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A Novel Role of Matrix Metalloproteinase-8 in Macrophage Differentiation and Polarization*

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Background: Macrophages differentiation and/or polarization have been implicated in many inflammatory diseases.
Results: Matrix metalloproteinase-8 (MMP8) promotes M2-macrophage differentiation and polarization through modulating TGF-β bioactivity.
Conclusion: MMP8 plays a novel role in macrophage differentiation and polarization.
Significance: The findings of this study significantly increased our understanding to biological functions of MMP8, the mechanisms for macrophage differentiation and polarization, and the pathogenesis of pathological conditions in which MMP8 is involved.

Matrix metalloproteinase-8 (MMP8) has been shown to influence various cellular functions. As monocytes and macrophages (Mϕ) express MMP8, we investigated if MMP8 played a role in macrophage differentiation and polarization. MMP8 expression was significantly increased during monocyte differentiation into Mϕ. Monocyte-derived Mϕ from MMP8-deficient mice expressed higher levels of M1-Mϕ markers but lower levels of M2-Mϕ markers than monocyte-derived Mϕ from wild-type mice. Although Mϕ from either MMP8-deficient or wild-type mice were inducible by interferon-γ into M1-Mϕ, only wild-type Mϕ but not MMP8-deficient Mϕ could be induced into M2-Mϕ by interleukin-4. However, MMP8-deficient Mϕ exposed to conditioned culture media of wild-type Mϕ developed a M2-Mϕ phenotype. Compared with conditioned culture media of wild-type Mϕ, conditioned culture media of MMP8-deficient Mϕ contained a lower concentration of active transforming growth factor-β (TGF-β), an M2-Mϕ inducer. Moreover, evidence also showed that the degradation of the TGF-β sequester, fibromodulin, was modulated by MMP8. The data indicate a previously unknown role of MMP8 in M2-Mϕ polarization by cleaving fibromodulin and therefore increasing the bioavailability of the M2-Mϕ inducer TGF-β.

Macrophages (Mϕ)[4] play important roles in many inflammatory diseases such as atherosclerosis. Studies have shown that the Mϕ phenotype is heterogeneous, plastic, and profoundly affected by local factors within the microenvironment (1–7). Lipopolysaccharides, interleukin-1β (IL-1β), and cytokines secreted by Th1 lymphocytes, such as interferon-γ (IFN-γ), induce a "classic" Mϕ phenotype (M1), whereas Th2 lymphocyte cytokines, such as IL-4 and IL-13, promote an "alternative" Mϕ phenotype (M2) (1–7). Activated M1-Mϕ produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6, and IL-12, whereas M2-Mϕ dampen the inflammatory state by producing anti-inflammatory molecules, such as IL-10, IL-1 receptor antagonist, and transforming growth factor (TGF)-β. In response to local factors in the microenvironment, M1-Mϕ can change to M2-Mϕ, and vice versa. Studies in recent years have revealed a number of signaling pathways, transcriptional networks, and epigenetic mechanisms that influence Mϕ differentiation and polarization (8, 9). These findings are important for understanding the pathogenesis of inflammatory diseases and can have potential therapeutic implications.

Matrix metalloproteinase-8 (MMP8), a relatively less studied member of the MMP family, has received increasing attention in recent years. The best known substrates of MMP8 are types I, II, and III collagens. However, a growing number of other proteins have been reported to be also susceptible to cleavage by MMP8, including angiotensin-1 (10, 11), chemokine (C-X-C motif) ligand 5 (CXCL5) (12), CXCL11 (13), and macrophage inflammatory protein-1 (14). Previously it was thought that only neutrophils produce MMP8, reflected by its alias names neutrophil collagenase and polymorphonuclear leukocyte collagenase. However, it is now known that various other types of cells also express this protease. Studies have shown that MMP8 can modulate the behavior and function of several cell types including neutrophils (14, 15), eosinophils (16), smooth muscle

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[4] The abbreviations used are: Mϕ, macrophages; MMP8, matrix metalloproteinase-8; MCP-1, monocyte chemoattractant protein-1; BMMϕ, bone marrow monocyte-derived macrophage; pMϕ, peritoneal naive macrophage; qPCR, quantitative PCR; LAP, latency-associated peptide; CM, culture medium.

cells (17), endothelial cells (18), and stem/progenitor cells (19), underlain by its proteolytic activity on matrix and non-matrix proteins. Furthermore, studies have shown that MMP8 is involved in a number of pathological conditions including atherosclerosis (11, 19), acute hepatitis (20), acute lung injury (14), airway inflammation (16), and cancers (21). Because Mϕ play important roles in many pathological conditions, and given that Mϕ express MMP8, we investigated in this present study if MMP8 had a role in Mϕ differentiation and/or polarization.

Experimental Procedures

Materials

Antibodies against arginase I (rabbit, H-52, sc-20150), arginase II (rabbit, H-64, sc-20151), F4/80 (rat, BM8, sc-52664), CD163 (goat, K18, sc-18796), CD206 (goat, C20, sc-34577), TGF-β (rabbit, sc-146), SMAD3 (mouse, sc-101154), LAP (goat, T-17, sc-34830), fibromodulin (rabbit, H50, sc-33772), and MMP8 (goat, sc-31741, used for double immunostaining with F4/80, 1:50) were purchased from Santa Cruz Biotechnology. Antibodies against MMP8 (rabbit, ab78423, used for Western blot, 1:500 or single immunostaining, 1:100), pSMad3 (phospho-Ser^{423}/Ser^{425}, rabbit, ab51451), and GAPDH (mouse, 6C5, Ab8245) were from Abcam, UK. Antibody against α-tubulin (mouse, T6074) was from Sigma. Cytokines (macrophage colony-stimulating factor (M-CSF), IL-4 and IFN-γ) were from ProSpec Bio, USA. All ELISA kits including TGF-β1, monocyte chemoattractant protein-1 (MCP-1), TNF-α, IFN-γ, IL-6, IL-10, and IL-12 were from Life Technologies. Other materials used in this study were purchased from Sigma unless specifically indicated.

Macrophage Culture and Polarization

Bone Marrow Monocyte-derived Macrophage Culture (BMMϕ)—Bone marrow monocytes were isolated from the tibias and femurs of mice 6–12 weeks of age. The bone marrow was triturated using an 18-gauge needle and passed through a 70-μm nylon mesh cell strainer (Becton Dickinson, Franklin Lakes, NJ) to make a single cell suspension in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 2% serum, 10 mM Hepes. Red blood cells in bone marrow cells were lysed with a lysis buffer (8.3 g of NH_4Cl, 1.0 g of $KHCO_3$, and 1.8 ml of 5% EDTA in 1000 ml). Bone marrow mononuclear cells were washed three times and cultured in the RPMI-1640 supplemented with 10% serum and 5 ng/ml of M-CSF for 7–14 days to induce BMMϕ differentiation and maturation. For each independent experiment, cells isolated from 6–8 WT (wild-type, $ApoE^{-/-}/MMP8^{+/+}$), MMP8KO (MMP8 knock-out, $ApoE^{-/-}/MMP8^{-/-}$), or C57BL/6 mice were pooled together, and subjected to different treatments. MMP8 WT and MMP8KO mice were generated in our previous study (11). C57BL/6 mice were purchased from Charles River. All the animal procedures were approved by Queen Mary University of London ethics review board, and performed to conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Peritoneal Naive Tissue Resident Mϕ (pMϕ)—pMϕ were isolated as described in the previous study (22). Briefly, 4 days before harvesting pMϕ, 1.5 ml of 4% (w/v) Brewer thioglycollate medium were injected into the peritoneal cavity of each mouse to induce macrophage infiltration and accumulation into peritoneal cavity. Mice were euthanized, cleaned with 70% ethanol, and mounted on the styrofoam block on its back. 5 ml of ice-cold PBS (with 3% serum) were injected into the peritoneal cavity using a 27-guage needle, and carefully collected using a plastic Pasteur pipette. Cells in the suspension were spun down for direct RNA extraction or re-suspended in the RPMI-1640 supplemented with 10% serum for macrophage culture. For each independent experiment, cells isolated from 6–8 WT or MMP8_KO mice were pooled together, and subjected to different treatments. In some experiments, an equal amount of PBS containing cells and peritoneal cavity fluid (4 ml) were collected from WT or MMP8KO mice and spun down to obtain cell-free supernatant. After mixing with a protease inhibitor mixture, the supernatant were concentrated using centrifugal concentrators (Millipore, UFC901024).

M1- and M2 Macrophage Polarization—Both BMMϕ or pMϕ were incubated with 10 ng/ml of IFN-γ or IL-4 for 48 h to induce M1- and M2 macrophage polarization, respectively, as described previously (23).

TGF-β and MMP8 Treatments—In separate experiments, BMMϕ or pMϕ were cultured for 48 h in RPMI culture medium in the presence or absence of 10 ng/ml of TGF-β or 50 ng/ml of MMP8, respectively.

Conditioned Culture Medium Swap—BMMϕ or pMϕ were differentiated or isolated as described previously, followed by IL-4 polarization for 24 h. The culture medium (3 ml per well for six-well plate) was collected and swapped as indicated in the figures, followed by another 24 h of culture.

Immunoblotting

Cells were harvested and lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0) supplemented with protease inhibitors and 0.5% Triton and sonicated to obtain whole cell lysate. 40 μg of protein was separated by SDS-PAGE with 4–20% Tris glycine gel (Invitrogen) and subjected to standard Western blot analysis. In some experiments, the blots were subjected to densitometric analysis with ImageJ software. Relative protein expression level was defined as the ratio of cleaved fragment to their respective full-length of fibromodulin protein expression level, of mature to precursor of TGF-β protein expression levels, of total TGF-β protein expression levels to GAPDH, or pSMAD3 to total SMAD3 expression levels, with that of the respective control sample set as 1.0.

