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A novel role of matrix metalloproteinase-8 in macrophage differentiation and polarization

Guanmei Wen<sup>1,2</sup>, Cheng Zhang<sup>1,3</sup>, Qishan Chen<sup>1</sup>, Le Anh Luong<sup>1</sup>, Arif Mustafa<sup>4</sup>, Shu Ye<sup>1\*</sup> and Qingzhong Xiao<sup>1\*</sup>

<sup>1</sup>Centre for Clinical Pharmacology and <sup>4</sup>Biological Service Unit, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, UK.

<sup>2</sup>Department of Pathophysiology, Guangzhou Medical University, 510182, Guangzhou, China.

<sup>3</sup>Department of Thoracic Surgery & Cardio Thoracic Surgery, The First Affiliated Hospital of Chongqing Medical University, 400016, Chongqing, China.

**Running Title:** MMP8 in macrophage differentiation and polarization

\*To whom correspondence should be addressed: *Professor Shu Ye* ([s.ye@qmul.ac.uk](mailto:s.ye@qmul.ac.uk)) or *Dr Qingzhong Xiao* ([q.xiao@qmul.ac.uk](mailto:q.xiao@qmul.ac.uk)), Centre for Clinical Pharmacology, William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Heart Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom. Tel: +44(0)2078826584;

**Key words:** Matrix metalloproteinase; Matrix metalloproteinase-8; Macrophage; Macrophage differentiation and polarization; Transforming growth factor beta; Inflammation.

**Background:** Macrophages differentiation and/or polarization have been implicated in many inflammatory diseases.

**Results:** MMP8 promotes M2-macrophage differentiation and polarization through modulating TGF- $\beta$  bioactivity.

**Conclusions:** 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.

## ABSTRACT

Matrix metalloproteinase-8 (MMP8) has been shown to influence various cellular functions. As monocytes and macrophages (M $\phi$ ) express MMP8, we investigated if MMP8 played a role in macrophage differentiation and polarization. MMP8 expression was significantly increased during monocyte differentiation into M $\phi$ . Monocyte-

derived M $\phi$  from MMP8-deficient mice expressed higher levels of M1-M $\phi$  markers but lower levels of M2-M $\phi$  markers, than monocyte-derived M $\phi$  from wildtype mice. While M $\phi$  from either MMP8-deficient or wildtype mice were inducible by interferon- $\gamma$  into M1-M $\phi$ , only wildtype M $\phi$  but not MMP8-deficient M $\phi$  could be induced into M2-M $\phi$  by interleukin-4. However, MMP8-deficient M $\phi$  exposed to conditioned culture media of wildtype M $\phi$  developed a M2-M $\phi$  phenotype. Compared with conditioned culture media of wildtype M $\phi$ , conditioned culture media of MMP8-deficient M $\phi$  contained a lower concentration of active transforming growth factor- $\beta$  (TGF- $\beta$ ), an M2-M $\phi$  inducer. Moreover, evidence also showed that the degradation of the TGF- $\beta$  sequester, fibromodulin, was modulated by MMP8. The data indicate a previously unknown role of MMP8 in M2-M $\phi$  macrophage polarization by cleaving fibromodulin and therefore increasing the bioavailability of the M2-M $\phi$  inducer TGF- $\beta$ .

## Introduction

Macrophages (M $\phi$ ) play important roles in many inflammatory diseases such as atherosclerosis. Studies have shown that M $\phi$  phenotype is heterogeneous, plastic and profoundly affected by local factors within the microenvironment(1-7). Lipopolysaccharides, interleukin-1 $\beta$  (IL-1 $\beta$ ), and cytokines secreted by Th1 lymphocytes, such as interferon- $\gamma$  (IFN- $\gamma$ ), induce a "classic" M $\phi$  phenotype (M1), while Th2 lymphocyte cytokines, such as IL-4 and IL-13, promote an "alternative" M $\phi$  phenotype (M2)(1-7). Activated M1-M $\phi$  produce proinflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-6, and IL-12, whereas M2-M $\phi$  dampen the inflammatory state by producing anti-inflammatory molecules, such as IL-10, IL-1 receptor antagonist, and transforming growth factor (TGF)- $\beta$ . In response to local factors in the microenvironment, M1-M $\phi$  can change to M2-M $\phi$ , and vice versa. Studies in recent years have revealed a number of signaling pathways, transcriptional networks and epigenetic mechanisms that influence M $\phi$  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 cell also express this protease. Studies have shown that MMP8 can modulate the behaviour and function of several cell types including

neutrophils(14,15), eosinophils(16), smooth muscle 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).

Since M $\phi$  play important roles in many pathological conditions, and given that M $\phi$  express MMP8, we investigated in this present study if MMP8 had a role in M $\phi$  differentiation and/or polarization.

## Materials and Methods

**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- $\beta$  (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 Biotech, USA. Antibodies against MMP8 (rabbit, ab78423, used for Western blot, 1:500 or single immunostaining, 1:100), pSMad3 (phospho-s423/s425, rabbit, ab51451) and GAPDH (mouse, 6C5, Ab8245) were from Abcam, UK. Antibody against  $\alpha$ -tubulin (mouse, T6074) was from Sigma. Cytokines [macrophage colony-stimulating factor (M-CSF), IL-4 and IFN- $\gamma$ ] were from ProSpec bio, USA. All ELISA kits including TGF- $\beta$ 1, Monocyte chemoattractant protein-1 (MCP-1), TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-10 and IL-12 was from Life Technologies, USA. Other materials used in this study were purchased from Sigma unless specifically indicated.

## Macrophage culture and polarization.

**Bone marrow monocyte derived macrophage culture (BMM $\phi$ )** 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  $\mu$ m nylon mesh cell strainer (Becton

Dickinson, Franklin Lakes, NJ, USA) to make a single cell suspension in RPMI-1640 supplemented with 2% fetal calf serum (FCS)/10mM Hepes. Red blood cells in bone marrow cells were lysed with a lysis buffer (8.3g NH<sub>4</sub>CL, 1.0g KHCO<sub>3</sub> and 1.8ml of 5%EDTA in 1000ml). Bone marrow mononuclear cells were washed three times and cultured in the Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% serum and 5ng/ml M-CSF for 7-14 days to induce BMM $\phi$  differentiation and maturation. For each independent experiment, cells isolated from 6~8 WT (wild type, ApoE<sup>-/-</sup>/MMP8<sup>+/+</sup>), MMP8KO (MMP8 knockout, ApoE<sup>-/-</sup>/MMP8<sup>-/-</sup>), 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 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 naïve tissue resident M $\phi$  (pM $\phi$ )** pM $\phi$  were isolated as described in the previous study(22). Briefly, four days before harvesting pM $\phi$ , 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. 5ml of ice cold PBS (with 3% FCS) were injected into the peritoneal cavity using a 27g needle, and carefully collected using a plastic Pasteur pipette. Cells in the suspension were spin 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, equal amount of PBS containing cells and peritoneal cavity fluid (4ml) were collected from WT or MMP8KO mice, spin down to obtain cell-

free supernatant. After mixing with a protease inhibitor cocktail, the supernatant were concentrated using centrifugal concentrators (Millipore, UFC901024).

