling pathway in both human and murine myeloid cells. Our results show that MMP9 production by fibronectin-stimulated monocytes and macrophages depends on autocrine thromboxane receptor signaling and that under normal conditions, this signaling pathway is attenuated by Arhgef1. Finally, we show that the expression of *ARHGEF1* by human peripheral blood monocytes varies between individuals and inversely correlates with fibronectin-mediated MMP9 production.

Inflammation is a host response to infection important for pathogen elimination but that also leads to tissue injury that must be repaired. Accordingly, this response must be tightly regulated as aberrant, or excessive inflammation can also result in tissue injury caused by responding leukocytes (1). Moreover, prolonged pro-inflammatory stimulation or an inability to resolve acute inflammation can contribute to the pathogenesis of a number of diseases that include chronic obstructive pulmonary disease, asthma, cancer, atherosclerosis, and autoimmunity (2). With regards to chronic obstructive pulmonary disease, continual stimulation through repeated cigarette smoke exposure leads to chronic inflammation that is perpetuated even years after cigarette smoke exposure has terminated (3). Thus, defining the molecular pathways that lead to inflammation, and the identification of possible points of intervention in these pathways is warranted.

The acute inflammatory response to pathogens initiates with tissue injury and/or exposure of pathogen-derived ligands that engage toll-like receptors expressed on resident tissue macrophages. Macrophages are innate immune cells that reside in diverse tissues and provide sentinel responses against pathogens or noxious substances by the production of pro-inflammatory vasoactive lipids, cytokines, and chemokines (4, 5). An immediate consequence of this tissue macrophage response is the recruitment of neutrophils that within hours release their granule contents at the site of infection in attempt to eradicate or neutralize pathogens. However, this response can also lead to local tissue injury. Recruited macrophages, differentiated from newly arriving monocytes, clear remaining pathogens and short-lived apoptotic neutrophils via phagocytosis and begin tissue repair through the production of angiogenic factors and proteolytic tissue enzymes such as matrix metalloproteases (MMPs)[2] (4, 5). Under normal circumstances, the acute inflammatory response to pathogen exposure is resolved within days.

In tissues, macrophages use integrins to adhere to integrin ligands found in extracellular matrix (ECM) proteins (*e.g.* collagen and fibronectin) or expressed on the cell surface of other cells (*e.g.* intercellular adhesion molecule-1 and vascular cell adhesion molecule-1). The adhesion of myeloid cells to ECM integrin ligands has repeatedly been shown to promote production of many pro-inflammatory mediators such as prostaglan-

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[2] The abbreviations used are: MMP, matrix metalloproteinase; GPCR, G-protein coupled receptor; S1P, sphingosine-1-phosphate; LPA, lysophosphatidic acid; TXB_2, thromboxane B_2; PTA_2, pinane-thromboxane A_2; PGE_2, prostaglandin E_2; FN, fibronectin; RGS, regulator of G-protein signaling; ECM, extracellular matrix; qPCR, quantitative PCR.

dins, inflammatory cytokines, chemokines, and multiple MMPs (6, 7). Although the production of prostaglandins is known to be dependent on cyclooxygenase activity, the integrin signaling pathways macrophages use to produce other pro-inflammatory mediators are not well understood, although mitogen-activated protein kinases, Src family, and Pyk2 nonreceptor tyrosine kinases are implicated in integrin signaling (6, 8). Work from our laboratory has recently found that macrophage adhesion to fibronectin via the $\alpha5\beta1$ integrin *in vitro* leads to MMP9 production and is normally inhibited by the Arhgef1 intracellular signaling molecule (9).

Arhgef1 (Lsc/p115RhoGEF) is an intracellular signaling molecule with expression predominantly restricted to hematopoietic cells. Arhgef1 has been biochemically and functionally characterized as both a regulator of G-protein signaling (RGS) and Rho guanine nucleotide exchange factor. RGS proteins act as GTPase-activating proteins for GTP-bound Gα subunits of heterotrimeric G-proteins (10). Arhgef1 specifically accelerates the inherent GTPase activity of $G\alpha_{12/13}$ subunits (11), thereby terminating signaling from GPCRs that associate with $G\alpha_{12/13}$-containing heterotrimeric G-proteins. Arhgef1 also contains a tandem Dbl and pleckstrin homology domain that functions as Rho guanine nucleotide exchange factor specific for RhoA (12). RhoA participates in a number of cell biological processes including regulating cytoskeletal organization, integrin adhesion, and integrin signaling (13). We have previously shown that in B-lymphocytes, Arhgef1 is required for resolving integrin adhesion (14, 15), and analyses of Arhgef1-deficient mouse mutants have further demonstrated a requirement for Arhgef1 in leukocyte migration and adhesion (14, 16), consistent with the reported role for Arhgef1 in fibroblast adhesion to fibronectin (17).

Previously, we have shown that Arhgef1-deficient mice spontaneously develop pulmonary pathology, including a loss of alveolar structure that is accompanied by an increased presence of pulmonary macrophages and exaggerated production of MMP2, MMP9, and MMP12 by *Arhgef1*$^{-/-}$ alveolar macrophages (9). Exaggerated MMP9 production by *Arhgef1*$^{-/-}$ macrophages was further found to be recapitulated *in vitro* when cells are cultured on fibronectin and largely dependent on the $\alpha5\beta1$ integrin. In this study, we have investigated the macrophage signaling pathway regulated by Arhgef1 that leads to MMP9 production upon integrin-fibronectin interaction. We find that fibronectin induction of MMP9 by murine macrophages, as well as human alveolar macrophages and peripheral blood monocytes, is dependent on autocrine thromboxane receptor signaling, and this $G\alpha_{12/13}$-associated GPCR signaling is normally inhibited by Arhgef1. We further show that the expression of *ARHGEF1* by human monocytes varies among individuals and is inversely correlated to *in vitro* fibronectin-mediated MMP9 production by these cells.

EXPERIMENTAL PROCEDURES

Murine Macrophage Isolation—Peritoneal elicited macrophages were obtained by peritoneal lavage with 5 ml of ice-cold Hanks' balanced salt solution with 5 mmol/liter EDTA from mice 5 days after intraperitoneal injection of 1 ml of sterile thioglycollate as previously described (9). Resident peritoneal macrophages were obtained in the same manner in the absence of thioglycollate treatment. The cells were counted using a Z2 particle count and size analyzer (Beckman Coulter, Fullerton, CA) as previously described (9). Macrophages were resuspended in DMEM with 5% heat-inactivated fetal calf serum and plated at the indicated cellular concentrations.

Macrophage/Monocyte Culture Conditions—Tissue-culture 96-well plates (Costar high binding catalogue number 3590) were coated with 10 μg/ml of murine fibronectin (Molecular Innovations, Nori, MN; catalogue number MFBN) or human fibronectin (Molecular Innovations; catalogue number HFBN) in Dulbecco's PBS without Ca^{2+} or Mg^{2+} (Mediatech, Inc., Manassas, VA) for 2 h at room temperature. The cells at the indicated concentrations were added to each well in a 100-μl volume. The plates were centrifuged at 40 $\times$ *g* for 1 min and then incubated for 24 h at 37 °C with 10% CO_2. Next, the wells were washed with PBS to remove nonadherent cells, and 100 μl of fresh DMEM without fetal calf serum was added to each well. In some experiments, the cells were treated at this point with either sphingosine 1-phosphate, lysophosphatidic acid (Avanti Polar Lipids, Alabama), U-46619, L-655,240, pinane-thromboxane A_2 (PTA_2), or aspirin (Cayman Chemical Company, Ann Arbor, MI) after resuspension as per manufacturer recommendations. The plates were then incubated for an additional 24 h at 37 °C with 10% CO_2. Next, conditioned medium was removed and stored at −80 °C for subsequent analysis. PGE_2 and TXB_2 were measured in conditioned medium by ELISA according to the manufacturer's instructions (ElisaTech, Aurora, CO). The cells were treated with TRIzol (Invitrogen) for RNA purification as previously described (18).