Real-time Quantitative PCR (RT-qPCR)

Real-time quantitative PCR (RT-qPCR) was performed as previously described (24). Briefly, total RNAs were extracted from cells using TRI Reagent (Sigma) according to the manufacturer's instructions. Reverse transcription was performed using an Improm-II^{TM} Reverse Transcription kit (Promega, Madison, WI) with RNase inhibitor (Promega), and Random primers (Promega). The resultant cDNA was diluted to a working concentration of 5 ng/μl and stored at -20 °C. Primers

TABLE 1
Primer sets used in the present study

Gene names	Forward (5′-3′)	Reverse (5′-3′)	Application
18S	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	Real-time RT-PCR (RT-qPCR)
Mmp8	GTCCCAAGTGGACACACACT	TCACTTCAGCCCTTGACAGC	RT-qPCR
Cd206	GTGGAGTGATGGAACCCCAG	CTGTCCGCCCAGTATCCATC	RT-qPCR
Arg I	AGCACTGAGGAAAGCTGGTC	CAGACCGTGGGTTCTTCACA	RT-qPCR
Arg II	TCTCCTCCACGGGCAAATTC	GCAAGCCAGCTTCTCGAATG	RT-qPCR
Cd163	GCGGATGATCTGGACTTGCT	GTGCCTCTGAATGACCCCTT	RT-qPCR
Mcp-1	CCCCAAGAAGGAATGGGTCC	TGCTTGAGGTGGTTGTGGAA	RT-qPCR
Irf5	TGGATGTGGCATGTAGTAGCC	CTGGGTACTGGCAGCTGTTC	RT-qPCR
Tnf-α	AGGCACTCCCCCAAAAGATG	TGAGGGTCTGGGCCATAGAA	RT-qPCR
Il-6	GTGGCTAAGGACCAAGACCA	TAACGCACTAGGTTTGCCGA	RT-qPCR
Il-12β	AGTGACATGTGGAATGGCGT	CAGTTCAATGGGCAGGGTCT	RT-qPCR
Tgf-β	TGCTAATGGTGGACCGCAA	CACTGCTTCCCGAATGTCTGA	RT-qPCR
Irf4	AATCCCCATTGAGCCAAGCA	CTCGTCGTGGTCAGCTCTTT	RT-qPCR
Ppar-γ	CGGGCTGAGAAGTCACGTT	TGCGAGTGGTCTTCCATCAC	RT-qPCR
Il-10	GCTGCCTGCTCTTACTGACT	CTGGGAAGTGGGTGCAGTTA	RT-qPCR
Fibromodulin	GTCCACCTACTACGACCCCT	GACAGTCGCATTCTTGGGGA	RT-qPCR

were designed using Primer Express software (Applied Biosystems) and the sequence for each primer was shown in Table 1. Relative mRNA expression level was defined as the ratio of target gene expression level to 18S expression level, respectively, with that of the control sample set as 1.0.

RAW264.7 Cell Culture and shRNA-mediated Stable Mmp8 Gene Silencing

Murine macrophage cell line, RAW264.7, was purchased from ATCC (ATCC® TIB-71™) and cultured in DMEM containing 10% fetal bovine serum. Non-target and *Mmp8* shRNA lentiviral particles were produced as described in our previous study (19). For lentiviral infection, RAW264.7 cells were plated 24 h prior to infection in 6-well plates at 37 °C. One transducing unit per cell (or 2–3 × 10^6/well) of *Mmp8* shRNA or control virus were added with 10 μg/ml of hexadimethrine bromide (H9268; Sigma). Viral constructs were incubated 24 h with the cells before the media was replaced with complete media containing 2–4 μg/ml of puromycin (P9620, Sigma). For selection of transductants, fresh media containing puromycin was added at 2–3-day intervals for 10–14 days. Stably infected cells were split and frozen for future experiments.

Fibromodulin Knockdown by siRNA and TGF-β Inhibition

A pool of small interfering RNAs (siRNAs) for fibromodulin (MISSION® esiRNA, esiRNA targeting mouse fibromodulin, EMU010381–20UG) and MISSION® siRNA Universal Negative Control number 1 (SIC001–10NMOL) were purchased from Sigma. Bone marrow-differentiated macrophages were cultured in 6-well plates for 7 days, and 6 μl of 10 μM siRNA (final concentration of siRNAs: 60 nM) was introduced into cells with TransIT-X2 Transfection Reagent (Geneflow Limited) according to the protocol provided. 24 h post-transfection, cells were incubated with 10 ng/ml of IL-4 for another 24 h for M2 polarization. Cells were harvested and RT-qPCR analyses were performed. In additional experiments, bone marrow-differentiated macrophages were pre-treated with TGF-β signaling specific inhibitor (SB-431542, 10 μM, Sigma) for 3 h, followed by 10 ng/ml of IL-4 incubation for another 24 h in the presence or absence of 10 μM SB-431542.

Immunofluorescence Staining for Cells

Cells were treated with various conditions as indicated in the figures and fixed, followed by immunohistological analyses with the respective antibody as described before (25, 26). Briefly, cells were blocked with 5% BSA in PBS for 1 h at room temperature in a humid chamber, and incubated with the respective primary antibody or IgG control overnight in the cold room. Followed by incubation with appropriate fluorescent dye-conjugated secondary antibodies, cells were then incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Sigma) for 5 min. Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR ×20, NA 0.5, objective lenses, AxioCam camera, and Axiovision software (all Carl Zeiss MicroImaging, Inc.) at room temperature, or examined with Zeiss LSM 510 confocal microscope with Plan-NEOFLUAR ×40 objective lenses and Zeiss ZEN microscope software (Carl Zeiss, Germany) at room temperature. All images were processed with Photoshop software (Adobe). The cells positive for M1- or M2-Mϕ markers were counted over 200 total Mϕ, and the mean fluorescence intensity (MFI) of the cells that were positive for the respective markers were analyzed using Photoshop software by two well trained independent investigators blinded to the treatments, from 20 random selected cells.

ELISA Analyses

The levels of individual cytokine in the conditioned culture medium or concentrated peritoneal cavity fluid (for active TGF-β) were measured using their respective ELISA kit purchased from Life Technologies, according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as mean ± S.E. and analyzed using a two-tailed Student's *t* test for two-group comparison or one-way analysis of variance followed by Newman-Keuls multiple comparison post-hoc test for comparing different groups. A value of $p < 0.05$ was considered as statistically significant.

Results

MMP8 Is Significantly Up-regulated during Macrophage Differentiation—To induce macrophage differentiation and maturation from BMMs, mouse BMMs were cultured in RPMI

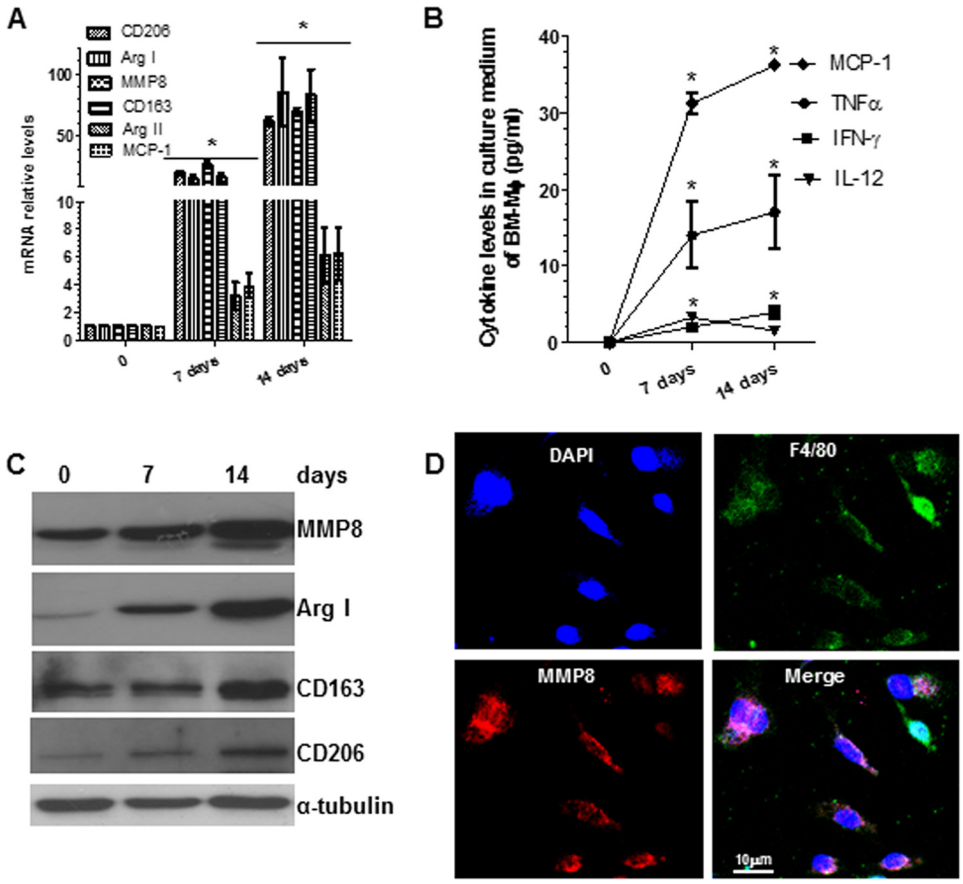
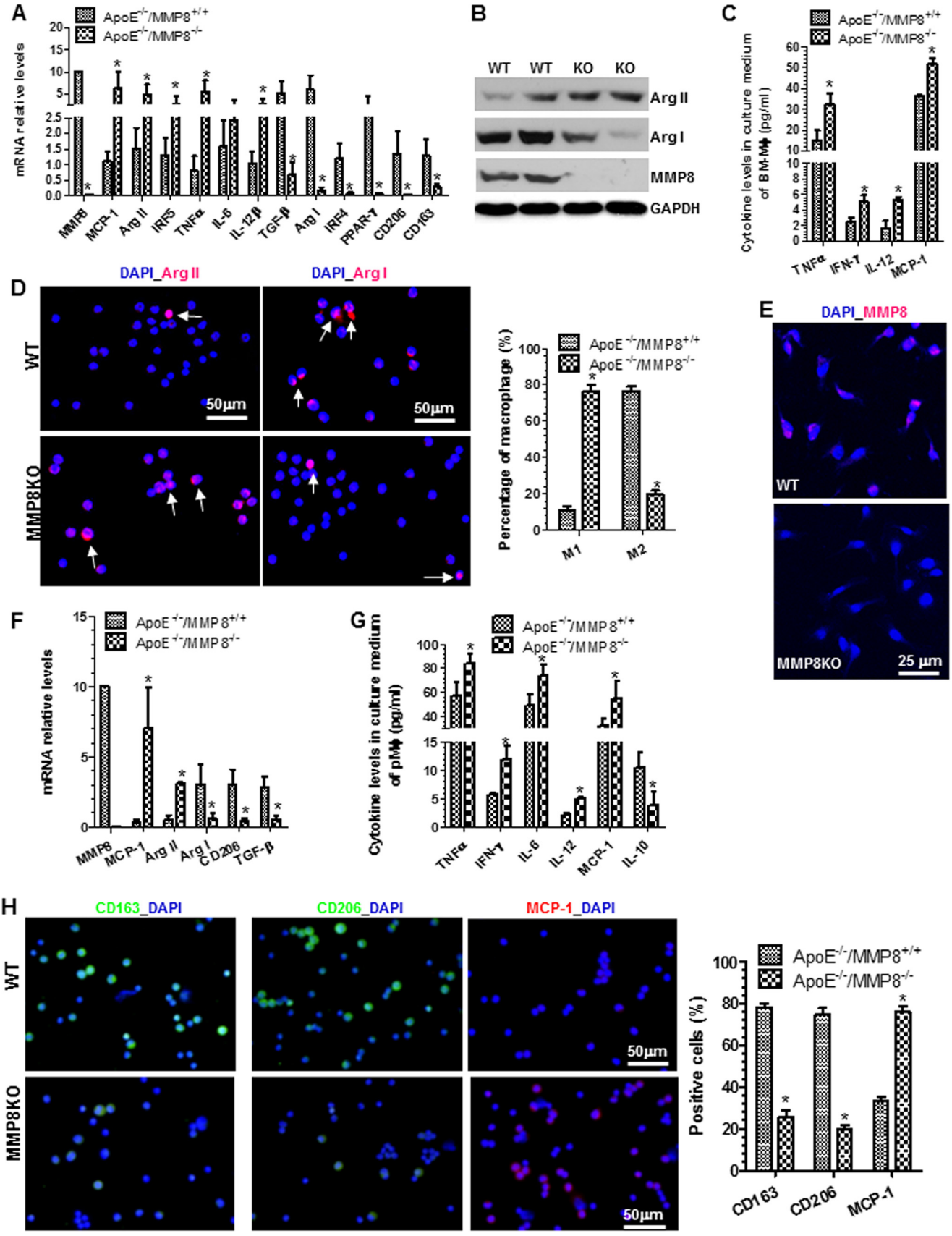