**M1- and M2 macrophage polarization** Both BMM $\phi$  or pM $\phi$  were incubated with 10ng/ml of IFN- $\gamma$  or IL-4 for 48 hours to induce M1- and M2 macrophage polarization, respectively, as described previously(23).

**TGF- $\beta$  and MMP8 treatments** In separate experiments, BMM $\phi$  or pM $\phi$  were cultured for 48 hours in RPMI culture medium in the presence or absence of 10ng/ml of TGF- $\beta$  or 50ng/ml of MMP8, respectively.

**Conditioned culture medium swap** BMM $\phi$  or pM $\phi$  were differentiated or isolated as described previously, followed by IL-4 polarization for 24 hours. The culture medium (3ml per well for six-well plate) was collected and swapped as indicated in the Figure, followed by another 24 hours of culture.

**Immunoblotting.** Cells were harvested and lysed in lysis buffer (50mM Tris-Cl pH 7.5, 150mM NaCl, 1 mM EDTA pH 8.0) supplemented with protease inhibitors and 0.5% Triton and sonicated to obtain whole cell lysate. 40  $\mu$ g of protein was separated by SDS-PAGE with 4%~20% Tris-Glycine gel (Invitrogen, Carlsbad, CA, USA) and subjected to standard Western blot analysis. In some experiments, the blots were subjected to densitometric analysis with Image J software. Relative protein expression level was defined as the ratio of cleaved fragment (CF) to their respective full length (FL) of fibromodulin protein expression level, of mature to precursor of TGF- $\beta$  protein expression levels, of total TGF- $\beta$  protein expression levels to GAPDH, or of 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<sup>TM</sup> Reverse Transcription kit (Promega, Madison, WI,

USA) with RNase inhibitor (Promega), and Random primers (Promega). The resultant cDNA was diluted to a working concentration of 5ng/ $\mu$ l and stored at -20°C. Primers were designed using Primer Express software (Applied Biosystems) and the sequence for each primer was shown in supplementary **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 hours prior to infection in 6 well-plates at 37°C. One transducing Unit per cell (or 2-3x10<sup>6</sup>/well) of MMP8 shRNA or control virus were added with 10 $\mu$ g/ml hexadimethrine bromide (H9268; Sigma). Viral constructs were incubated 24 hours with the cells before the media was replaced with complete media containing 2~4 $\mu$ g/ml 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- $\beta$ inhibition**

A pool of small interfering RNAs (siRNAs) for fibromodulin (MISSION® esiRNA, esiRNA targeting mouse fibromodulin, EMU010381-20UG) and MISSION® siRNA Universal Negative Control #1 (SIC001-10NMOL) were purchased from Sigma. Bone marrow differentiated macrophages were cultured in 6-well plates for 7 days, and 6  $\mu$ l of 10 $\mu$ M siRNA (final concentration of siRNAs: 60nM) was introduced into cells with TransIT-X2 Transfection Reagent (Geneflow Limited) according to the protocol provided. 24 hours post-transfection, cells were incubated with

10ng/ml IL-4 for another 24 hours for M2 polarization. Cells were harvested and RT-qPCR analyses were performed. In additional experiments, bone marrow differentiated macrophages were pre-treated with TGF- $\beta$  signaling specific inhibitor (SB-431542, 10  $\mu$ M, Sigma) for 3 hours, followed by 10ng/ml IL-4 incubation for another 24 hours in the presence or absence of 10 $\mu$ M SB-431542.

#### **Immunofluorescence staining for cells.**

Cells were treated with various conditions as indicated in the figures and fixed, followed by immunohistological analyses with respective antibody as described before(25,26). Briefly, cells were blocked with 5% BSA in PBS for 1 hour at room temperature (RT) in a humid chamber, and incubated with respective primary antibody or IgG control overnight in the cold room. Followed by incubation with appropriate fluorescent dyes conjugated secondary antibodies, cells were then incubated with 4',6-diamidino-2-phenylindole (DAPI) (1:1000, Sigma) for 5 minutes. Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 20 $\times$ , 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 $\times$  objective lenses and Zeiss ZEN microscope software (Carl Zeiss, Germany) at room temperature. All the images were processed with Photoshop software (Adobe). The cells positive for M1- or M2-M $\phi$  markers were counted over 200 total M $\phi$ , and the mean fluorescence intensity (MFI) of the cells that were positive for 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- $\beta$ ) were measured using their respective ELISA kit purchased from Life Technologies, according to the manufacturer's instructions.

**Statistical analysis.** Data were expressed as mean $\pm$ SEM and analyzed using a two-tailed student's *t*-test for two-group comparison or one-way ANOVA 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 bone marrow derived monocytes (BMMs), mouse BMMs were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 5ng/ml macrophage colony-stimulating factor (M-CSF) for the indicated experimental time-points. Total RNA, conditioned culture media 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- M $\phi$  markers [arginase (Arg) I, mannose receptor C type 1 (MRC1)/CD206, and CD163], but much lower levels of M1-M $\phi$  markers [Arg II and monocyte chemoattractant protein-1 (MCP-1)], during M $\phi$  differentiation (**Figure 1A**). Similarly, the ELISA analyses showed significant increases in several inflammatory cytokines [MCP-1, TNF- $\alpha$ , IFN- $\gamma$ , and IL-12] in conditioned culture media, over the course of M $\phi$  differentiation and maturation (**Figure 1B**), and Western blot analyses showed significant induction in three M2-M $\phi$  markers examined in this study (**Figure 1C**). Immunofluorescence staining showed that more than 95% of differentiated cells were positive for the M $\phi$  marker F4/80 after 10-14 days (**Figure 1D**). These data suggest that M-CSF promotes M $\phi$  differentiation and maturation from BMMs with a predominant M2-M $\phi$  phenotype, as suggested in previous studies(1).

Importantly, we also observed that MMP8 expression significantly increased during M $\phi$  differentiation and maturation

(**Figures 1A and 1C**). Double immunostaining demonstrated co-expression of MMP8 with M $\phi$  specific markers F4/80 (**Figure 1D**).