Human Alveolar Macrophages—Human alveolar macrophages were isolated from the bronchoalveolar lavage of healthy volunteers using protocols approved by the National Jewish Health Human Subjects Institutional Review Board. Six individuals (two males and four females) between the ages of 52 and 67 years of age participated in the study. The lavage cells were enumerated as described above for murine macrophages. The cells were then resuspended in DMEM with 5% FCS and plated at the indicated cellular concentrations in 96-well plates. Human cells were cultured as described for murine macrophages.

Human Peripheral Blood Monocytes—Peripheral blood monocytes were isolated from whole blood of healthy volunteers using protocols approved by the National Jewish Health Human Subjects Institutional Review Board. Ten individuals (five males and five females) between the ages of 45 and 68 years of age participated in the study. Whole blood was obtained by venipuncture and collected in an 8-ml BD Vacutainer™ CPT™ tube (BD Biosciences, Franklin Lakes, NJ). Mononuclear cells were isolated according to the manufacturer's instructions and enumerated as previously described for murine macrophages. After resuspension in DMEM with 5% FCS, the cells were plated at the indicated cellular concentrations in 96-well plates and incubated at 37 °C with 10% CO_2. After 24 h, the wells were washed with PBS to remove nonadherent cells, and 100 μl of fresh DMEM without fetal calf serum was added to each well. This procedure consistently yields

monocyte purities of >80% as assessed by flow cytometry (19, 20).

Real Time RT-PCR—Total RNA was purified using TRIzol according to the manufacturer's instructions, and gene expression was determined using real time RT-PCR as previously described (9). Differences in expression between samples was determined using the comparative threshold cycle ($\Delta\Delta C_T$) as suggested by the manufacturer (Applied Biosystems, Foster City, CA), normalizing each sample to either murine or human GAPDH (catalogue numbers 435293E and Hs99999905_m1, respectively). Murine MMP9 (catalogue number Mm00442991_m1), human MMP9 (catalogue number Hs00957562_m1), and human ARHGEF1 (catalogue number Hs00180327_m1) expression was determined using primers and probe sets purchased from Assays on Demand (Applied Biosystems).

MMP9 Quantitation—MMP9 was quantitated using gelatin zymography as described below for all murine experiments and the human studies displayed in Figs. 4, 5, and 6*B*. For subsequent human studies, we quantitated MMP9 by ELISA in conditioned medium (ELISA TECH, Aurora, CO).

Gelatin Zymography—Novex (Invitrogen) zymogram 10% gelatin gels were used for zymography as indicated per the manufacturer's instructions on conditioned media. Protease activity was detected as a loss of gelatin at the molecular weight of the indicated MMP and quantified by densitometric analysis using ImageJ (National Institutes of Health) software.

RESULTS

Fibronectin-mediated MMP9 Production by Macrophages Is Cell Concentration-dependent—Macrophages are induced to express MMP9 when cultured on ECM proteins such as fibronectin (6, 21–23). We have previously reported that the intracellular signaling molecule Arhgef1 functions in macrophages to suppress this protease production *in vivo* and *in vitro* and that $\alpha5$ and $\beta1$ integrin signaling is largely required for this *in vitro* MMP9 production (9). In the course of that study, we noted a macrophage cell concentration-dependent effect on MMP9 induction. To investigate this in more detail, we cultured thioglycollate-elicited peritoneal macrophages, isolated from wild type or *Arhgef1*$^{-/-}$ mice, over a range of cell concentrations. The cells were cultured for 48 h on either plastic or fibronectin, and *Mmp9* expression was measured. Fig. 1*A* shows that fibronectin promotes *Mmp9* expression by wild type and mutant macrophages over a range of cell concentrations ($0.03–0.5 \times 10^6$ cells/ml) that is maximal at 0.13×10^6 cells/ml for both genotypes. As previously reported (9), in the absence of Arhgef1, fibronectin induction of *Mmp9* by macrophages is significantly elevated (Fig. 1*A*; note the *y axis* is log scale). At the highest cell concentration of 2.0×10^6 cells/ml, fibronectin did not appear to induce *Mmp9* expression. To determine whether these changes in *Mmp9* mRNA were similarly reflected by changes in MMP9 protein, we also measured protease activity in conditioned media. These results show that MMP9 activity is significantly elevated in conditioned media from cells cultured on fibronectin compared with cells cultured on plastic and at all cell concentrations evaluated (Fig. 1, *B* and *C*). Furthermore, Arhgef1-deficient macrophages exhibited significantly higher levels of MMP9 activity compared with wild type cells at 0.25×10^6 cells/ml. However, this difference in MMP9 activity between *Arhgef1*$^{-/-}$ and wild type samples diminishes as cell concentration increases, possibly reflecting a limited linear range when quantitating gelatin zymography.

These results indicate that *Mmp9* mRNA expression reflects the status of the macrophages at the time of harvest, whereas MMP9 activity reflects the cumulative production of MMP9 over the culture period. The differences between wild type and Arhgef1-deficient samples are most pronounced at the lower cellular concentrations as analyzed by both qPCR and enzyme activity. Therefore, we used these cell concentrations to investigate how the absence of Arhgef1 alters the expression and production of MMP9 by macrophages cultured on fibronectin.

We initially explored whether the cell concentration dependence could be attributed to cell-to-cell contact. To accomplish this, the cells were fixed after culture on either plastic or fibronectin and stained with crystal violet to determine the extent of cell-to-cell contact. We found minimal cell-to-cell contact between macrophages at cell concentrations that induce MMP9 expression and production (data not shown). At the highest cell concentration (2.0×10^6 cells/ml), essentially all macrophages are in contact with adjacent cells, yet MMP9 production remains elevated in the samples cultured on fibronectin (data not shown). Thus, we conclude that cell-to-cell contact does not inhibit MMP9 production at the higher cell concentrations and, conversely, is not required for *Mmp9* induction at the lower concentrations. We next considered whether an autocrine/paracrine factor(s) might contribute to macrophage *Mmp9* expression.

Thromboxane and PGE_2 Are Produced by Macrophages Cultured on Fibronectin—In addition to MMP9 production, ECM proteins have also been shown to promote prostanoid production by myeloid cells (24–27). Thus, we questioned whether fibronectin was similarly stimulating production of PGE_2 and thromboxane A_2 by macrophages in our *in vitro* cultures. In particular, PGE_2 has been reported to function as an autocrine factor for MMP9 production by murine macrophages stimulated with ECM components (28). We measured PGE_2 in our culture supernatant from macrophages incubated on plastic or fibronectin at the cell concentration (0.13×10^6 cells/ml) that maximally induced *Mmp9* expression. These findings reveal that although conditioned media from macrophages cultured on plastic failed to contain measureable levels of PGE_2, conditioned media from macrophages cultured on fibronectin harbor readily detectable levels of this prostaglandin (Fig. 1*D*).