FIGURE 1. **MMP8 is up-regulated during Mϕ differentiation.** Bone marrow monocytes were induced to differentiate into Mϕ by M-CSF. Total RNAs, conditioned culture medium (*CM*), and cell lysate were harvested at the indicated times, and subjected to RT-qPCR (*A*), ELISA (*B*), and Western blot (*C*) analyses, respectively. M1 (MCP-1 and *Arg II*) or M2 (CD206, Arg I and CD163)-Mϕ genes/proteins were examined along with MMP8. *D,* immunofluorescence staining analyses of MMP8 expression in the 14 days of differentiated Mϕ. The data presented here are an average or representative of three independent experiments. *, $p < 0.05$ (*versus* day 0).

1640 medium supplemented with 10% fetal bovine serum and 5 ng/ml of M-CSF for the indicated experimental time points. Total RNA, conditioned culture medium, and proteins were harvested and subjected to RT-qPCR, ELISA, and Western blot analyses, respectively. The RT-qPCR analyses showed significant increases in the expression levels of M2-Mϕ markers (arginase (*Arg*) I, mannose receptor C type 1 (*Mrc1*)/*Cd206*, and *Cd163*), but much lower levels of M1-Mϕ markers (*Arg II* and monocyte chemotactic protein-1 (*Mcp-1*)), during Mϕ differentiation (Fig. 1*A*). Similarly, ELISA showed significant increases in several inflammatory cytokines (MCP-1, TNF-α, IFN-γ, and IL-12) in conditioned culture media, over the course of Mϕ differentiation and maturation (Fig. 1*B*), and Western blot analyses showed significant induction in three M2-Mϕ markers examined in this study (Fig. 1*C*). Immunofluorescence staining showed that more than 95% of differentiated cells were positive for the Mϕ marker F4/80 after 10–14 days (Fig. 1*D*). These data suggest that M-CSF promotes Mϕ differentiation and maturation from BMMs with a predominant M2-Mϕ phenotype, as suggested in previous studies (1).

Importantly, we also observed that MMP8 expression significantly increased during Mϕ differentiation and maturation (Fig. 1, *A* and *C*). Double immunostaining demonstrated co-expression of MMP8 with Mϕ specific markers F4/80 (Fig. 1*D*).

MMP8 Deficiency Leads to a M1 Macrophage Phenotype—To investigate whether MMP8 plays a role in Mϕ differentiation and maturation from BMMs, BMMs isolated from MMP8 knock-out (MMP8KO) mice and WT control littermates were induced to differentiate into Mϕ by M-CSF stimulation for 14 days. RT-qPCR analyses showed that MMP8 deficiency significantly increased the expression levels of M1-Mϕ genes (Mcp-1, *Arg II*, *Irf5*, *Tnf-α*, *Il-6*, and *Il-12β*), but reduced M2-Mϕ gene expression (*Tgf-β*, *Arg I*, *Irf4*, peroxisome proliferator-activated receptor-γ, *Cd206*, and *Cd163*), in differentiated Mϕ (Fig. 2*A*). Western blot analyses showed corresponding changes in Arg I and Arg II protein levels, and that the MMP8 protein expression was absent in MMP8-deficient Mϕ (Fig. 2*B*). Conditioned culture media of MMP8KO Mϕ contained higher levels of inflammatory cytokines including TNF-α, IFN-γ, IL-12, and MCP-1, as demonstrated by ELISA analyses (Fig. 2*C*). Importantly, at day 14, the percentage of M1-Mϕ was significantly higher and the percentage of M2-Mϕ was much lower, in Mϕ differentiated from MMP8KO BMMs than in Mϕ differentiated from WT BMMs, although there was no significant differences in the total numbers of Mϕ (F4/80-positive cells, data not shown) (Fig. 2*D*). Expectedly, whereas MMP8 was clearly expressed in Mϕ differentiated from WT BMMs, no apparent signal for MMP8 was detected in Mϕ dif-


A
ApoE-/-/MMP8+/+
ApoE-/-/MMP8-/-
mRNA relative levels
MMP8
MCP-1
Arg II
IRF5
TNFα
IL-6
IL-12β
TGF-β
Arg I
IRF4
PPAR-γ
CD206
CD163
B
WT
WT
KO
KO
Arg II
Arg I
MMP8
GAPDH
C
ApoE-/-/MMP8+/+
ApoE-/-/MMP8-/-
Cytokine levels in culture medium of BMMΦ (pg/ml)
TNFα
IFN-γ
IL-12
MCP-1
D
DAPI_Arg II
DAPI_Arg I
WT
MMP8KO
50μm
Percentage of macrophage (%)
M1
M2
E
DAPI_MMP8
WT
MMP8KO
25 μm
F
mRNA relative levels
MMP8
MCP-1
Arg II
Arg I
CD206
TGF-β
G
Cytokine levels in culture medium of pMΦ (pg/ml)
TNFα
IFN-γ
IL-6
IL-12
MCP-1
IL-10
H
CD163_DAPI
CD206_DAPI
MCP-1_DAPI
WT
MMP8KO
50μm
Positive cells (%)
CD163
CD206
MCP-1

ferentiated from MMP8KO BMMs (Fig. 2*E*). A similar effect of MMP8 deficiency was observed in naive pMϕ (peritoneal cavity macrophages) (Fig. 2, *F–H*). Briefly, compared with WT pMϕ, an increased expression level of M1-Mϕ markers, but a decreased expression level of M2-Mϕ markers, was observed in MMP8-deficient pMϕ (Fig. 2, *F* and *G*). Similarly, more M1-Mϕ (percentage of MCP-1-positive cells) and less M2-Mϕ (the percentage of CD163- or CD206-positive cells) were observed in MMP8-deficient pMϕ (Fig. 2*G*).

MMP8 Is Required for M2-Mϕ Polarization—To investigate whether MMP8 is involved in Mϕ polarization, BMMϕ from MMP8KO and WT mice were incubated with Mϕ polarization inducers (10 ng/ml of IFN-γ for M1 priming, and 10 ng/ml of IL-4 for M2, respectively) for 48 h. The M1 priming by IFN-γ increased the expression of M1-Mϕ markers, but this effect was significantly greater in MMP8KO BMMϕ than in WT BMMϕ (Fig. 3, *A* and *B*). In contrast, the M2 inducer IL-4 significantly up-regulated the expression of M2-Mϕ markers in WT BMMϕ, but such an effect was not observed in MMP8KO BMMϕ (Fig. 3, *C* and *D*), indicating that MMP8 deficiency significantly reduces M2-Mϕ polarization. This was confirmed using the naive pMϕ (Fig. 3, *E–H*).

It has been reported that ApoE can affect Mϕ polarization (27). The MMP8KO and WT mice used in the above experiments were apoE deficient. To investigate if the effect of MMP8 on M2-Mϕ polarization is apoE independent, murine macrophage cells, RAW264.7 (apoE wild-type), were infected with either *Mmp8*-specific shRNA lentivirus and non-target control lentivirus, followed by incubation with either the M1-Mϕ (IFN-γ) or the M2-Mϕ (IL-4) inducer for 48 h. RT-qPCR analyses showed that MMP8 knockdown significantly down-regulated M2-Mϕ genes and up-regulated M1-Mϕ genes (Fig. 4*A*). Furthermore, MMP8 knockdown diminished IL-4 induction of M2-Mϕ genes (*Arg I*, *Cd163*, and *Cd206*) in RAW264.7 cells (Fig. 4*B*) but did not affect IFN-γ-induced expression of M1-Mϕ genes (*Arg II*, *Mcp-1*, and *Tnf-α*) (Fig. 4*C*), suggesting that the effect of MMP8 on Mϕ polarization is ApoE independent. To further confirm the functional importance of MMP8 in macrophage polarization in a truly wild-type genetic background, BMMϕ from C57BL/6 were incubated with non-target shRNA or *Mmp8* shRNA lentivirus and primed to M1- or M2-Mϕ as described previously. A similar effect of *Mmp8* knockdown was observed in these BMMϕ with a truly wild-type genetic background (Fig. 4, *D–F*), further confirming the functional involvements of MMP8 in M2-Mϕ polarization is ApoE independent.