### *MMP8 deficiency leads to a M1 macrophage phenotype*

To investigate whether MMP8 plays a role in M $\phi$  differentiation and maturation from BMMs, BMMs isolated from MMP8 knockout (MMP8KO) mice and wildtype (WT) control littermates were induced to differentiate into M $\phi$  by M-CSF stimulation for 14 days. RT-qPCR analyses showed that MMP8-deficiency significantly increased the expression levels of M1-M $\phi$  genes (MCP-1, Arg II, IRF5, TNF- $\alpha$ , IL-6 and IL-12 $\beta$ ), but reduced M2-M $\phi$  gene expression (TGF- $\beta$ , Arg I, IRF4, PPAR- $\gamma$ , CD206 and CD163), in differentiated M $\phi$  (**Figure 2A**). Western blot analyses showed corresponding changes in Arg I and Arg II protein levels, and that the MMP8 protein expression was absent in the MMP8-deficient M $\phi$  (**Figure 2B**). Conditioned culture media of MMP8KO M $\phi$  contained higher levels of inflammatory cytokines including TNF- $\alpha$ , IFN- $\gamma$ , IL-12 and MCP-1, as demonstrated by ELISA analyses (**Figure 2C**). Importantly, at day 14, the percentage of M1-M $\phi$  was significantly higher and the percentage of M2-M $\phi$  was much lower, in M $\phi$  differentiated from MMP8KO BMMs than in M $\phi$  differentiated from WT BMMs, although there was no significant difference in the total numbers of M $\phi$  (F4/80-positive cells, **data not shown**) (**Figure 2D**). Expectedly, while MMP8 was clearly expressed in M $\phi$  differentiated from WT BMMs, no apparent signal for MMP8 was detected in M $\phi$  differentiated from MMP8KO BMMs (**Figure 2E**). A similar effect of MMP8 deficiency was observed in naïve pM $\phi$  (peritoneal cavity macrophages) (**Figures 2F-2H**). Briefly, compared to the WT pM $\phi$ , an increased expression level of M1-M $\phi$  markers, but a decreased expression level of M2-M $\phi$  markers, was observed in MMP8-deficient pM $\phi$  (**Figures 2F-2G**). Similarly, more M1-M $\phi$  (percentage of MCP-1-positive cells) and less M2-M $\phi$  (the percentage of CD163- or CD206-positive cells) were

observed in MMP8-deficient pM $\phi$  (Figures 2G).

### ***MMP8 is required for M2-M $\phi$ polarization***

To investigate whether MMP8 is involved in M $\phi$  polarization, bone marrow derived macrophages (BMM $\phi$ ) from MMP8KO and WT mice were incubated with M $\phi$  polarization inducers (10ng/ml IFN- $\gamma$  for M1 priming, and 10ng/ml IL-4 for M2, respectively) for 48 hours. The M1 priming by IFN- $\gamma$  increased the expression of M1-M $\phi$  markers, but this effect was significantly greater in MMP8KO BMM $\phi$  than in WT BMM $\phi$  (Figures 3A and 3B). In contrast, the M2 inducer IL-4 significantly up-regulated the expression of M2-M $\phi$  markers in WT BMM $\phi$ , but such effect were not observed in MMP8KO BMM $\phi$  (Figures 3C and 3D), indicating that MMP8 deficiency significantly reduces M2-M $\phi$  polarization. This was confirmed using the naïve pM $\phi$  (Figure 3E-3H).

It has been reported that apoE can affect M $\phi$  polarization(27). The MMP8KO and WT mice used in the above experiments were apoE deficient. To investigate if the effect of MMP8 on M2-M $\phi$  polarization is apoE independent, murine macrophage cells, RAW264.7 (apoE wildtype), were infected with either MMP8-specific shRNA lentivirus and non-target control lentivirus, followed by incubation with either the M1-M $\phi$  (IFN- $\gamma$ ) or the M2-M $\phi$  (IL-4) inducer for 48 hours. RT-qPCR analyses showed that MMP8 knockdown significantly down-regulated M2-M $\phi$  genes and up-regulated M1-M $\phi$  genes (Figure 4A). Furthermore, MMP8 knockdown diminished IL-4 induction of M2-M $\phi$  genes (Arg I, CD163 and CD206) in RAW264.7 cells (Figure 4B) but did not affect IFN- $\gamma$  induced expression of M1-M $\phi$  genes (Arg II, MCP-1 and TNF- $\alpha$ ) (Figure 4C), suggesting that the effect of MMP8 on M $\phi$  polarization is ApoE independent. To further confirm the functional importance of MMP8 in macrophage polarization in a truly wild-type genetic background, BMM $\phi$  from C57BL/6 were incubated with non-target shRNA or MMP8 shRNA lentivirus and

primed to M1- or M2-M $\phi$  as described previously. A similar effect of MMP8 knockdown was observed in these BMM $\phi$  with a truly wild-type genetic background (Figure 4D-4F), further confirming the functional involvements of MMP8 in M2-M $\phi$  polarization is ApoE independent.

### ***Conditioned media of wildtype M $\phi$ stimulated with IL-4 induce M2-M $\phi$ gene expression in MMP8KO M $\phi$***

To investigate whether the difference in M $\phi$  polarization between MMP8KO and WT arises from an effect of MMP8 on concentrations of molecules involved M $\phi$  polarization, WT BMM $\phi$  were cultured with conditioned media of MMP8KO BMM $\phi$ , and MMP8KO BMM $\phi$  were cultured with conditioned media of WT BMM $\phi$ . The experiment showed that culturing WT BMM $\phi$  with conditioned media of MMP8KO BMM $\phi$  resulted in a significant decrease in M2-M $\phi$  genes/proteins (Figures 5A and 5B). In contrast, replacing the culture medium for MMP8KO BMM $\phi$  with conditioned media of WT BMM $\phi$  almost restored the expression levels of M2-M $\phi$  genes/proteins in MMP8-deficient M $\phi$  (Figures 5A and 5B). An experiment using pM $\phi$  showed similar results (Figures 5C and 5D). These data suggest that the effect of MMP8 on M2-M $\phi$  polarization is mediated by certain biological molecule(s) in the media.

### ***The effect of MMP8 on M2 M $\phi$ polarization is mediated by TGF- $\beta$ 1***

It has been reported that TGF- $\beta$  signalling plays a critical role in promoting IL-4 induced alternative M $\phi$  activation (M2-M $\phi$  polarization)(28). We found that TGF- $\beta$ 1 mRNA expression levels were significantly lower in MMP8-deficient BMM $\phi$  and pM $\phi$  than in WT BMM $\phi$  and pM $\phi$  (Figures 2A and 2F). To investigate whether the effect of MMP8 on M2 M $\phi$  polarization was mediated by TGF- $\beta$ 1, the concentrations of total and active TGF- $\beta$ 1 in conditioned culture media of BMM $\phi$  and pM $\phi$  were measured by ELISA. The experiment showed that the levels of total and active TGF- $\beta$ 1 increased during M $\phi$  differentiation (Figure 6A), and that

active TGF- $\beta$ 1 levels, but not total TGF- $\beta$ 1 levels, were lower in conditioned media of MMP8-deficient BMM $\phi$  and pM $\phi$  than in conditioned media of WT BMM $\phi$  and pM $\phi$  (**Figures 6B and 6C**). Importantly, a higher level of active TGF- $\beta$ 1 was also observed in the peritoneal cavity fluid of WT mice induced by Brewer thioglycollate medium injection compared to MMP8KO mice (**Figure 6D**).