Thromboxane is another prostanoid produced by peritoneal macrophages when cultured on ECM proteins (24, 26) and was also measured in the supernatant from our macrophage cultures. Similar to PGE_2, the thromboxane metabolite TXB_2 was also modestly but reproducibly induced by fibronectin in both wild type and mutant macrophages (Fig. 1*E*). Thus, fibronectin not only induces increased macrophage MMP9 production but also leads to synthesis of the inflammatory prostanoids PGE_2 and TXB_2. Furthermore, although both prostanoids were elevated in Arhgef1-deficient samples, these increases were not statistically significant, and we conclude that production of these prostanoids does not depend on Arhgef1 expression.

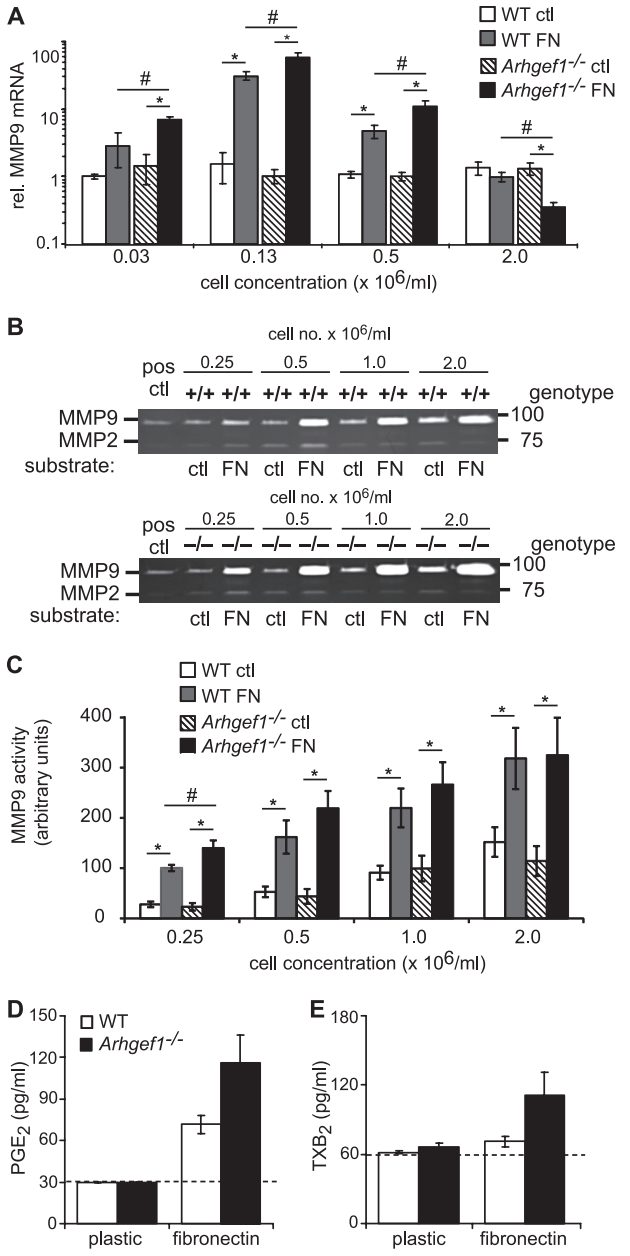

FIGURE 1. **Fibronectin induces macrophages to produce MMP9, PGE_2, and TXB_2.** *A*, *Mmp9* expression was measured by qPCR in peritoneal macrophages cultured on FN (10 μg/ml) or plastic (*ctl*) plated at the indicated cellular concentrations and cultured for 48 h. *Mmp9* expression is shown on a log scale as fold induction over expression of wild type cells cultured on plastic (*ctl*) at each respective concentration. The number of experiments at each concentration (0.03, 0.13, 0.5, and 2.0 $\times$ 10^6 cells/ml) for wild type cells on plastic (*open bars*) are represented by $n = 3$, 5, 10, and 3, respectively; wild type cells on fibronectin (*gray bars*) are represented by $n = 3$, 12, 12, and 3; *Arhgef1*$^{-/-}$ cells on plastic (*hatched bars*) are represented by $n = 3$, 6, 11, and 3; *Arhgef1*$^{-/-}$ cells on fibronectin (*black bars*) are represented by $n = 3$, 12, 12, and 3. The data represent the means $\pm$ S.E. *B*, representative zymogram of conditioned media from peritoneal macrophages cultured on either plastic (*ctl*) or FN at the indicated cellular concentrations from wild type (+/+) and *Arhgef1*$^{-/-}$ (−/−) samples. Molecular weight standards and respective enzymatic activities of MMP9 and MMP2 are shown. *C*, quantitation of MMP9 activity as determined by densitometric analyses of zymograms. MMP9 activity is shown in arbitrary units and represents $n = 7$ for wild type cells on plastic (*open bars*) and on fibronectin (*gray bars*) at all cellular concentrations. For *Arhgef1*$^{-/-}$ samples, $n = 6$ for cells on plastic (*hatched bars*) and on fibronectin (*black bars*) at all cellular concentrations. The data represent the means $\pm$ S.E. *D*, PGE_2 was measured by ELISA in conditioned media from macrophages cultured for 48 h on either plastic or fibronectin. For wild type (*open bars*), $n = 4$, and for *Arhgef1*$^{-/-}$ (*black bars*), $n = 6$. The data represent the means $\pm$ S.E. The *dotted line* indicates the limit of detection. *E*, TXB_2 was measured by ELISA in conditioned media from macrophages cultured for 48 h on either plastic or fibronectin. For wild type (*open bars*), $n = 4$, and for *Arhgef1*$^{-/-}$ (*black bars*), $n = 6$. The data represent the means $\pm$ S.E. The *dotted line* indicates the limit of detection. *, $p < 0.05$ Student's two-tailed *t* test comparing MMP9 expression/activity on fibronectin to respective cells on plastic. #, $p < 0.05$ Student's two-tailed *t* test comparing wild type to *Arhgef1*$^{-/-}$ cells under identical conditions.

Cyclooxygenase Activity and Thromboxane Receptor Signaling Are Required by Macrophages for Fibronectin-mediated MMP9 Production—Prostaglandins and thromboxane are generated from arachidonic acid metabolism by cyclooxygenases; thus we asked whether cyclooxygenase activity was similarly required for fibronectin-mediated induction of MMP9. To address this, we treated macrophages cultured on fibronectin with a cyclooxygenase inhibitor, aspirin, followed by measurement of MMP9 production and activity by gelatin zymography. These data revealed that aspirin inhibited MMP9 activity in a dose-dependent manner in both wild type and Arhgef1-deficient macrophages maximally inhibiting MMP9 activity by 70–75% (Fig. 2, *A* and *B*). We note that the 20 mM maximal concentration of aspirin used is >4-fold above the IC_{50} for COX1 and COX2 (29). Therefore, cyclooxygenase activity is required for optimal fibronectin-induced macrophage MMP9 production, although our results also indicate a minor contribution to MMP9 production via a cyclooxygenase-independent pathway.