Conditioned Media of Wild-type Mϕ Stimulated with IL-4 Induce M2-Mϕ Gene Expression in MMP8KO Mϕ—To investigate whether the difference in Mϕ polarization between MMP8KO and WT arises from an effect of MMP8 on concen-

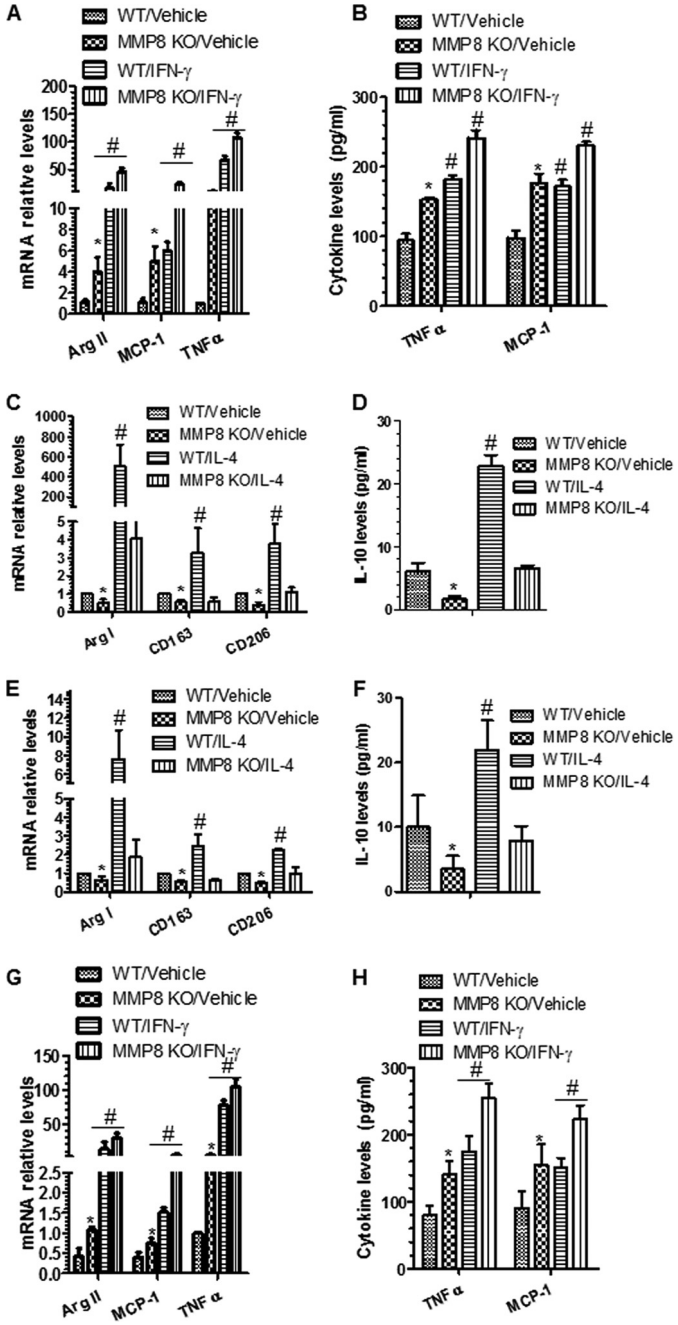


FIGURE 3. **MMP8 is required for M2-Mϕ polarization.** *A* and *B*, IFN-γ significantly up-regulated M1-Mϕ gene (*A*) and protein (*B*) expression levels in both WT and MMP8-deficient BMMϕ. *C* and *D*, IL-4 significantly up-regulated M2-Mϕ gene (*C*) and IL-10 protein (*D*) expression levels in WT, but not in MMP8-deficient BMMϕ. *E* and *F*, IL-4 significantly increased M2-Mϕ gene (*E*) and IL-10 protein (*F*) expression levels in WT, but not in MMP8 KO pMϕ. *G* and *H*, IFN-γ up-regulated M1-Mϕ gene (*G*) and protein (*H*) expression levels in both WT and MMP8KO pMϕ. The data presented here are an average of three to four independent experiments. *, $p < 0.05$ (MMP8KO *versus* WT); #, $p < 0.05$ (Mϕ inducers *versus* control).

FIGURE 2. **MMP8 is required for M2-Mϕ differentiation.** *A*, RT-qPCR analysis of expression levels of M1- and M2-Mϕ genes in the day 14 of differentiated bone marrow Mϕ (BMMϕ). *B*, Western blot analyses show that the protein expression levels of Arg I, Arg II, and MMP8 in WT and MMP8KO BMMϕ. *C*, ELISA analysis of the inflammatory cytokine levels in the culture medium conditioned by BMMϕ. *D*, immunofluorescence staining shows that the percentage of M2-Mϕ in MMP8KO (ApoE$^{-/-}$/MMP8$^{-/-}$) differentiated BMMϕ is much lower than that of WT (ApoE$^{-/-}$/MMP8$^{+/+}$) BMMϕ. *Arrows* indicate M1-Mϕ (cells are positive for Arg II) or M2-Mϕ (cells are positive for Arg I). *E*, immunofluorescence staining of MMP8 in WT and MMP8KO BMMϕ. *F*, RT-qPCR analysis of M1- and M2-Mϕ gene expression levels in the naive peritoneal Mϕ (pMϕ) isolated from the peritoneal cavity of WT and MMP8KO mice. *G*, ELISA analysis of the inflammatory cytokine levels in the culture medium conditioned by WT and MMP8-deficient pMϕ. *H*, immunofluorescence staining shows that MMP8 deficiency results in a M1-Mϕ phenotype in pMϕ. The data presented here are representative or an average of three to six independent experiments. *, $p < 0.05$ (*versus* WT). Shown in *panels D* and *H* are representative images each from three independent experiments, and column charts of the percentage of M1- and M2-Mϕ. *, $p < 0.05$ (*versus* WT).

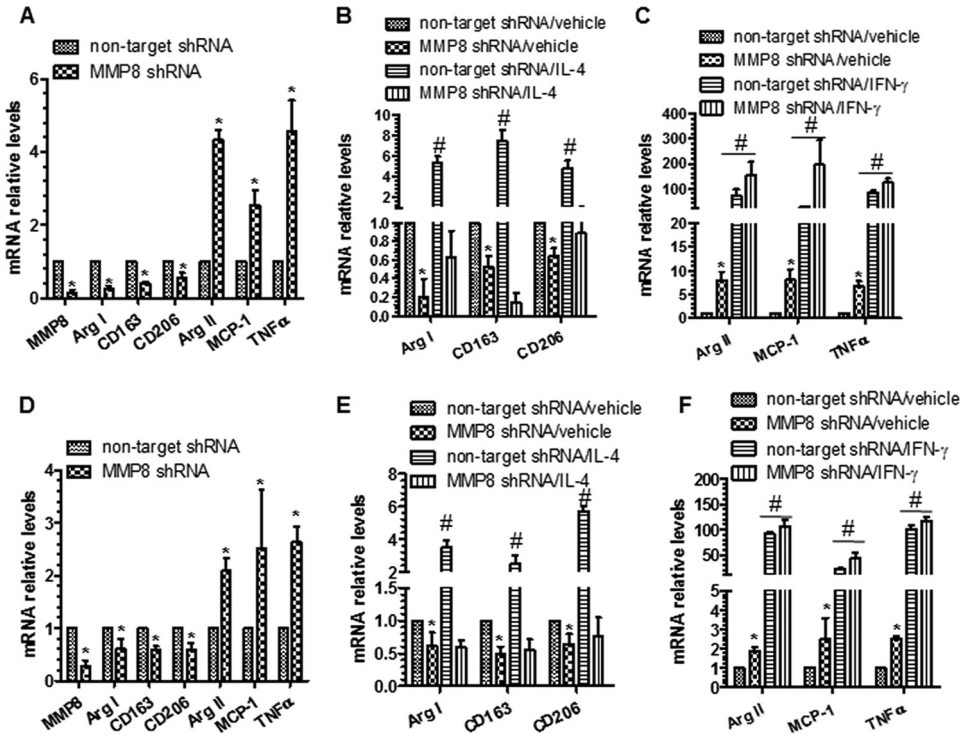


FIGURE 4. **MMP8 plays a similar role in M2-Mϕ polarization of Raw264.7 cells and BMMϕ differentiated from C57BL/6 bone marrow monocytes.** *A,* RT-qPCR analyses. RAW264.7 cells were infected with non-target or *Mmp8* shRNA lentivirus and cultured in the presence of 5 ng/ml of M-CSF for 3 days. Gene expression levels for *Mmp8*, M1 (*Arg II*, *Mcp-1*, and *Tnf-*α), and M2-Mϕ (*Arg I*, *Cd163*, and *Cd206*) genes were analyzed. *B*, IL-4 significant up-regulated M2-Mϕ genes in control Raw264.7 cells, but not in *Mmp8* knockdown Raw264.7 cells. *C,* IFN-γ significant up-regulated M1-Mϕ genes. *D*, RT-qPCR analyses. BMMϕ differentiated from C57BL/6 bone marrow monocytes were infected with non-target or *Mmp8* shRNA lentivirus and cultured for another 2 days. Gene expression levels for *Mmp8*, M1, and M2-Mϕ genes were analyzed. *E,* IL-4 significant up-regulated M2-Mϕ genes in control BMMϕ, but not in *Mmp8* knockdown BMMϕ. *F,* IFN-γ significant up-regulated M1-Mϕ genes in both control and *Mmp8* knockdown BMMϕ. The data presented here are an average of three to four independent experiments. *, $p < 0.05$ (*Mmp8* shRNA *versus* non-target shRNA); #, $p < 0.05$ (Mϕ inducers *versus* control).

trations of molecules involved Mϕ polarization, WT BMMϕ was cultured with conditioned medium of MMP8KO BMMϕ, and MMP8KO BMMϕ was cultured with conditioned medium of WT BMMϕ. The experiment showed that culturing WT BMMϕ with conditioned medium of MMP8KO BMMϕ resulted in a significant decrease in M2-Mϕ genes/proteins (Fig. 5, *A* and *B*). In contrast, replacing the culture medium for MMP8KO BMMϕ with conditioned medium of WT BMMϕ almost restored the expression levels of M2-Mϕ genes/proteins in MMP8-deficient Mϕ (Fig. 5, *A* and *B*). An experiment using pMϕ showed similar results (Fig. 5, *C* and *D*). These data suggest that the effect of MMP8 on M2-Mϕ polarization is mediated by certain biological molecule(s) in medium.