TGF- $\beta$ 1 is synthesized as a latent precursor and secreted from cells as a latent complex (an inactive form) containing latent TGF- $\beta$  binding protein (LTBP) and TGF- $\beta$  pro-peptide called latency-associated peptide (LAP)(29,30). Activation of latent TGF- $\beta$  involved cleavage of LAP(29,30). We found that IL-4 priming induced a significant increase in the levels of active TGF- $\beta$ 1 in WT BMM $\phi$  and pM $\phi$ , but not in MMP8-deficient BMM $\phi$  and pM $\phi$  (**Figures 6E and 6F**). Immunostaining with an antibody against LAP showed that MMP8-deficient BMM $\phi$  and pM $\phi$  expressed higher levels of LAP protein than both WT BMM $\phi$  and pM $\phi$  (**Figure 6G-6J**). Western blot analyses showed a much higher level of TGF- $\beta$  cleavage (ratio of the mature to precursor of TGF- $\beta$ ) occurred in the WT BMM $\phi$ , which was further increased by IL-4. No such induction by IL-4 was observed in MMP8-deficient BMM $\phi$  (**Figures 6K and 6L**). A moderate but significant lower level of total TGF- $\beta$  was observed in the cell lysate of MMP8-deficient BMM $\phi$  (**Figures 6K and 6L**). Importantly, the protein level of phosphorylated SMAD3 (pSMAD3) was much lower in MMP8-deficient BMM $\phi$  than that of WT BMM $\phi$ , and IL-4 further increased pSMAD3 protein level in WT BMM $\phi$ , but not in MMP8-deficient BMM $\phi$  (**Figures 6K and 6L**), suggesting that IL-4 induces M2-M $\phi$  polarization through activation of TGF- $\beta$  signalling, and such signalling is at least partially responsible for MMP8-mediated M2-M $\phi$  polarization.

Furthermore, culturing MMP8-deficient BMM $\phi$  and pM $\phi$  with conditioned media of WT BMM $\phi$  and pM $\phi$  for 24 hours increased active TGF- $\beta$ 1 levels similar to

those in conditioned media of WT BMM $\phi$  and pM $\phi$  (**Figure 7A and 7B**). To further determine if TGF- $\beta$ 1 mediates the effect of MMP8 on M2-M $\phi$  polarization, MMP8KO and WT BMM $\phi$  were incubated with a recombinant active TGF- $\beta$ 1, followed by analyses of M2-M $\phi$  genes. The experiment showed that incubation with the recombinant active TGF- $\beta$ 1 significantly increased the expression of M2-M $\phi$  genes in both WT and MMP8KO BMM $\phi$  (**Figures 7C and 7D**). Taken together, the above data suggest that the effect of MMP8 deficiency on M2 M $\phi$  polarization is, at least in part, due to reduced levels of active TGF- $\beta$ 1.

### ***MMP8 increases TGF- $\beta$ 1 bioavailability and regulates M2-M $\phi$ polarization by increasing fibromodulin cleavage***

It has been reported that the matrix protein fibromodulin can interact with TGF- $\beta$ 1 and regulate TGF- $\beta$ 1 bioavailability/activity through sequestering TGF- $\beta$ 1 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 M $\phi$  (**Figure 8A**) and that IL-4 priming induced fibromodulin cleavage in WT BMM $\phi$  but not in MMP8-deficient M $\phi$ . Further experiments showed that incubation of M $\phi$  with 50ng/ml recombinant activated MMP8 significantly increased fibromodulin cleavage as well as the levels of active TGF- $\beta$ 1, in conditioned culture media of both WT and MMP8-deficient M $\phi$  (**Figures 8B and 8C**). Furthermore, incubation of M $\phi$  with the recombinant activated MMP8 significantly up-regulated the expression of M2-M $\phi$  genes in both WT and MMP8-deficient M $\phi$ , as demonstrated by RT-qPCR (**Figures 8D**) and immunostaining (**Figure 9**), suggesting that MMP8 induces M2-M $\phi$  polarization via cleavage of fibromodulin that affects TGF- $\beta$ 1 bioavailability.



**Functional involvements of fibromodulin and TGF- $\beta$  signalling in IL-4-mediated M2-M $\phi$  polarization**

To further establish the causal link between fibromodulin and M2-M $\phi$  polarization induced by IL-4, fibromodulin knockdown in BMM $\phi$  was conducted by using specific fibromodulin siRNA. As expected, the endogenous expression level of fibromodulin in BMM $\phi$  was successfully knocked down by fibromodulin specific siRNAs (**Figure 10A**). Consequently, M2-M $\phi$  specific gene expression levels (Arg I, CD163 and CD206) were significantly up-regulated (**Figure 10A, 3<sup>rd</sup> bar versus 1<sup>st</sup> bar**), such inductions were further increased in the presence of IL-4 (**Figure 10A, 3<sup>rd</sup> bar versus 4<sup>th</sup> bar**), suggesting a functional role of fibromodulin in M2-M $\phi$  polarization. Moreover, to investigate if TGF- $\beta$  signalling plays a causal role in IL-4 induced M2-M $\phi$  polarization, TGF- $\beta$  signalling specific inhibitor, SB-431542, was used to block the activation of TGF- $\beta$  signal pathway in the BMM $\phi$ . As shown in **Figure 10B**, the expression level of phosphorylated SMAD3 (pSMAD3), one of the key downstream effect genes of TGF- $\beta$  signal pathway, was significantly up-regulated by IL-4, while its induction was almost abolished by incubation with SB-431542. Expectedly, incubation with SB-431542 in BMM $\phi$  resulted in a decrease of M2-M $\phi$  gene expressions, and importantly inhibition of TGF- $\beta$  signal pathway almost abrogated M2-M $\phi$  gene expressions induced by IL-4 (**Figure 10C**), implying a functional role of TGF- $\beta$  signalling in IL-4 induced M2-M $\phi$  phenotype.