We find that fibronectin induces PGE_2 production by primary murine macrophages (Fig. 1*D*), and it has previously been reported that PGE_2 signaling via the EP4 receptor is required for production of MMP9 by a murine macrophage cell line after stimulation with a mixture of ECM components (28). To assess whether a similar dependence on EP4 signaling was also required for fibronectin-induced MMP9 production by primary mouse macrophages, we inhibited EP4 receptor signaling with the EP4 antagonist L-161,982 (30). Treatment of macrophages cultured on fibronectin with 0.4–10 μM of the L-161,982 EP4 receptor antagonist maximally suppressed fibronectin-induced MMP9 production by mouse macrophages by only ~40% (Fig. 2*C* and data not shown) despite completely inhibiting EP4 signaling at these same concentrations (31). Thus, although EP4 signaling may be necessary for maximal fibronectin-mediated MMP9 production by murine macrophages, there is not an absolute requirement for signaling by this receptor to produce MMP9.

Our data thus far show that fibronectin-induced macrophage MMP9 production is largely dependent on cyclooxygenase activity (Fig. 2, *A* and *B*) but less so on EP4 signaling (Fig. 2*C*). Macrophages also express thromboxane receptors (32), and because thromboxane is also produced when macrophages are cultured on fibronectin (Fig. 1*E*), we next assessed whether autocrine thromboxane receptor signaling contributed to MMP9 production and might partially account for the cyclooxygenase dependence. To test this, we inhibited macrophage thromboxane receptor signaling with two different receptor antagonists: PTA_2 and L-655,240 (33, 34). Treatment of mouse macrophages with PTA_2 resulted in a reproducible and dose-dependent suppression of MMP9 production and

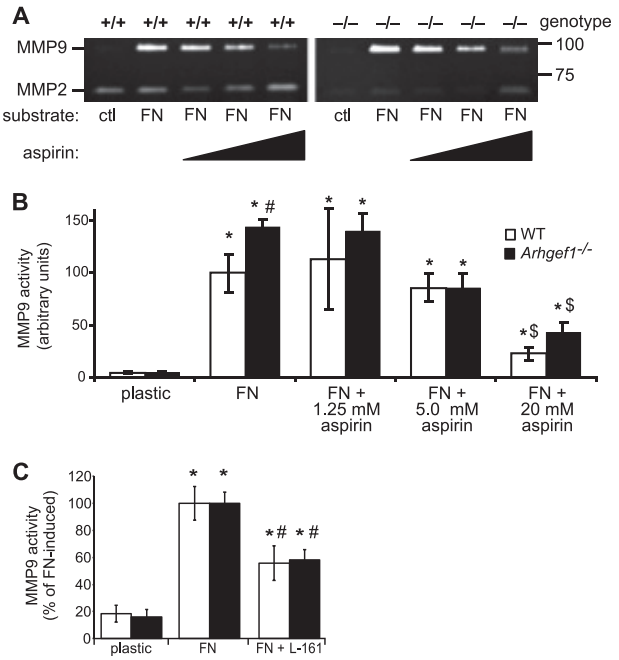

FIGURE 2. **Cyclooxygenase activity but not EP4 receptor signaling is required for MMP9 production by murine macrophages cultured on fibronectin.** *A*, representative MMP9 gelatin zymograms of conditioned media from peritoneal macrophages cultured on either plastic (*ctl*) or FN and treated with increasing concentrations of aspirin (1.25, 5.0, and 20 mM, respectively). Wild type (+/+) and *Arhgef1*$^{-/-}$ (−/−) samples from separate zymograms are shown. Molecular weight standards and respective enzymatic activities of MMP9 and MMP2 are shown. *B*, quantitation of MMP9 activity as determined by densitometric analysis of zymograms in *A*. MMP9 activity is shown in arbitrary units and represents $n = 6$ for both wild type (*open bars*) and *Arhgef1*$^{-/-}$ (*black bars*) samples. The results are compiled from two independent experiments. The data represent the means ± S.E. *, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on plastic. #, $p < 0.05$ Student's two-tailed *t* test comparing conditioned media from wild type cells to *Arhgef1*$^{-/-}$ cells cultured under identical conditions. $, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on fibronectin. *C*, quantitation of MMP9 activity in conditioned media from macrophages cultured on fibronectin in the presence of the EP4 antagonist L161,982 (10 μM). MMP9 activity was normalized to fibronectin response for each genotype. The results are compiled from two independent experiments with wild type (*open bars*, $n = 5$) and *Arhgef1*$^{-/-}$ (*black bars*, $n = 5$) samples. The data represent the means ± S.E. *, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on plastic. #, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on fibronectin.

activity reaching 80–85% inhibition in both wild type and Arhgef1-deficient macrophages (Fig. 3, *A* and *B*). Similarly, the L-655,240 thromboxane receptor antagonist also inhibited MMP9 production and activity by ~90% regardless of macrophage genotype (Fig. 3, *C* and *D*). In contrast, MMP2 activity was not affected by either fibronectin stimulation or treatment with 20 mM aspirin or 28 μM L-655,240, indicating that this fibronectin signaling pathway was specific for MMP9 and that neither the cyclooxygenase nor the thromboxane receptor antagonists influenced cell viability (Fig. 3*E*). Furthermore, because aspirin, L-161,982, PTA_2, and L-655,240 were all dissolved in DMSO with a maximal final concentration of <0.5%, we compared the MMP9 activity in samples cultured on fibronectin and treated with 0.5% DMSO or 28 μM L-655,240 (Fig. 3*F*). Although treatment with 28 μM L-655,240 significantly reduced MMP9 activity as previously shown (Fig. 3*D*), the addition of 0.5% Me_2SO alone did not significantly alter MMP9 activity compared with untreated cells (Fig. 3*F*).

These findings demonstrate that when thromboxane receptor signaling is prevented, fibronectin-mediated production of MMP9 by mouse macrophages is largely abolished. Considered collectively, thus far these results show that fibronectin induces the production of PGE_2, TXB_2, and MMP9 by macrophages and Arhgef1 attenuates the production of MMP9. Furthermore, fibronectin-mediated MMP9 production by macrophages appears completely dependent on thromboxane receptor signaling and partially dependent on signaling by the EP4 receptor.

Thromboxane Receptor Signaling Is Exaggerated in Arhgef1-deficient Macrophages—Prostanoids signal via GPCRs (35), and Arhgef1 harbors an RGS domain that has been characterized to specifically attenuate signaling by GPCRs that associate with $G\alpha_{12/13}$ heterotrimeric G-protein subunits (11). Of the nine identified prostanoid receptors, only the thromboxane receptor signals via $G\alpha_{12/13}$ (36–40), and Arhgef1 has been shown to inhibit thromboxane receptor signaling in leukocytes (38, 40).

Our results show that fibronectin induces MMP9 production by macrophages in a signaling pathway dependent on thromboxane receptor signaling (Fig. 3, *A–D*) and that Arhgef1-deficient macrophages display elevated MMP9 production compared with wild type cells (Fig. 1 and Ref. 9). Therefore, we hypothesized that if Arhgef1 functioned normally to attenuate thromboxane receptor autocrine signaling, then Arhgef1-deficient macrophages would lack this repression and might account for the elevated MMP9 production displayed by *Arhgef1*$^{-/-}$ macrophages *in vitro* and *in vivo*. To directly test this, we pharmacologically manipulated thromboxane receptor signaling *in vitro* with the thromboxane receptor agonist U-46619 (41). The results from these experiments reveal that although wild type macrophage *Mmp9* expression was similar in the presence and absence of U-46619, Arhgef1-deficient macrophages expressed significantly more *Mmp9* after U-46619 agonist treatment compared with untreated cells (Fig. 3*G*).