The Effect of MMP8 on M2 Mϕ Polarization Is Mediated by TGF-β1—It has been reported that *Tgf-β* signaling plays a critical role in promoting IL-4-induced alternative Mϕ activation (M2-Mϕ polarization) (28). We found that TGF-β1 mRNA expression levels were significantly lower in MMP8-deficient BMMϕ and pMϕ than in WT BMMϕ and pMϕ (Figs. 2, *A* and *F*). To investigate whether the effect of MMP8 on M2 Mϕ polarization was mediated by TGF-β1, the concentrations of total and active TGF-β1 in conditioned culture media of BMMϕ and pMϕ were measured by ELISA. The experiment showed that the levels of total and active TGF-β1 increased during Mϕ differentiation (Fig. 6*A*), and that active TGF-β1 levels, but not total TGF-β1 levels, were lower in conditioned media of MMP8-deficient BMMϕ and pMϕ than in conditioned media of WT BMMϕ and pMϕ (Fig. 6, *B* and C). Importantly, a higher level of active TGF-β1 was also observed in the peritoneal cavity fluid of WT mice induced by Brewer thioglycollate medium injection compared with MMP8KO mice (Fig. 6*D*).

TGF-β1 is synthesized as a latent precursor and secreted from cells as a latent complex (an inactive form) containing latent TGF-β-binding protein and TGF-β pro-peptide called latency-associated peptide (LAP) (29, 30). Activation of latent TGF-β involved cleavage of LAP (29, 30). We found that IL-4 priming induced a significant increase in the levels of active TGF-β1 in WT BMMϕ and pMϕ, but not in MMP8-deficient BMMϕ and pMϕ (Fig. 6, *E* and *F*). Immunostaining with an antibody against LAP showed that MMP8-deficient BMMϕ and pMϕ expressed higher levels of LAP protein than both WT BMMϕ and pMϕ (Fig. 6, *G–J*). Western blot analyses showed a much higher level of TGF-β cleavage (ratio of the mature to precursor of TGF-β) occurred in the WT BMMϕ, which was further increased by IL-4. No such induction by IL-4 was observed in MMP8-deficient BMMϕ (Fig. 6, *K* and *L*). A moderate but significant lower level of total TGF-β was observed in the cell lysate of MMP8-deficient BMMϕ (Fig. 6, *K* and *L*). Importantly, the protein level of phosphorylated SMAD3 (pSMAD3) was much lower in MMP8-deficient BMMϕ than that of WT BMMϕ, and IL-4 further increased the pSMAD3 protein level in WT BMMϕ, but not in MMP8-deficient BMMϕ (Fig. 6, *K* and *L*), suggesting that IL-4 induces M2-Mϕ

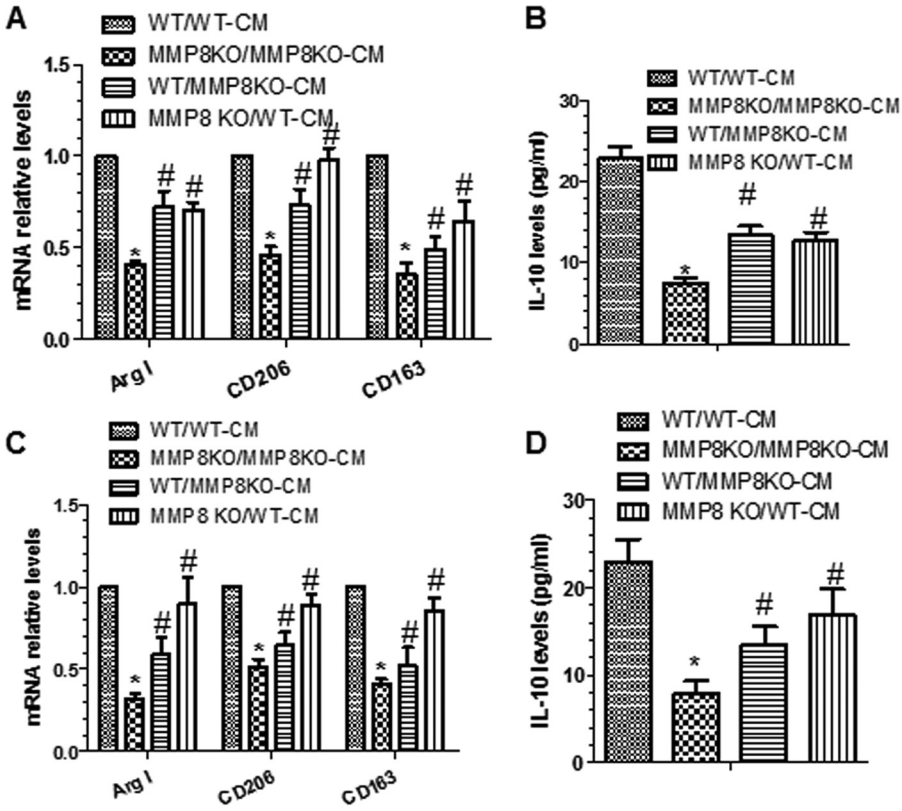


FIGURE 5. **Conditioned medium from WT Mϕ stimulated with IL-4 rescues M2-Mϕ gene expression in MMP8KO Mϕ.** *A* and *B,* monocytes isolated from WT and MMP8KO bone marrow were cultured in the complete medium containing 5 ng/ml of M-CSF for 7 days, followed by IL-4 polarization for 24 h, then subjected to culture medium swapping (*WT/MMP8KO-CM* indicates that the culture medium for WT Mϕ was replaced with the conditioned medium harvested from MMP8KO Mϕ, whereas *KO/WT-CM* indicates that the culture medium for MMP8KO Mϕ was replaced with the conditioned medium harvested from WT Mϕ; *WT/WT-CM* and *KO/KO-CM* indicate that there were no culture medium swapping) and cultured for a further 24 h. Total RNA and culture medium were harvested at the end of medium swapping experiments and subjected to RT-qPCR (*A*) and ELISA (*B*) analyses, respectively. *C* and *D,* peritoneal macrophages isolated from WT and MMP8KO mice were cultured overnight in the complete medium containing 5 ng/ml of M-CSF, followed by IL-4 polarization for 24 h, then subjected to culture medium swapping as described above and cultured for a further 24 h. Total RNA and culture medium were harvested and subjected to RT-qPCR (*C*) and ELISA (*D*) analyses, respectively. The data presented here are an average of five independent experiments. *, $p < 0.05$ (MMP8KO *versus* WT); #, $p < 0.05$ (after versus before medium swapping).

polarization through activation of TGF-β signaling, and such signaling is at least partially responsible for MMP8-mediated M2-Mϕ polarization.

Furthermore, culturing MMP8-deficient BMMϕ and pMϕ with conditioned media of WT BMMϕ and pMϕ for 24 h increased active TGF-β1 levels similar to those in conditioned media of WT BMMϕ and pMϕ (Fig. 7, *A* and *B*). To further determine whether TGF-β1 mediates the effect of MMP8 on M2-Mϕ polarization, MMP8KO and WT BMMϕ were incubated with a recombinant active TGF-β1, followed by analyses of M2-Mϕ genes. The experiment showed that incubation with the recombinant active TGF-β1 significantly increased the expression of M2-Mϕ genes in both WT and MMP8KO BMMϕ (Fig. 7, *C* and *D*). Taken together, the above data suggest that the effect of MMP8 deficiency on M2-Mϕ polarization is, at least in part, due to reduced levels of active TGF-β1.

MMP8 Increases TGF-β1 Bioavailability and Regulates M2-Mϕ Polarization by Increasing Fibromodulin Cleavage—It has been reported that the matrix protein fibromodulin can interact with TGF-β1 and regulate TGF-β1 bioavailability/activity through sequestering TGF-β1 in the extracellular matrix (31). It has been suggested that MMPs can cleave fibromodulin (32). In this study, we found reduced cleavage of fibromodulin (lower ratio of the cleaved fragment to full-length fibromodulin) in MMP8-deficient Mϕ (Fig. 8*A*) and that IL-4 priming induced fibromodulin cleavage in WT BMMϕ but not in MMP8-deficient Mϕ. Further experiments showed that incubation of Mϕ with 50 ng/ml of recombinant activated MMP8 significantly increased fibromodulin cleavage as well as the levels of active TGF-β1, in conditioned culture media of both WT and MMP8-deficient Mϕ (Fig. 8, *B* and *C*). Furthermore, incubation of Mϕ with the recombinant activated MMP8 significantly up-regulated the expression of M2-Mϕ genes in both WT and MMP8-deficient Mϕ, as demonstrated by RT-qPCR (Fig. 8*D*) and immunostaining (Fig. 9), suggesting that MMP8 induces M2-Mϕ polarization via cleavage of fibromodulin that affects TGF-β1 bioavailability.