## Discussion

The key novel finding of this study is that MMP8 deficiency has an effect on M $\phi$  differentiation and polarization, favouring M1 over M2, suggesting a particular role of MMP8 in M2-M $\phi$  differentiation and polarization. The study indicates that this effect is related to fibromodulin cleavage and therefore increased bioavailability of TGF- $\beta$ 1 that induces M2-M $\phi$ . This finding is relevant for the understanding of regulatory mechanisms for M $\phi$

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 behaviour 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 be also related to its role in M2-M $\phi$  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-M $\phi$  polarization seems at odds with the finding that MMP8 promotes atherosclerosis, since inflammation plays an important role in atherogenesis and since M2-M $\phi$  are anti-inflammatory. However, atherogenesis 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 M $\phi$

polarization(23,33). A recent study showed that MMP28 gene inactivation impaired M2-M $\phi$  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-M $\phi$  polarization within *helicobacter pylori*-infected stomachs, and M $\phi$  isolated from MMP7-deficient mice infected with *helicobacter pylori* expressed significantly higher levels of the M1-M $\phi$  marker IL-1 $\beta$  compared with M $\phi$  isolated from WT mice(34). Furthermore, MMP1, MMP3 and MMP10 have been found to be highly expressed in M1-M $\phi$ , while MMP12 has been found to be strongly expressed in M2-M $\phi$ (35). It has also been shown that classical activation of mouse M $\phi$  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 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-M $\phi$  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, while 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 M $\phi$  than that of WT M $\phi$ , while MMP7 and MMP19 were mildly increased in MMP8-deficient M $\phi$  compared to that in WT M $\phi$  (**Data not shown**), suggesting a potential role for these MMPs in MMP8-mediated M2-M $\phi$  polarization, which warrants a further investigation.

TGF- $\beta$  signalling has recently been suggested to play a central role in promoting M2-M $\phi$  activation(28). TGF-

$\beta$ 1 is synthesized as a latent precursor and secreted from cells as a latent complex. Once secreted from cells, the TGF- $\beta$ 1 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- $\beta$  and allow it to interact with TGF- $\beta$  receptors and induce transcription of TGF- $\beta$ -responsive genes by initiating the canonical Smad-dependent pathway and/or non-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-M $\phi$  differentiation and polarization through activating TGF- $\beta$  signal pathway. Firstly, our data showed that the gene expression levels (**Figure 2A and 2F**) and total cellular protein levels (**Figure 6K and 6L**) of TGF- $\beta$  were much higher in WT M $\phi$  than that of MMP8KO M $\phi$ . Secondly, compared to WT, a decreased level of TGF- $\beta$ , particularly the active form of TGF- $\beta$ , was observed in the conditioned culture medium of MMP8KO M $\phi$  (**Figure 6B and 6C**) and in the peritoneal cavity of MMP8KO mice (**Figure 6D**). Thirdly, compared to WT M $\phi$  the level of pSMAD3 was much lower in MMP8-deficient M $\phi$  (**Figure 6K and 6L**), suggesting that the TGF- $\beta$  signalling is suppressed in MMP8-deficient M $\phi$ . Fourthly, exogenous TGF- $\beta$  could restore the inhibitory effects of MMP8 gene inactivation on M2-M $\phi$  gene expression (**Figure 7C and 7D**). Finally, our data also revealed that TGF- $\beta$  signal pathway at least partially was responsible for IL-4-induced M2-M $\phi$  polarization (**Figure 10B and 10C**). Interestingly, we observed a more significant inhibitory effect of MMP8 gene inactivation on TGF- $\beta$  mRNA expression (**Figure 2A and 2F**) than total TGF- $\beta$  protein level in conditioned culture medium (**Figure 6B and 6C**), indicating a role for MMP8 in TGF- $\beta$  post-transcription and/or post-translational regulation, or in modulating TGF- $\beta$  protein secretion from cells, which remains to be investigated in our future study. Furthermore, it would be interesting to investigate if MMP8 could

affect TGF- $\beta$  dimer formation. For such purpose, we have applied different non-reduced and/or non-denatured conditions in our Western blot analyses (e.g. omitting  $\beta$ -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 TGF $\beta$ 1 dimer formation, theoretically, the TGF $\beta$ 1 protein dimer level will be much lower in MMP8KO macrophage due to the facts that 1) a much lower level of TGF $\beta$ 1 monomer have been observed in MMP8KO macrophages (**Figure 6K**); and b) there has no report to suggest that MMP8 plays a role in the protein dimer formation through disulphide bond(s).

Studies have indicated that the matrix protein fibromodulin can interact with TGF- $\beta$ 1 and regulate TGF- $\beta$ 1 bioavailability/activity through sequestering TGF- $\beta$ 1 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-M $\phi$  differentiation and polarization via degrading fibromodulin and increasing the bioavailability of the M2-M $\phi$  inducer

TGF- $\beta$ 1. It is noteworthy to mention that one most recent study has suggested an opposite role for MMP8 in regulating TGF- $\beta$  signalling 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 suppressing TGF- $\beta$  signalling via cleaving and releasing another matrix protein, decorin. Subsequently, the cleaved decorin by MMP8 can bind to soluble TGF- $\beta$  and prevent its interaction with TGF- $\beta$  receptors. Therefore, it would be plausible to speculate that MMP8 may play a distinct role in the TGF- $\beta$  signal pathway in different cellular context or the regulatory role of MMP8 in TGF- $\beta$  signal pathway is likely substrate-dependent. It also would be very interesting to investigate the expression levels and cleavage of decorin by MMP8 in M $\phi$ , 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-M $\phi$  differentiation and polarization. The findings of this study are useful for understanding to biological functions of MMP8, the mechanisms for M $\phi$  differentiation and polarization, and the pathogenesis of pathological conditions in which MMP8 is involved.

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**Conflict-of-interest disclosure:**

None

**Author contributions**

G.W. designed and performed the experiments and analyzed the data. C.Z and Q.C. performed and analysed RT-qPCR experiments. L.A.L. performed microscopy experiments. A.M. isolated and cultured peritoneal M $\phi$ . 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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**Figure legend:**

**Figure 1. MMP8 is up-regulated during M $\phi$  differentiation.** Bone marrow monocytes were induced to differentiate into M $\phi$  by M-CSF. Total RNAs, conditioned culture medium (CM) and cell lysate were harvested at 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)-M $\phi$  genes/proteins were examined along with MMP8. (D) Immunofluorescence staining analyses of MMP8 expression in the 14 days of differentiated M $\phi$ . The data presented here are an average or representative of three independent experiments. \* $p < 0.05$  (v.s. day 0).

**Figure 2. MMP8 is required for M2-M $\phi$  differentiation.** (A) RT-qPCR analysis of expression levels of M1- and M2-M $\phi$  genes in the day 14 of differentiated bone marrow M $\phi$  (BMM $\phi$ ). (B) Western blot analyses show that the protein expression levels of Arg I, Arg II and MMP8 in WT and MMP8KO BMM $\phi$ . (C) ELISA analysis of the inflammatory cytokine levels in the culture medium conditioned by BMM $\phi$ . (D) Immunofluorescence staining shows that the percentage of M2-M $\phi$  in MMP8KO (ApoE<sup>-/-</sup>/MMP8<sup>-/-</sup>) differentiated BMM $\phi$  is much lower than that of WT (ApoE<sup>-/-</sup>/MMP8<sup>+/+</sup>) BMM $\phi$ . Arrows indicate M1-M $\phi$  (cells are positive for Arg II) or M2-M $\phi$  (cells are positive for Arg I). (E) Immunofluorescence staining of MMP8 in WT and MMP8KO BMM $\phi$ . (F) RT-qPCR analysis of M1- and M2-M $\phi$  gene expression levels in the naïve peritoneal M $\phi$  (pM $\phi$ ) 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 pM $\phi$ . (H) Immunofluorescence staining shows that MMP8 deficiency results in a M1-M $\phi$  phenotype in pM $\phi$ . The data presented here are representative or an average of three to six independent experiments. \* $p < 0.05$  (v.s. 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-M $\phi$ . \* $p < 0.05$  (versus WT).