Besides the thromboxane receptor, macrophages also express $G\alpha_{12/13}$-associated GPCRs specific for the sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) lysophospholipids (42–44) that have been shown to be inhibited by Arhgef1 (14, 45, 46). However, S1P or LPA stimulation of macrophages cultured on fibronectin promoted a slight decrease in *Mmp9* expression by both wild type or mutant macrophages (Fig. 3*G*) consistent with previous reports that these lysophospholipids blunt *in vitro* macrophage inflammatory responses to LPS stimulation (47, 48). These results indicate that Arhgef1 attenuation of MMP9 production is specific for thromboxane receptor-mediated signaling. Based on these findings, we conclude that fibronectin induces macrophages to produce MMP9 via thromboxane receptor-dependent autocrine signaling, and Arhgef1 normally functions to attenuate this signaling pathway.

Human Alveolar Macrophages Produce MMP9 and TXB_2 in a Cell Concentration-dependent Manner—We have observed that Arhgef1-deficient lungs display pathophysiology reminiscent of the lungs from emphysematous individuals. Thus, we were interested to know whether fibronectin-mediated production of MMP9 and prostanoids was also recapitulated with

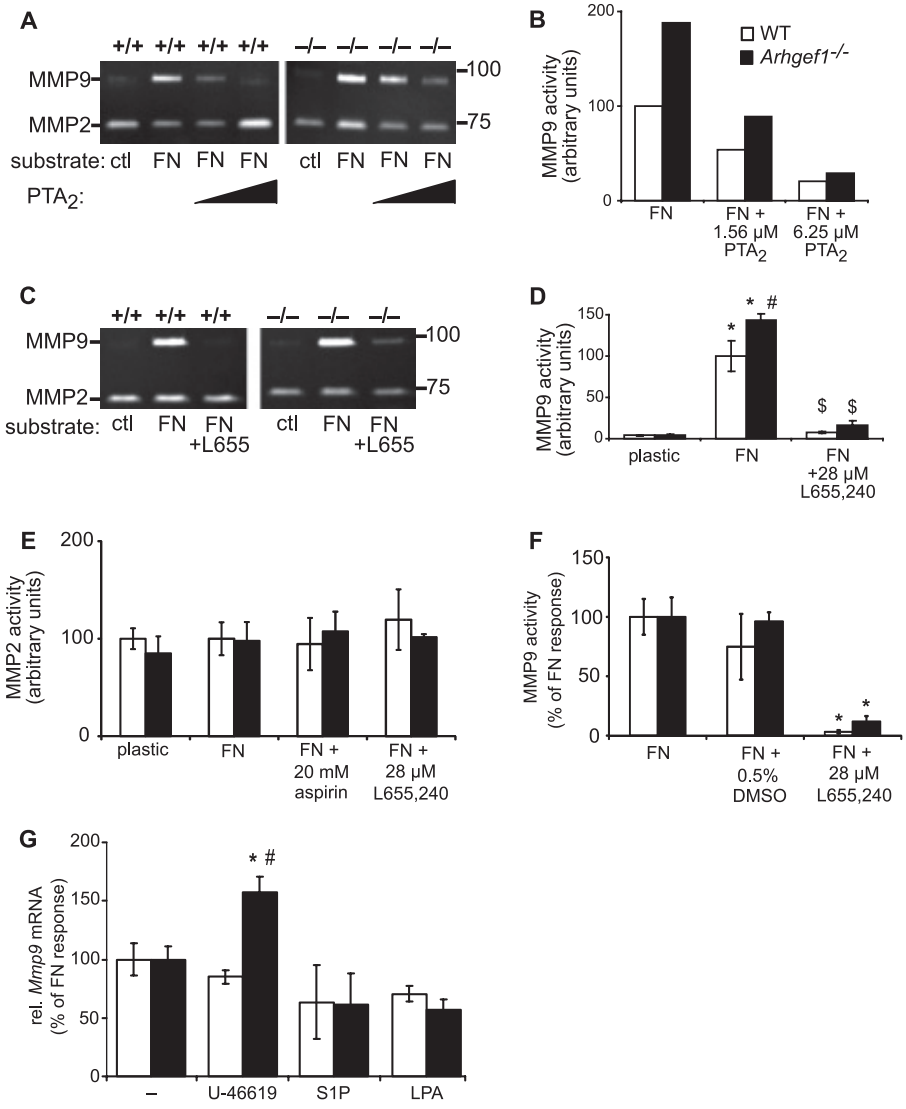

FIGURE 3. **Thromboxane receptor signaling is necessary for fibronectin-induced MMP9 production by macrophages and is attenuated by Arhgef1.** *A*, representative gelatin zymograms of conditioned media from macrophages cultured on plastic (*ctl*) or FN and treated with 1.56 or 6.25 μM of the thromboxane receptor antagonist PTA_2. Wild type (+/+) and *Arhgef1*$^{-/-}$ (−/−) samples from separate zymograms are shown. *B*, quantitation of MMP9 activity as determined by densitometric analysis of zymograms in *A*. The results are representative of two independent experiments. *C*, representative zymograms from conditioned media from macrophages cultured on plastic or fibronectin and treated with 28 μM of the thromboxane receptor antagonist L-655,240. *D*, quantitation of MMP9 activity as determined by densitometric analysis of zymograms in *C*. The results are compiled from two independent experiments with $n = 4$ for both wild type (*open bars*) and *Arhgef1*$^{-/-}$ (*black bars*) samples. The data represent the means ± S.E. *, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on plastic. #, $p < 0.05$ Student's two-tailed *t* test comparing conditioned media from wild type cells to *Arhgef1*$^{-/-}$ cells cultured under identical conditions. $, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on fibronectin. *E*, MMP2 activity was quantitated in conditioned media as previously described for MMP9. The results are compiled from four independent experiments with $n = 10$ for wild type (*open bars*) and *Arhgef1*$^{-/-}$ (*black bars*) samples cultured on either plastic or fibronectin. For cells treated with 20 mM aspirin, $n = 6$ for wild type (*open bars*) and *Arhgef1*$^{-/-}$ (*black bars*) samples. For cells treated with 28 μM L-655,240, $n = 4$ for wild type (*open bars*) and *Arhgef1*$^{-/-}$ (*black bars*) samples. The data represent the means ± S.E. *F*, MMP9 activity was quantitated as previously described and normalized to a percentage of fibronectin response for each genotype. The cells were either untreated or treated with 0.5% DMSO or 28 μM L-655,240. The results are compiled from two independent experiments with $n = 3$ for all conditions and genotypes. The data represent the means ± S.E. *, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on fibronectin. *G*, relative *Mmp9* expression was measured by qPCR in macrophages cultured on fibronectin in the presence of the thromboxane receptor agonist (U-46619, 10 nM), S1P (40 nM), or LPA (10 μM). *Mmp9* expression is displayed as a percentage of fibronectin response for each genotype. Wild type (*open bars*, $n = 12, 6, 3$, and 3 for fibronectin, +U-46619, +S1P, and +LPA, respectively) and *Arhgef1*$^{-/-}$ (*black bars*, $n = 14, 6, 3$, and 2, respectively) from at least two independent experiments. The data represent the means ± S.E. *, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on fibronectin. #, $p < 0.05$ Student's two-tailed *t* test comparing conditioned media from wild type cells to *Arhgef1*$^{-/-}$ cells cultured under identical conditions.

human macrophages. Similar to murine peritoneal macrophages, we observed a strong cell concentration-dependent effect on the induction *MMP9* mRNA in human alveolar macrophages cultured on fibronectin with maximal induction when macrophages are plated at 0.13×10^6 cells/ml (Fig. 4*A*). Measurement of MMP9 activity in the conditioned media from these cultures by gelatin zymography confirmed an increase in MMP9 production and activity in the samples cultured on fibronectin compared with those cultured on plastic at the same concentration (Fig. 4*B*). Furthermore, analysis of culture supernatant from macrophages isolated from six individuals confirmed that fibronectin induces human alveolar macrophages to significantly increase the production of active MMP9 (Fig. 4*C*).