Functional Involvements of Fibromodulin and TGF-β Signaling in IL-4-mediated M2-Mϕ Polarization—To further establish the causal link between fibromodulin and M2-Mϕ polarization induced by IL-4, fibromodulin knockdown in BMMϕ was conducted by using specific *Fibromodulin* siRNA. As expected, the endogenous expression level of *Fibromodulin* in BMMϕ was successfully knocked down by *Fibromodulin*-specific siRNAs (Fig. 10A). Consequently, M2-Mϕ-specific gene expression levels (*Arg I*, *Cd163*, and *Cd206*) were significantly

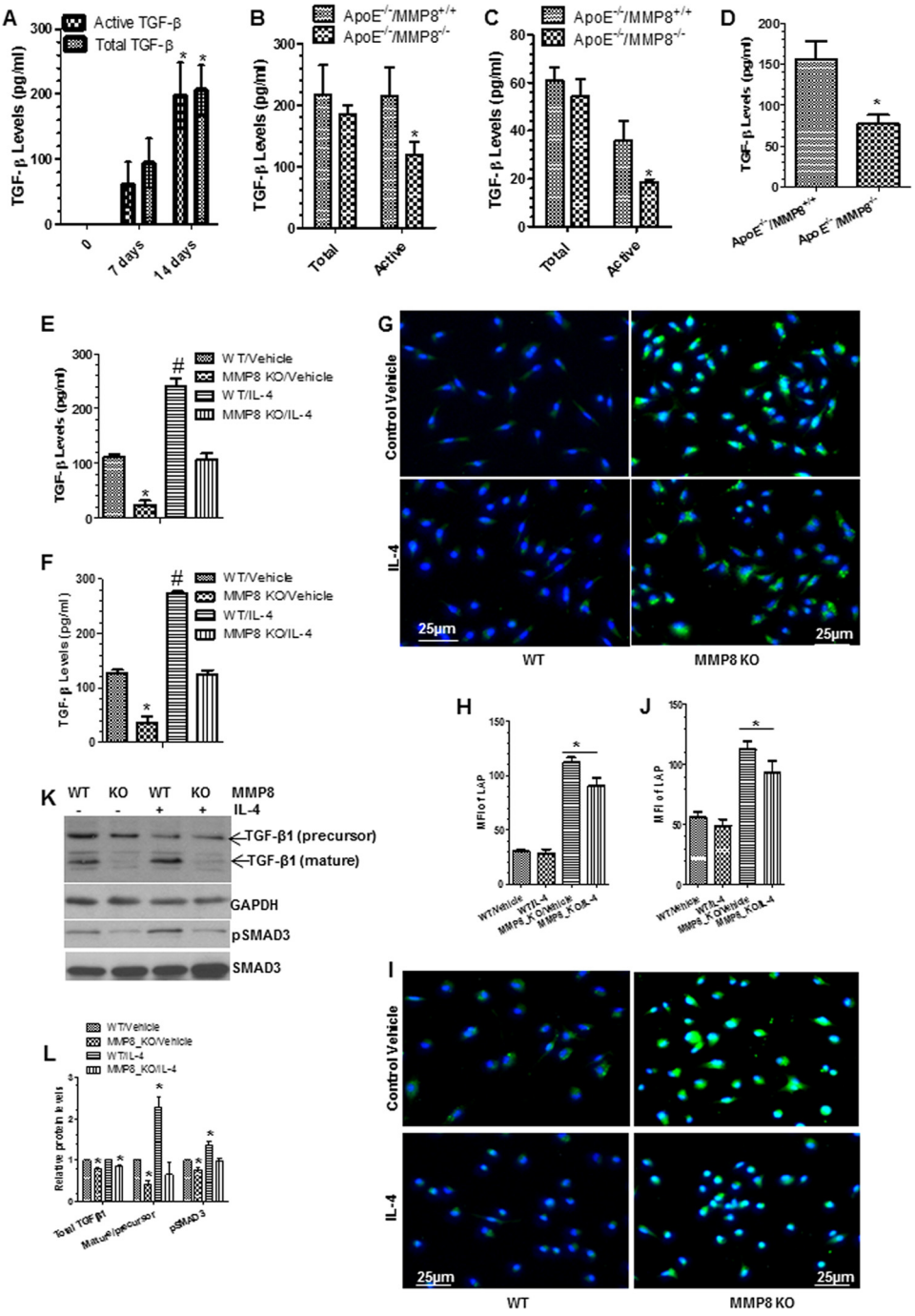


FIGURE 6. **Bioavailability of TGF-β was mediated by MMP8 during macrophage differentiation and polarization.** Cells were cultured and treated as described previously. Conditioned culture medium (*CM*) were harvested and subjected to ELISA analyses. *A,* both total and active TGF-β levels were significantly increased during bone marrow macrophage differentiation from monocytes. *B* and *C*, active, not total TGF-β levels in MMP8-deficient BMMϕ (*B*) and pMϕ (*C*) were lower than that of WT macrophages. *D,* active TGF-β levels in the peritoneal cavity fluid of MMP8-deficient mice were lower than that of WT mice. *E* and *F*, active TGF-β levels in CM of WT and MMP8-deficient BMMϕ (*E*) and pMϕ (*F*) in response to IL-4 polarization. The data presented here are an average of three to four independent experiments. *, $p < 0.05$ (day 14 *versus* 7 or MMP8KO *versus* WT); #, $p < 0.05$ (Mϕ inducers *versus* control). *G-J*, MMP8-deficiency results in higher amount of LAP on BMMϕ (*G* and *H*) and pMϕ (*I* and *J*). Cells were fixed and subjected to immunofluorescence staining analyses with antibody against LAP (N terminus of TGF-β1). Shown in the figure are representative images each from three independent experiments, and column charts of mean fluorescence intensity (mean ± S.E., $n = 20$). *, $p < 0.05$ (*versus* controls). *K* and *L*, Western analyses of the expression levels of TGF-β and pSMAD3. Proteins were harvested and subjected to Western blot analyses with the antibodies against the C terminus of TGF-β and pSMAD3 (phospho-Ser^{423}/Ser^{425}). Note: the molecular mass for the bands of TGF-β1 (precursor) and TGF-β1 (mature) in *panel K* are ~37.5 and ~12.5 kDa, respectively. GAPDH and total SMAD3 were included as internal controls. Shown in the figure are representative images each from three independent experiments, and column charts of relative protein levels (mean ± S.E., $n = 3$). *, $p < 0.05$ (*versus* WT/vehicle).

up-regulated (Fig. 10*A*, *3rd bar versus 1st bar*), such inductions were further increased in the presence of IL-4 (Fig. 10*A*, *3rd bar versus 4th bar*), suggesting a functional role of fibromodulin in M2-Mϕ polarization. Moreover, to investigate if TGF-β signaling plays a causal role in IL-4-induced M2-Mϕ polarization, the TGF-β signaling specific inhibitor, SB-431542, was used to

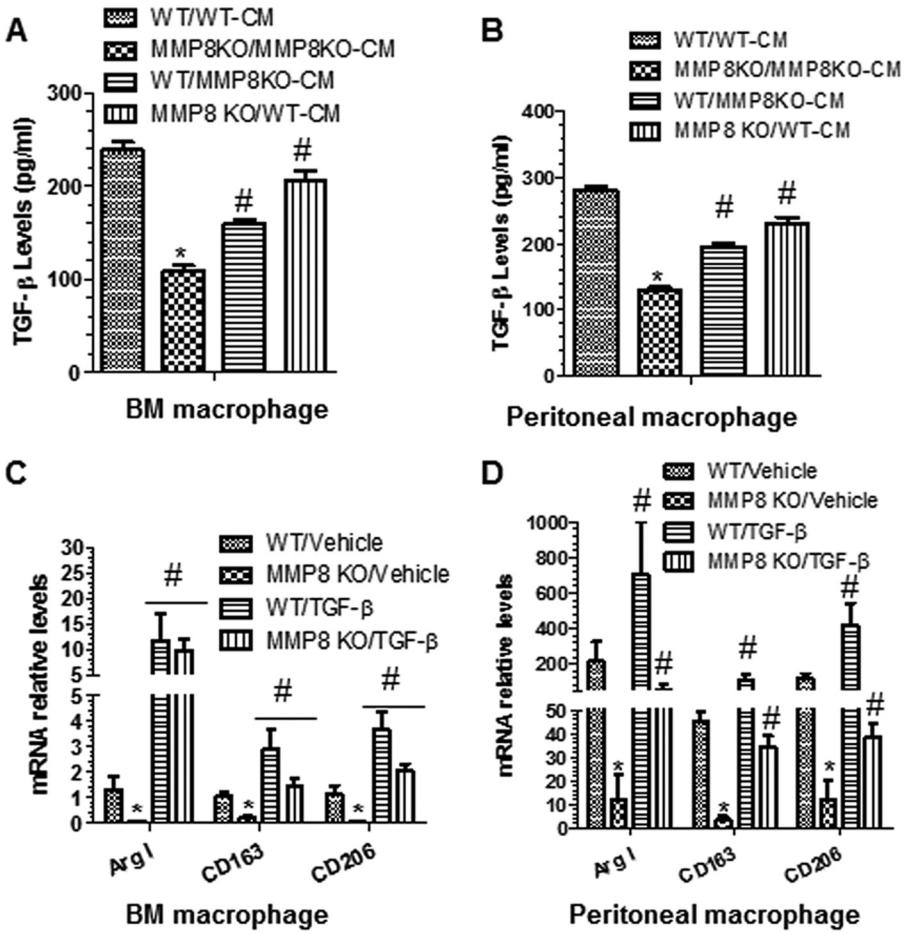


FIGURE 7. **TGF-β activity is responsible for MMP8-mediated M2 macrophage polarization.** *A* and *B,* the protein levels of active TGF-β in CM of WT and MMP8-deficient BMMϕ (*A*) and pMϕ (*B*) after medium swapping as described in the legend to Fig. 5. *C* and *D,* exogenous active TGF-β significantly up-regulated M2-Mϕ gene expression levels in both WT and MMP8-deficient BMMϕ (*C*) and pMϕ (*D*). The data presented here are an average of three to five independent experiments. *, $p < 0.05$ (MMP8KO *versus* WT); #, $p < 0.05$ (after *versus* before medium swapping, or Mϕ inducers *versus* control).

block activation of the TGF-β signal pathway in the BMMϕ. As shown in Fig. 10*B*, the expression level of phosphorylated SMAD3 (pSMAD3), one of the key downstream effect genes of the TGF-β signal pathway, was significantly up-regulated by IL-4, whereas its induction was almost abolished by incubation with SB-431542. Expectedly, incubation with SB-431542 in BMMϕ resulted in a decrease of M2-Mϕ gene expressions, and importantly inhibition of the TGF-β signaling pathway almost abrogated M2-Mϕ gene expressions induced by IL-4 (Fig. 10*C*), implying a functional role of TGF-β signaling in IL-4-induced M2-Mϕ phenotype.