**Figure 3. MMP8 is required for M2-M $\phi$  polarization.** (A-B) IFN- $\gamma$  significantly up-regulated M1-M $\phi$  gene (A) and protein (B) expression levels in both WT and MMP8-deficient BMM $\phi$ . (C-D) IL-4 significantly up-regulated M2-M $\phi$  gene (C) and IL-10 protein (D) expression levels in WT, but not in MMP8-deficient BMM $\phi$ . (E-F) IL-4 significantly increased M2-M $\phi$  gene (E) and IL-10 protein (F) expression levels in WT, but not in MMP8 KO pM $\phi$ . (G-H) IFN- $\gamma$  up-regulated M1-M $\phi$  gene (G) and protein (H) expression levels in both WT and MMP8KO pM $\phi$ . The data presented here are an average of three to four independent experiments. \* $p < 0.05$  (MMP8KO v.s. WT), #  $p < 0.05$  (M $\phi$  inducers v.s. control).

**Figure 4. MMP8 plays a similar role in M2-M $\phi$  polarization of Raw264.7 cells and BMM $\phi$  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 5ng/ml M-CSF for 3 days. Gene expression levels for MMP8, M1 (Arg II, MCP-1 and TNF- $\alpha$ ) and M2-M $\phi$  (Arg I, CD163 and CD206) genes were analysed. (B) IL-4 significant up-regulated M2-M $\phi$  genes in control Raw264.7 cells, but not in MMP8 knockdown Raw264.7 cells. (C) IFN- $\gamma$  significant up-regulated M1-M $\phi$  genes. (D) RT-qPCR analyses. BMM $\phi$  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-M $\phi$  genes were analysed. (E) IL-4 significant up-regulated M2-M $\phi$  genes in control BMM $\phi$ , but not in MMP8 knockdown BMM $\phi$ . (F) IFN- $\gamma$  significant up-regulated M1-M $\phi$  genes in both control and MMP8 knockdown BMM $\phi$ . The data presented here are an average of three to four independent experiments. \* $p < 0.05$  (MMP8 shRNA v.s. non-target shRNA), #  $p < 0.05$  (M $\phi$  inducers v.s. control).

**Figure 5. Conditioned medium from WT M $\phi$  stimulated with IL-4 rescues M2-M $\phi$  gene expression in MMP8KO M $\phi$ .** (A-B) Monocytes isolated from WT and MMP8KO bone marrow were cultured in the complete medium containing 5 ng/ml M-CSF for 7 days, followed by IL-4 polarization for 24 hours, then subjected to culture medium swapping (WT/MMP8KO-CM indicates that the culture medium for WT M $\phi$  was replaced with the



conditioned medium harvested from MMP8KO M $\phi$ , while KO/WT-CM indicates that the culture medium for MMP8KO M $\phi$  was replaced with the conditioned medium harvested from WT M $\phi$ ; WT/WT-CM and KO/KO-CM indicate that there were no culture medium swapping) and cultured for further 24 hours. 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-D) Peritoneal macrophages isolated from WT and MMP8KO mice were cultured overnight in the complete medium containing 5ng/ml M-CSF, followed by IL-4 polarization for 24 hours, then subjected to culture medium swapping as described above and cultured for further 24 hours. 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 v.s. WT), #  $p < 0.05$  (after v.s. before medium swapping).

**Figure 6. Bioavailability of TGF- $\beta$  was mediated by MMP8 during macrophage differentiation and polarization.** Cell were cultured and treated as described previously. Conditioned culture medium (CM) were harvested and subjected to ELISA analyses, respectively. (A) Both total and active TGF- $\beta$  levels were significantly increased during bone marrow macrophage differentiation from monocytes. (B-C) Active, not total TGF- $\beta$  levels in MMP8-deficient BMM $\phi$  (B) and pM $\phi$  (C) were lower than that of WT macrophages. (D) Active TGF- $\beta$  levels in the peritoneal cavity fluid of MMP8-deficient mice were lower than that of WT mice. (E-F) Active TGF- $\beta$  levels in CM of WT and MMP8-deficient BMM $\phi$  (E) and pM $\phi$  (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 v.s 7 or MMP8KO v.s. WT), #  $p < 0.05$  (M $\phi$  inducers v.s. control). (G-J) MMP8-deficiency results in higher amount of LAP on BMM $\phi$  (G and H) and pM $\phi$  (I and J). Cells were fixed and subjected to immunofluorescence staining analyses with antibody against LAP (N-terminus of TGF- $\beta$ 1). Shown in figure are representative images each from three independent experiments, and column charts of mean fluorescence intensity (MFI; mean $\pm$ SEM, n=20). \* $p < 0.05$  (versus controls). (K and L) Western analyses of the expression levels of TGF- $\beta$  and pSMAD3. Proteins were harvested and subjected to western blot analyses with the antibodies against c-terminus of TGF- $\beta$  and pSMAD3 (phospho-s423/s425). Note: the molecular weight for the bands of TGF- $\beta$ 1 (precursor) and TGF- $\beta$ 1 (mature) in panel K are ~37.5KD and ~12.5KD, respectively. GAPDH and total SMAD3 were included as internal controls. Shown in figure are representative images each from three independent experiments, and column charts of relative protein levels (mean $\pm$ SEM, n=3). \* $p < 0.05$  (versus WT/vehicle).

**Figure 7. TGF- $\beta$  activity is responsible for MMP8 mediated M2 macrophage polarization.** (A-B) The protein levels of active TGF- $\beta$  in CM of WT and MMP8-deficient BMM $\phi$  (A) and pM $\phi$  (B) after medium swapping as described in Figure 5. (C-D) Exogenous active TGF- $\beta$  significantly up-regulated M2-M $\phi$  gene expression levels in both WT and MMP8-deficient BMM $\phi$  (C) and pM $\phi$  (D). The data presented here are an average of three to five independent experiments. \* $p < 0.05$  (MMP8KO v.s. WT), #  $p < 0.05$  (after v.s. before medium swapping, or M $\phi$  inducers v.s. control).