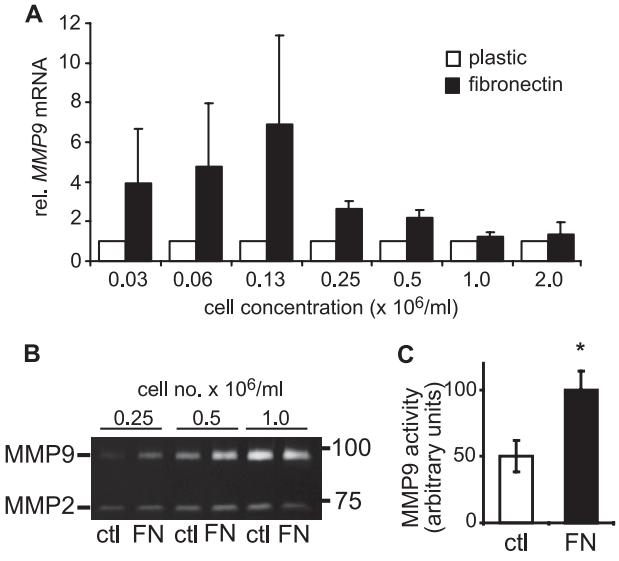

FIGURE 4. **Human alveolar macrophages are induced to express *MMP9* when cultured on fibronectin.** *A*, induction of *MMP9* expression by human macrophages cultured on plastic (*open bars*) or fibronectin (*closed bars*) as measured by qPCR at the indicated cell concentrations. MMP9 expression on fibronectin is shown as fold overexpression of cells cultured on plastic at each respective concentration from the same individual. The results are compiled from cells obtained from four individuals. The data represent the means ± S.E. *B*, representative gelatin zymogram of conditioned media from human macrophages cultured on either plastic (*ctl*) or FN at the indicated cell concentrations. *C*, quantitation of MMP9 activity in conditioned media from cells cultured on FN or plastic (*ctl*) at 0.125 × 10^6 cells/ml. The results are compiled from cells obtained from six individuals. The data represent the means ± S.E. *, $p < 0.05$ Student's two-tailed *t* test compared with cells cultured on plastic.

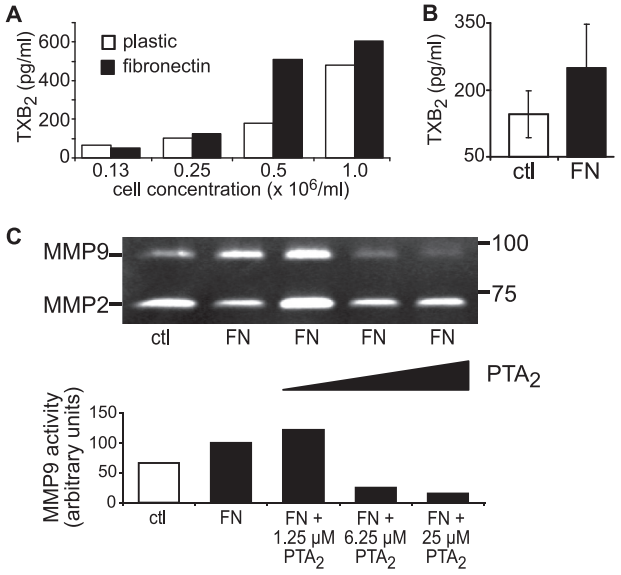

FIGURE 5. **Human alveolar macrophages produce TXB_2 when cultured on fibronectin and thromboxane receptor signaling is required for MMP9 production.** *A*, TXB_2 production measured in conditioned media from human macrophages cultured on fibronectin (*closed bars*) or plastic (*open bars*) at the indicated cellular concentrations. *B*, TXB_2 measured in conditioned media from macrophages cultured at 0.125 × 10^6 cells/ml on either plastic (*ctl*) or FN from four individuals. The data represent the means ± S.E. *C*, gelatin zymogram of conditioned media from cells cultured as indicated and in the presence of increasing doses of the thromboxane receptor antagonist PTA_2 (at 1.25, 6.25, and 25 μM, respectively). Below the zymogram is quantitation of MMP9 activity by densitometric analysis.

We also determined whether PGE_2 and TXB_2 were present in the supernatant of human alveolar macrophages cultured on plastic or fibronectin. These results show that although TXB_2 was produced by cultured human macrophages in a cell concentration-dependent manner (Fig. 5*A*), unlike murine macrophages, PGE_2 was not produced at measureable levels at any cell concentration and when assessed from macrophages isolated from four different individuals (data not shown). This difference between human and mouse macrophages in the ability to produce PGE_2 upon stimulation has been previously reported (49). Although human macrophages cultured on fibronectin reproducibly generated more TXB_2 compared with that produced when cultured on plastic, we found that this production varied between macrophages isolated from different individuals (Fig. 5*B*). Importantly, and similar to that observed with murine macrophages, Fig. 5*C* shows that treatment with the thromboxane receptor antagonist PTA_2 resulted in a dose-dependent inhibition (approaching 90%) of MMP9 production. These results demonstrate that thromboxane receptor signaling is also required for fibronectin-induced MMP9 production by human alveolar macrophages. Thus, both human and murine macrophages are induced to express MMP9 and thromboxane when cultured on fibronectin, and MMP9 production is dependent on thromboxane receptor autocrine signaling.

Fibronectin Induces MMP9 and Thromboxane in Peripheral Blood Monocytes and MMP9 Production Is Dependent on Thromboxane Receptor Signaling—Human peripheral blood monocytes have also been shown to increase MMP9 production when cultured on fibronectin (21). Therefore, we repeated our *in vitro* culture with human peripheral blood monocytes and, consistent with both murine peritoneal (Fig. 1*A*) and human alveolar macrophages (Fig. 4*A*), a cell concentration-dependent effect was documented for the induction of MMP9 by human monocytes cultured on fibronectin (Fig. 6, *A* and *B*). In experiments with human monocytes, we noted variation in MMP9 activity between cultures established from different individuals. Thus, to alleviate concerns of a limited linear range of activity detected by gelatin zymography, we turned to measuring MMP9 protein by ELISA, and that similarly demonstrated fibronectin was able to induce human monocytes to produce significant amounts of MMP9 over control (Fig. 6*C*).