Discussion

The key novel finding of this study is that MMP8 deficiency has an effect on Mϕ differentiation and polarization, favoring M1 over M2, suggesting a particular role of MMP8 in M2-Mϕ differentiation and polarization. This study indicates that this effect is related to fibromodulin cleavage and therefore increased bioavailability of TGF-β1 that induces M2-Mϕ. This finding is relevant for the understanding of regulatory mechanisms for Mϕ differentiation and polarization, and for the understanding of the pathogenesis of inflammatory conditions in which MMP8 is implicated.

Previous studies have shown that MMP8 has a protective effect against acute hepatitis (20), acute lung injury (14), airway inflammation (16), and cancers (21). These studies have demonstrated that the protective effect of MMP8 in these conditions is related to its influence on the behavior and function of neutrophils (14, 16, 21) or of eosinophils in the case of airway inflammation (16). The finding of the present study raises the possibility that the effect of MMP8 in some of these conditions might also be related to its role in M2-Mϕ polarization, which warrants further investigations.

There is also substantial evidence from experimental and clinical studies indicating MMP8 promotes atherosclerosis and a number of atherosclerosis-related conditions including atherosclerotic plaque rupture leading to myocardial infarction, heart failure after myocardial infarction, neointima formation following angioplasty, and abdominal aortic aneurysm. The pathogenesis of atherosclerosis involves a number of cell types, with endothelial cells, macrophages, lymphocytes, and smooth muscle cells having been studied most. The finding of the present study that MMP8 promotes M2-Mϕ polarization seems at odds with the finding that MMP8 promotes atherosclerosis, because inflammation plays an important role in atherogenesis and because M2-Mϕ are anti-inflammatory. However, athero-

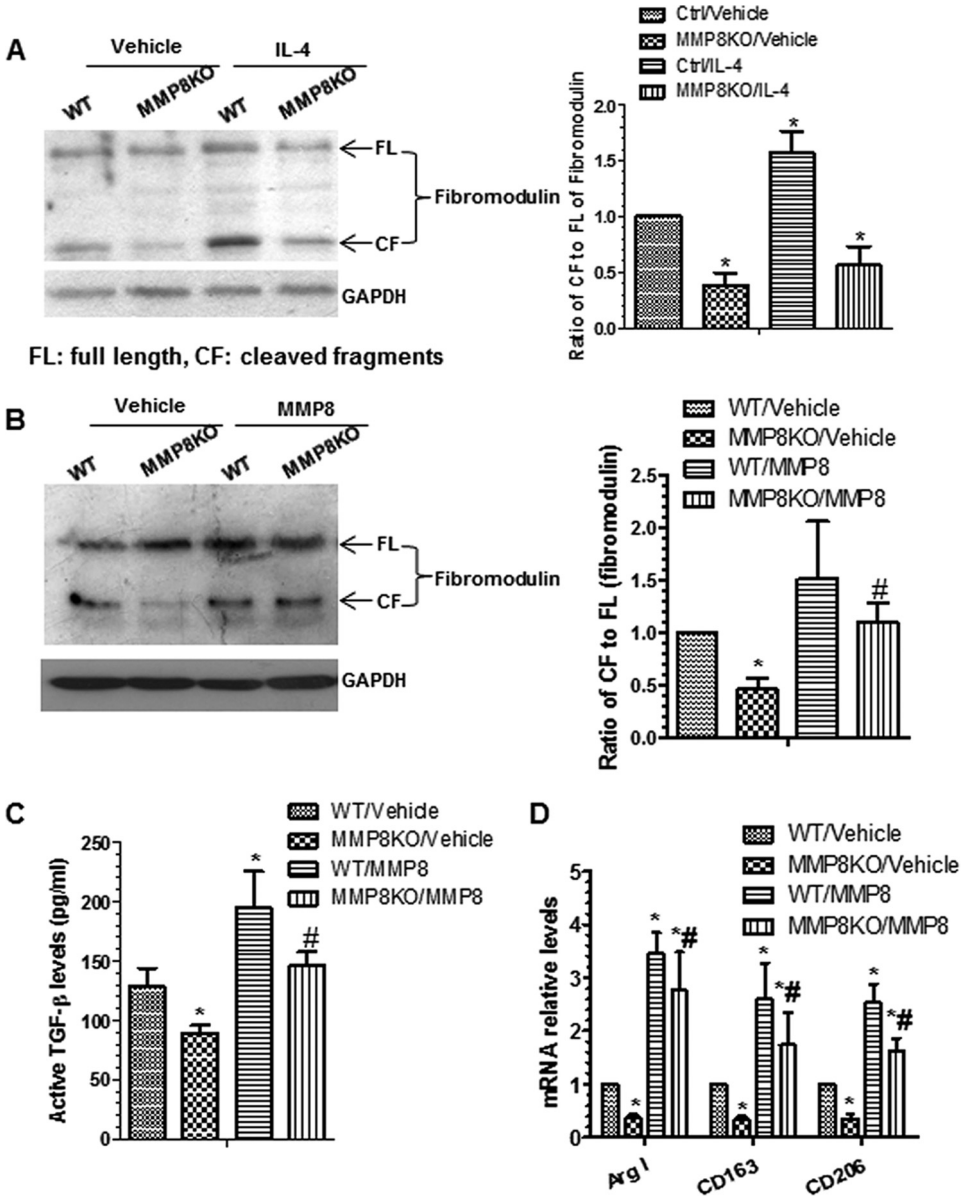


FIGURE 8. **MMP8 increases TGF-β1 bioavailability and regulates M2-Mϕ polarization by increasing fibromodulin cleavage.** *A,* WT and MMP8KO BMMϕ were incubated with IL-4 for 48 h. Proteins were harvested and subjected to Western blot analyses with antibodies against the C terminus of fibromodulin. Shown are representative images each from three independent experiments, and column charts of relative protein levels (mean ± S.E., $n = 3$). *, $p < 0.05$ (*versus* controls). *B-D,* MMP8 increases fibromodulin cleavage, TGF-β bioavailability, and rescues the M2-Mϕ phenotype of MMP8-deficient Mϕ. WT and MMP8KO BMMϕ were incubated with vehicle or 50 ng/ml of MMP8 for 48 h. Cell lysates, conditioned culture medium, and total RNAs were harvested and subjected to Western blot (*B*), ELISA (*C*), and RT-qPCR (*D*) analyses, respectively. The data presented here are an average of three independent experiments. *, $p < 0.05$ (treatments *versus* WT/vehicle); #, $p < 0.05$ (MMP8 *versus* vehicle in MMP8KO Mϕ).

genesis also involves a number of other cell types, and previous studies have indicated that MMP8 can promote atherosclerosis partly via its influences on migration and proliferation of stem/progenitor cells (19), smooth muscle cells (17), and endothelial cells (18), as well as leukocyte recruitment (11). Thus, it appears that MMP8 can influence atherosclerosis via multiple mechanisms.

Several other MMPs have been reported to have an effect on Mϕ polarization (23, 33). A recent study showed that *Mmp28* gene inactivation impaired M2-Mϕ polarization and resulted in an aggravated cardiac dysfunction after myocardial infarction in mice (23). Another study showed that loss of MMP28 reduced M2 polarization and protection from bleomycin-induced fibrosis (33). It has also been shown that loss of MMP7 resulted in M1-Mϕ polarization within *Helicobacter pylori*-infected stomachs, and Mϕ isolated from MMP7-deficient mice infected with *H. pylori* expressed significantly higher levels of the M1-Mϕ marker IL-1β compared with Mϕ isolated from WT mice (34). Furthermore, MMP1, MMP3, and MMP10 have been found to be highly expressed in M1-Mϕ, whereas MMP12 has been found to be strongly expressed in M2-Mϕ (35). It has also been shown that classical activation of mouse Mϕ increased the expression of MMP13, MMP14, and MMP25 but decreased MMP19 and TIMP2, whereas alternative activation with IL-4 increased MMP19 expression (36). Taken together, findings from previous and present studies indicate that various