**Figure 8. MMP8 increases TGF- $\beta$ 1 bioavailability and regulates M2-M $\phi$  polarization by increasing fibromodulin cleavage.** (A) WT and MMP8KO BMM $\phi$  were incubated with IL-4 for 48 hours. Proteins were harvested and subjected to western blot analyses with antibodies against c-terminus of fibromodulin. FL: full length, CF: cleaved fragments. Shown in figure are representative images each from three independent experiments, and column charts of relative protein levels (mean $\pm$ SEM, n=3). \* $p < 0.05$  (versus controls). (B-D) MMP8 increases fibromodulin cleavage, TGF- $\beta$  bioavailability and rescues the M2-M $\phi$  phenotype of MMP8-deficient M $\phi$ . WT and MMP8KO BMM $\phi$  were incubated with vehicle or 50ng/ml MMP8 for 48 hours. 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 v.s. WT/vehicle), #  $p < 0.05$  (MMP8 v.s. vehicle in MMP8KO M $\phi$ ).

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

**Figure 10. Fibromodulin and TGF- $\beta$  signalling play a role in IL-4-mediated M2-M $\phi$  polarization.** (A) Knockdown of fibromodulin promoted M2-M $\phi$  polarization. BMM $\phi$  were transfected control or fibromodulin specific siRNAs, followed by IL-4 priming. Total RNAs were harvested and subjected to RT-qPCR analyses. (B-C) TGF- $\beta$  activation is required for IL-4 induced M2-M $\phi$  gene expression. BMM $\phi$  were pre-incubated with 10 $\mu$ M SB-431542 for three hours, 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).

**Table 1:** Primer sets used in the present study

<b>Gene names</b>	<b>Forward (5'-3')</b>	<b>Reverse (5'-3')</b>	<b>Application</b>
18s	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	Real-time RT-PCR (RT-qPCR)
MMP8	GTCCCAAGTGGACACACACT	TCACTTCAGCCCTTGACAGC	RT-qPCR
CD206	GTGGAGTGATGGAACCCAG	CTGTCCGCCAGTATCCATC	RT-qPCR
Arg I	AGCACTGAGGAAAGCTGGTC	CAGACCGTGGGTCTTCACA	RT-qPCR
Arg II	TCTCCTCCACGGGCAAATTC	GCAAGCCAGCTTCTCGAATG	RT-qPCR
CD163	GCGGATGATCTGGACTTGCT	GTGCCTCTGAATGACCCCTT	RT-qPCR
MCP-1	CCCCAAGAAGGAATGGGTCC	TGCTTGAGGTGGTTGTGGAA	RT-qPCR
IRF5	TGGATGTGGCATGTAGTAGCC	CTGGGTACTGGCAGCTGTT	RT-qPCR
TNF-alpha	AGGCAC TCCCCAAAAGATG	TGAGGGTCTGGGCCATAGAA	RT-qPCR
IL-6	GTGGCTAAGGACCAAGACCA	TAACGCACTAGGTTTGCCGA	RT-qPCR
IL-12beta	AGTGACATGTGGAATGGCGT	CAGTTC AATGGGCAGGGTCT	RT-qPCR
TGF-beta	TGCTAATGGTGGACCGCAA	CACTGCTTCCCGAATGTCTGA	RT-qPCR
IRF4	AATCCCCATTGAGCCAAGCA	CTCGTCGTGGTCAGCTCTTT	RT-qPCR
PPAR-gamma	CGGGCTGAGAAGTCACGTT	TGCGAGTGGTCTTCCATCAC	RT-qPCR
IL-10	GCTGCCTGCTTACTGACT	CTGGGAAGTGGGTGCAGTTA	RT-qPCR
Fibromodulin	GTCCACCTACTACGACCCCT	GACAGTCGCATTCTGGGGA	RT-qPCR

Figure 1

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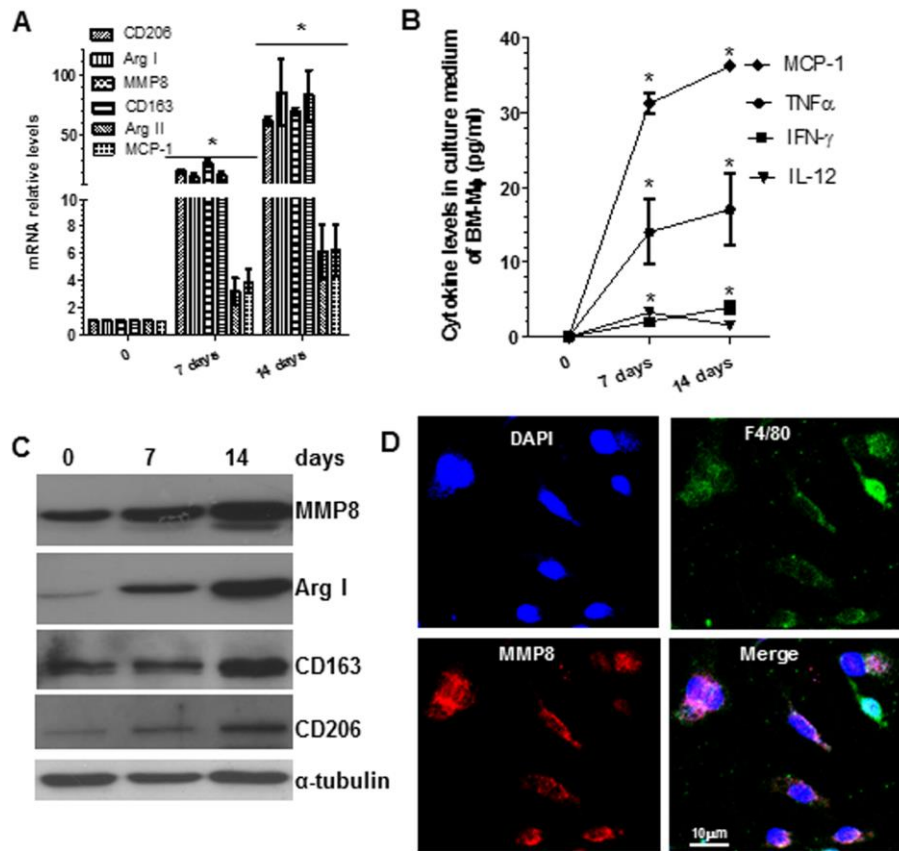
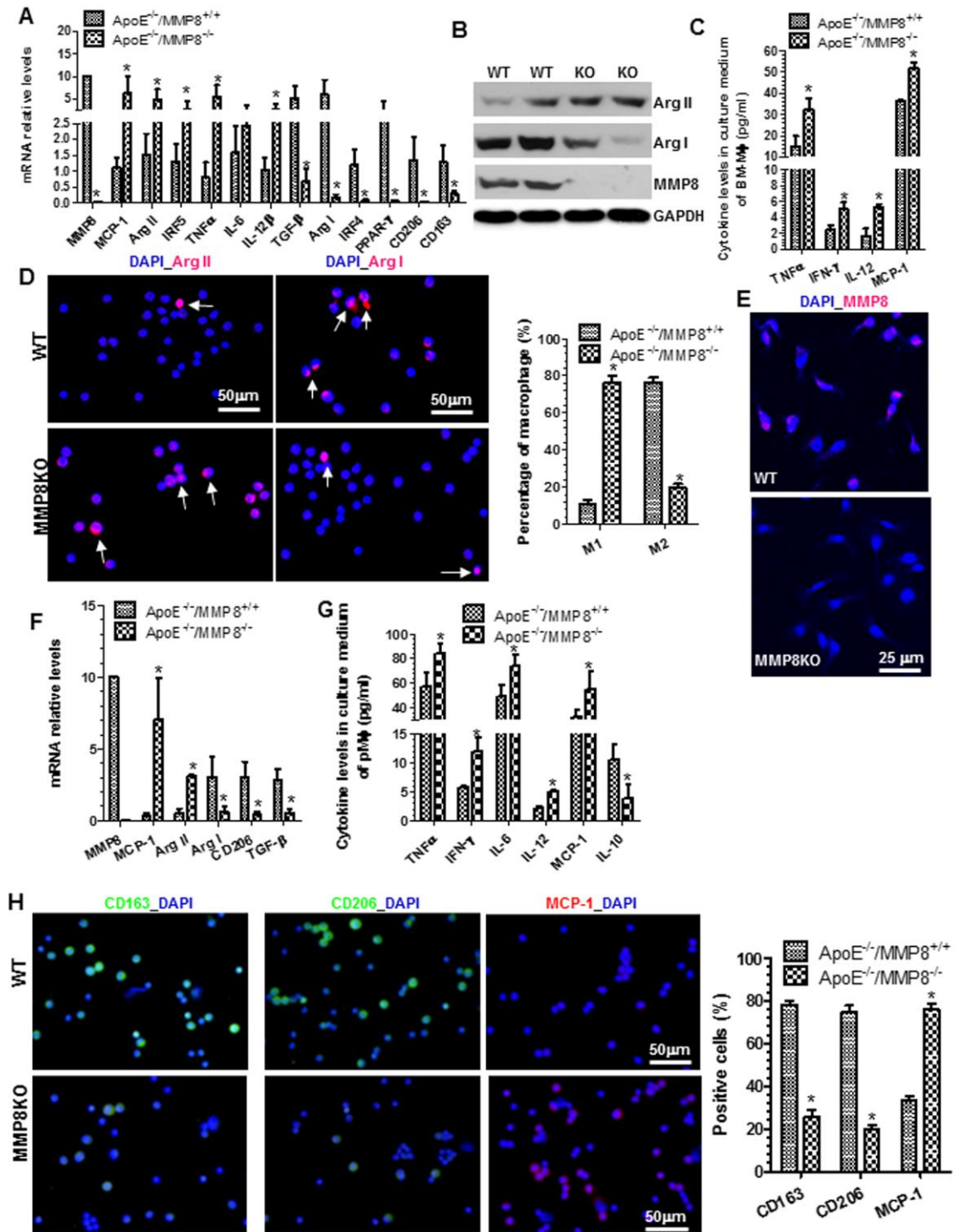