As observed with alveolar macrophages, although production of PGE_2 was not measureable in the supernatant in multiple subjects over a range of cellular concentrations (data not shown), a cell concentration-dependent increase in TXB_2 production by monocytes cultured on fibronectin was observed (Fig. 6, *D* and *E*). Fibronectin-induced MMP9 production by human monocytes was again found to be dependent on thromboxane receptor signaling (Fig. 6, *F* and *G*) as shown by treating cultures with two separate thromboxane receptor antagonists (L-655,240 and PTA_2), respectively.

Considering our data collectively, we show that human and murine myeloid cells are stimulated by fibronectin to produce both thromboxane and MMP9, whereas fibronectin-mediated PGE_2 production appears restricted to mouse macrophages. Furthermore, the production of MMP9 induced by fibronectin in both human and mouse myeloid cells proceeds by a thromboxane receptor-dependent signaling pathway.

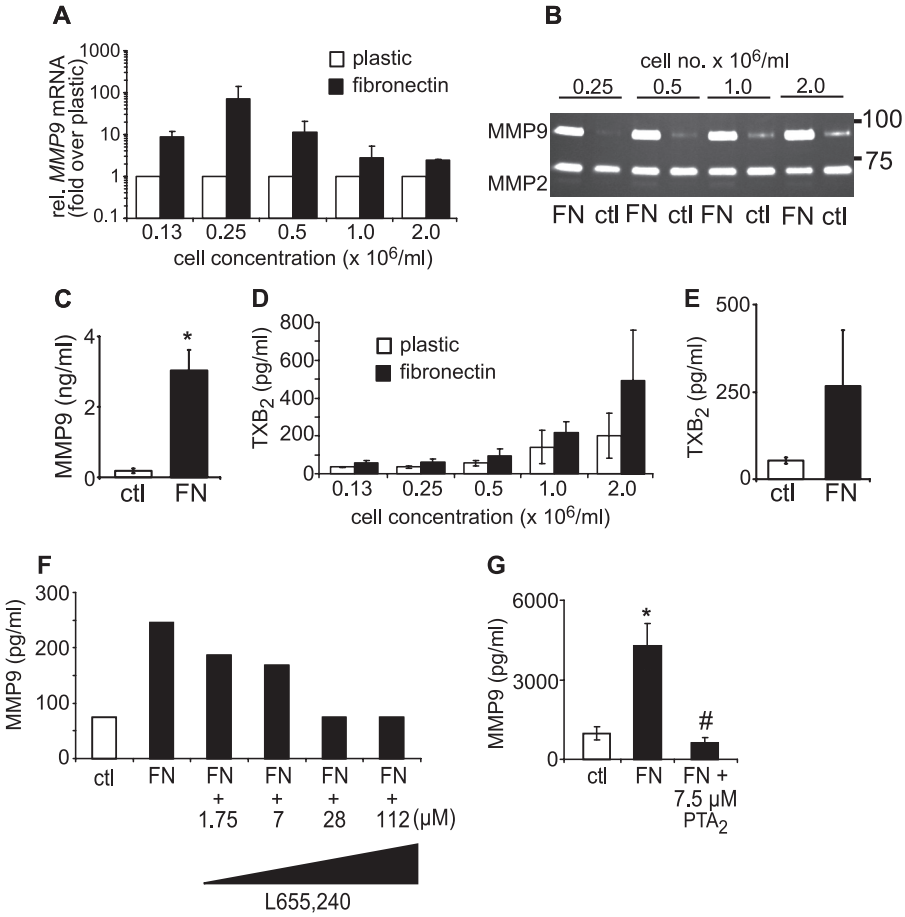

FIGURE 6. **Human peripheral blood monocytes cultured on fibronectin are induced to express *MMP9* and that is dependent on thromboxane receptor signaling.** *A*, MMP9 induction was measured by qPCR at the indicated cell concentrations. *MMP9* expression on fibronectin (*closed bars*) is represented as fold overexpression of cells cultured on plastic at each respective concentration from the same individual. The results are compiled from cells obtained from two individuals. The data represent the means ± S.E. *B*, representative zymogram of conditioned media from monocytes cultured on either plastic (*ctl*) or FN at the indicated cellular concentrations. *C*, MMP9 production as measured by ELISA in conditioned media from monocytes cultured on plastic (*ctl*) or FN at 0.25×10^6 cells/ml. The results are compiled from cells obtained from eight individuals. The data represent the means ± S.E. *D*, TXB_2 production in conditioned media from monocytes cultured on either plastic or FN at the indicated cellular concentrations. The results are compiled from cells obtained from three individuals. The data represent the means ± S.E. *E*, TXB_2 in conditioned media from monocytes cultured on either plastic (*ctl*) or FN at 0.25×10^6 cells/ml. The results are compiled from cells obtained from 10 individuals. The data represent the means ± S.E. *F*, MMP9 levels as measured by ELISA in conditioned media from monocytes cultured as indicated with increasing concentrations of the thromboxane receptor antagonist L-655,240 (1.75, 7, 28, and 112 μM). *G*, MMP9 as measured by ELISA in conditioned media from monocytes cultured as indicated with the thromboxane receptor antagonist PTA_2 (7.5 μM). The results are compiled from cells obtained from six individuals. *, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on plastic. #, $p < 0.05$ Student's two-tailed *t* test compared with conditioned media from cells cultured on fibronectin.

ARHGEF1 Expression Is Negatively Correlated with MMP9 Production in Human Monocytes—As previously noted, although human monocytes are stimulated to produce MMP9 when cultured on fibronectin, a wide range was observed in the amount of MMP9 produced by monocytes from different individuals (*e.g.* Fig. 6, *C* and *G*). Based on the observation that murine macrophages deficient in Arhgef1 demonstrate exaggerated MMP9 production, we hypothesized that differences in ARHGEF1 expression between individuals may account for some of this variation. To directly test this hypothesis, we compared ARHGEF1 expression with MMP9 production in monocytes and cultured supernatant, respectively, from nine individuals. Specifically, *ARHGEF1* expression was measured by qPCR in monocytes, and MMP9 was measured in conditioned media of monocytes cultured on fibronectin. Subsequently, we plotted the relative *ARHGEF1* expression in monocytes from a given individual against the amount of MMP9 produced by the same monocytes when cultured on fibronectin (Fig. 7). Consistent with our hypothesis, we found a significant negative correlation between *ARGHEF1* expression and MMP9 production in human peripheral blood monocytes (Fig. 7). From these data, we conclude that the level of MMP9 produced by myeloid cell interaction with integrin ligands is at least partially dependent on the level of *ARHGEF1* expressed by these leukocytes.