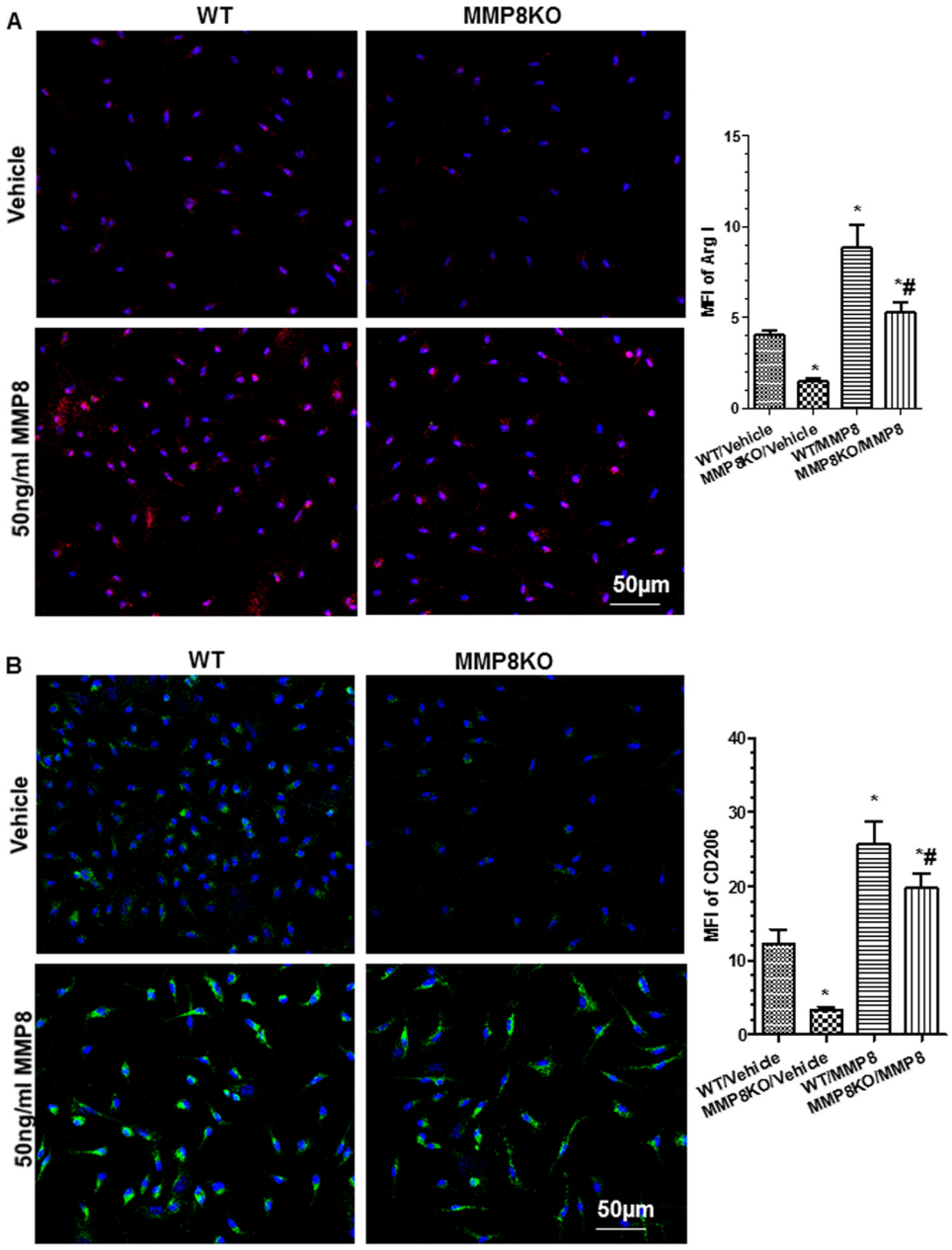


FIGURE 9. **Exogenous MMP8 restores M2-Mϕ polarization (functional properties) of MMP8-deficient Mϕ.** WT and MMP8 knock-out (*MMP8KO*) bone marrow (*BM*) Mϕ were incubated with vehicle or 50 ng/ml of MMP8 for 48 h. Cells were fixed and subjected to immunofluorescence staining with antibodies against Arg I (*A*) and CD206 (*B*), respectively. Shown in the figure are representative images each from three independent experiments, and column charts of mean fluorescence intensity (*MFI*; mean $\pm$ S.E., $n = 20$) of Arg I (*A*) or CD206 (*B*) on Mϕ. *, $p < 0.05$ (*versus* controls).

MMPs play important and divergent roles in macrophage polarization. It would be very interesting to study the relationships between these reported MMPs and MMP8 in IL-4-induced M2-Mϕ polarization. To this aim, we first examined if the expression levels of these MMPs were regulated by IL-4 in our cell culture system. Our data showed that both *Mmp12* and *Mmp19* were significantly up-regulated by IL-4 treatment, whereas the expression levels of *Mmp7* and *Mmp28* were not dramatically changed upon IL-4 treatment (data not shown). Interestingly, the expression levels of *Mmp12* and *Mmp28* were much lower in MMP8-deficient Mϕ than that of WT Mϕ, whereas *Mmp7* and *Mmp19* were mildly increased in MMP8-deficient Mϕ compared with that in WT Mϕ (data not shown), suggesting a potential role for these MMPs in MMP8-mediated M2-Mϕ polarization, which warrants further investigation.

TGF-β signaling has recently been suggested to play a central role in promoting M2-Mϕ activation (28). TGF-β1 is synthesized as a latent precursor and secreted from cells as a latent complex. Once secreted from cells, the TGF-β1 latent complex is sequestered in the extracellular matrix through binding to matrix proteins (29, 30). Proteolytic degradation of the LAP or the bound matrix proteins by specific proteolytic enzymes such as MMPs, or mechanical stretching of cell-surface integrins, release active TGF-β and allow it to interact with TGF-β receptors and induce transcription of TGF-β-responsive genes by initiating the canonical Smad-dependent pathway and/or non-

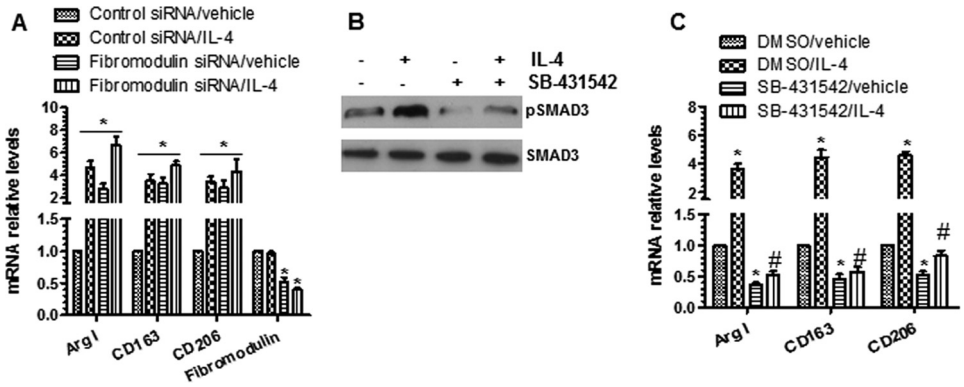


FIGURE 10. **Fibromodulin and TGF-β signaling play a role in IL-4-mediated M2-Mϕ polarization.** *A,* knockdown of fibromodulin-promoted M2-Mϕ polarization. BMMϕ were transfected as control or *Fibromodulin*-specific siRNAs, followed by IL-4 priming. Total RNAs were harvested and subjected to RT-qPCR analyses. *B* and *C*, TGF-β activation is required for IL-4-induced M2-Mϕ gene expression. BMMϕ were preincubated with 10 μM SB-431542 for 3 h, followed by IL-4 priming. Cell lysate and total RNAs were harvested and subjected to Western blot (*B*) and RT-qPCR (*C*) analyses, respectively. The data presented here are an average or representative of three independent experiments. *, $p < 0.05$ (*versus* control siRNA/vehicle or DMSO/vehicle); #, $p < 0.05$ (*versus* DMSO/IL-4).

canonical pathways involving MAPK and RhoA (37). In the current study, we provided several lines of evidence to support the notion that MMP8 mediated M2-Mϕ differentiation and polarization through activating the TGF-β signal pathway. First, our data showed that the gene expression levels (Fig. 2, *A* and *F*) and total cellular protein levels (Fig. 6, *K* and *L*) of TGF-β were much higher in WT Mϕ than that of MMP8KO Mϕ. Second, compared with WT, a decreased level of TGF-β, particularly the active form of TGF-β, was observed in the conditioned culture medium of MMP8KO Mϕ (Fig. 6, *B* and *C*) and in the peritoneal cavity of MMP8KO mice (Fig. 6*D*). Third, compared with the WT Mϕ the level of pSMAD3 was much lower in MMP8-deficient Mϕ (Fig. 6, *K* and *L*), suggesting that TGF-β signaling is suppressed in MMP8-deficient Mϕ. Fourth, exogenous TGF-β could restore the inhibitory effects of *Mmp8* gene inactivation on M2-Mϕ gene expression (Fig. 7, *C* and *D*). Finally, our data also revealed that the TGF-β signalling pathway at least partially was responsible for IL-4-induced M2-Mϕ polarization (Fig. 10, *B* and *C*). Interestingly, we observed a more significant inhibitory effect of *Mmp8* gene inactivation on *Tgf-β* mRNA expression (Fig. 2, *A* and *F*) than the total TGF-β protein level in conditioned culture medium (Fig. 6, *B* and *C*), indicating a role for MMP8 in TGF-β post-transcription and/or post-translational regulation, or in modulating TGF-β protein secretion from cells, which remains to be investigated in a future study. Furthermore, it would be interesting to investigate if MMP8 could affect TGF-β dimmer formation. For such a purpose, we have applied different non-reduced and/or non-denatured conditions in our Western blot analyses (*e.g.* omitting β-mercaptoethanol and/or SDS in loading buffer or running buffer, boiling or not boiling samples), which is prerequisite for detecting the protein dimers in whole cell lysate, but we were unsuccessful to obtain any meaningful data under these conditions. Although we have failed to show the effects of MMP8 deficiency on TGFβ1 dimmer formation, theoretically, the TGFβ1 protein dimer level will be much lower in the MMP8KO macrophage due to the facts that 1) a much lower level of TGFβ1 monomer has been observed in MMP8KO macrophages (Fig. 6*K*); and 2) there has been no report to suggest that MMP8 plays a role in protein dimmer formation through disulfide bond(s).

Studies have indicated that the matrix protein fibromodulin can interact with TGF-β1 and regulate TGF-β1 bioavailability/activity through sequestering TGF-β1 in the extracellular matrix (31, 38–40). Other studies have shown that MMP2, MMP8, MMP9, and MMP13 can cleave fibromodulin (32). In line with these findings, our present study indicates that MMP8 induces M2-Mϕ differentiation and polarization via degrading fibromodulin and increasing the bioavailability of the M2-Mϕ inducer TGF-β1. It is noteworthy to mention that one most recent study (41) has suggested an opposite role for MMP8 in regulating TGF-β signaling in breast cancer cells and metastasis progression. Soria-Valles *et al.* (41) reported that MMP8 inhibited tumor growth and breast cancer cell lung metastasis through supressing TGF-β signaling via cleaving and releasing another matrix protein, decorin. Subsequently, the cleaved decorin by MMP8 can bind to soluble TGF-β and prevent its interaction with TGF-β receptors. Therefore, it would be plausible to speculate that MMP8 may play a distinct role in the TGF-β signaling pathway in different cellular contexts or the regulatory role of MMP8 in the TGF-β signal pathway is likely substrate-dependent. It also would be very interesting to investigate the expression levels and cleavage of decorin by MMP8 in Mϕ, however, such investigations are beyond the remit of the current study.

In conclusion, we have identified a novel role of MMP8, *i.e.* in M2-Mϕ differentiation and polarization. The findings of this study are useful for understanding the biological functions of MMP8, the mechanisms for Mϕ differentiation and polarization, and the pathogenesis of pathological conditions in which MMP8 is involved.

Author Contributions—G. W. designed and performed the experiments and analyzed the data. C. Z. and Q. C. performed and analyzed RT-qPCR experiments. L. A. L. performed microscopy experiments. A. M. isolated and cultured peritoneal Mϕ. S. Y. supervised the study and wrote the manuscript. Q. X. conceived the study, analyzed the data, supervised the study and wrote the manuscript.

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