DISCUSSION

Arhgef1-deficient mice display unprovoked pulmonary pathophysiology resembling a chronic inflammatory disorder, and $Arhgef1^{-/-}$ pulmonary leukocytes, the vast majority which are macrophages, are able to transfer this phenotype to wild type lungs (9). Possibly contributing to alveolar tissue destruction in $Arhgef1^{-/-}$ lungs, we found that Arhgef1-deficient alveolar macrophages expressed significantly higher levels of several MMPs, including MMP9 that was increased over 100-fold in expression compared with wild type cells (9). To better understand the signaling pathways that lead to pulmonary

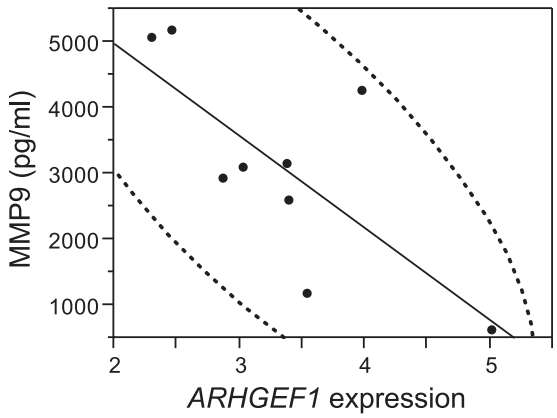

FIGURE 7. **ARHGEF1 expression negatively correlates with MMP9 production by monocytes cultured on fibronectin.** Relative *ARHGEF1* expression as measured by RT-PCR is expressed on the *x axis*. *ARHGEF1* expression was normalized to GAPDH expression and displayed relative to the lowest expressing individual. MMP9 production was measured in conditioned media by ELISA and is expressed on the *y axis*. Shown are the results from monocytes obtained from nine individuals cultured under identical conditions where each point represents the values obtained from a separate individual. A Pearson product moment correlation analysis was performed, and a correlation coefficient of -0.737 was obtained with a $p = 0.0234$ between *ARHGEF1* expression and MMP9 production. The *dotted lines* denote bivariate normal ellipse for 95% of the values. The *solid line* represents the linear fit.

pathology in the absence of Arhgef1, we identified an *in vitro* macrophage culture condition that recapitulated the enhanced MMP9 production by *Arhgef1*$^{-/-}$ macrophages observed *in vivo*. Using this system and myeloid cells from both human and mouse, in this study we show that fibronectin-mediated MMP9 production by myeloid cells depends on autocrine thromboxane receptor signaling. Furthermore, Arhgef1 functions normally to attenuate this signaling. Thus, these findings identify a previously unrecognized signaling pathway used by macrophages to produce MMP9 in response to fibronectin.

Adhesion to fibronectin induces Arhgef1-deficient macrophages to produce elevated levels of MMP9 and prostanoids compared with Arhgef1-sufficient wild type cells. Because the RGS activity of Arhgef1 has been characterized to attenuate signaling from GPCRs associated with $G\alpha_{12/13}$ heterotrimeric G-proteins (11), we sought to identify a putative $G\alpha_{12/13}$-associated GPCR expressed by macrophages that could signal in an autocrine manner and could account for this difference. GPCRs for three different lipids, LPA, S1P, and thromboxane, met this criteria and were expressed by macrophages, and each lipid ligand could feasibly be produced in an autocrine manner. However, when directly tested, the LPA and S1P lysophospholipids modestly repressed MMP9 production, and only thromboxane receptor stimulation led to enhanced MMP9 production by *Arhgef1*$^{-/-}$ macrophages relative to controls. Together, these results demonstrate that Arhgef1 functions in macrophages to attenuate thromboxane receptor signaling and that when Arhgef1-deficient macrophages are stimulated with fibronectin, exaggerated thromboxane receptor signaling leads to elevated MMP9 production.

An important conclusion from our results is that fibronectin-stimulated production of MMP9 by both human and mouse macrophages is dependent on thromboxane receptor signaling (Figs. 3, 5, and 6) and less dependent on cyclooxygenase activity (Fig. 2 and data not shown). Cyclooxygenase activity also is required for the production of PGE_2, and contrary to our findings, it was previously suggested that PGE_2-EP4 autocrine signaling was necessary for MMP9 production by mouse macrophages (28). The basis for the apparent difference with our findings is not clear but may reflect our measurement of MMP9 activity produced by primary macrophages after culture on fibronectin *versus* the stimulation of the RAW246.7 macrophage cell line with ECM components derived from vascular smooth muscle cells and measuring MMP9 by Western blot analysis (28). Perhaps more importantly, although we reproducibly measured elevated levels of PGE_2 in the supernatant of fibronectin-stimulated mouse macrophages, we were unable to detect PGE_2 in the supernatants of similarly stimulated human macrophages or monocytes that were isolated from multiple individuals and over a range of cellular concentrations. In contrast, thromboxane production was always induced and detected after fibronectin stimulation of both murine and human macrophages. Together, these data suggest differences in prostanoid metabolism between integrin-stimulated murine and human macrophages with regards to PGE_2 production. These data are supported by a recent investigation using mass spectrometry to identify prostanoids from toll-like receptor-stimulated human and murine macrophages (49). In that study, stimulated murine macrophages were found to produce PGE_2, whereas stimulated human macrophages only produced thromboxane and did not produce detectable levels of PGE_2. Despite these interspecies differences in PGE_2 production, fibronectin induces both human and murine macrophages to produce thromboxane and MMP9 and supports our identification that thromboxane receptor signaling is required for the fibronectin induction of MMP9 by both human and mouse myeloid cells.

Human and murine macrophages have long been known to produce thromboxane (50–53) in response to zymosan and a number of other stimuli including LPS, platelet-activating factor, complement components, and CD44 ligation (54–58). Myeloid cells also express thromboxane receptors (32), and thromboxane autocrine or paracrine signaling by human monocytes has been shown to participate in the production of the TNFα and IL-1β pro-inflammatory cytokines (59, 60). Our data show that macrophage autocrine thromboxane receptor signaling is also required for MMP9 production after adhesion to fibronectin. MMP9 is another pro-inflammatory mediator produced by macrophages during the inflammatory response (5) where it acts on chemokines and extracellular matrix proteins to regulate the recruitment of inflammatory cells and tissue remodeling. Thus, an autocrine thromboxane receptor signaling pathway appears to contribute to macrophage production of pro-inflammatory mediators during inflammation. Whether this pathway is used physiologically by macrophages to generally promote inflammation remains to be established. However, the chronic pulmonary inflammatory environment promoted by Arhgef1-deficient leukocytes (9), which cannot repress thromboxane receptor signaling, would support this notion. Indeed, inhibiting thromboxane synthesis or receptor signaling attenuates both *in vitro* macrophage production of the TNF-α and IL-1β pro-inflammatory cytokines (57, 60, 61) and *in vivo* features of inflammation (62–65).

Our results identify a common human and murine macrophage thromboxane receptor signaling pathway induced by fibronectin and, in mouse cells, attenuated by Arhgef1. However, as opposed to results obtained with genetically identical wild type and *Arhgef1*$^{-/-}$ macrophages, we found variation in fibronectin-induced MMP9 production by human macrophages and monocytes. Thus, we considered whether this variation reflected differences in *ARHGEF1* expression by myeloid cells from different individuals and found a >2.5-fold variation in monocyte *ARHGEF1* expression from nine individuals. Importantly, a significant inverse correlation was shown between *ARHGEF1* expression and *in vitro* fibronectin-induced MMP9 production. Thus, as with mouse macrophages, ARHGEF1 also appears to function in human myeloid cells to attenuate thromboxane receptor signaling.

Although macrophages play an instrumental role in the initiation, maintenance, and resolution of inflammation, they are also associated with the pathogenesis of many diseases associated with chronic inflammatory disorders such as chronic obstructive pulmonary disease, atherosclerosis, and cancer (1). The data presented in this study not only identify a novel macrophage signaling pathway that may contribute to chronic inflammatory processes that drive these diseases but also reveal potential therapeutic points for intervention.

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