Figure 2

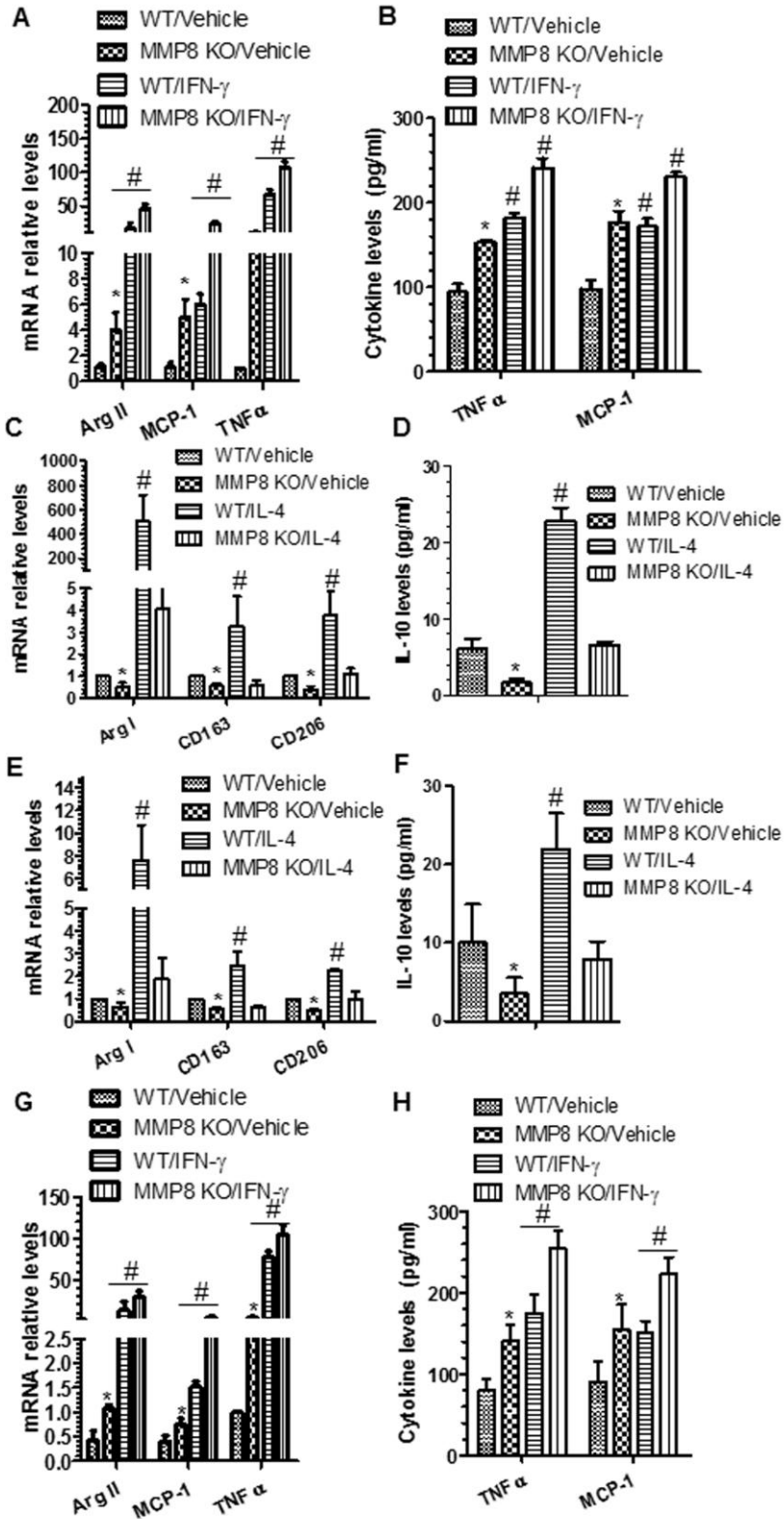
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Figure 3

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Figure 4

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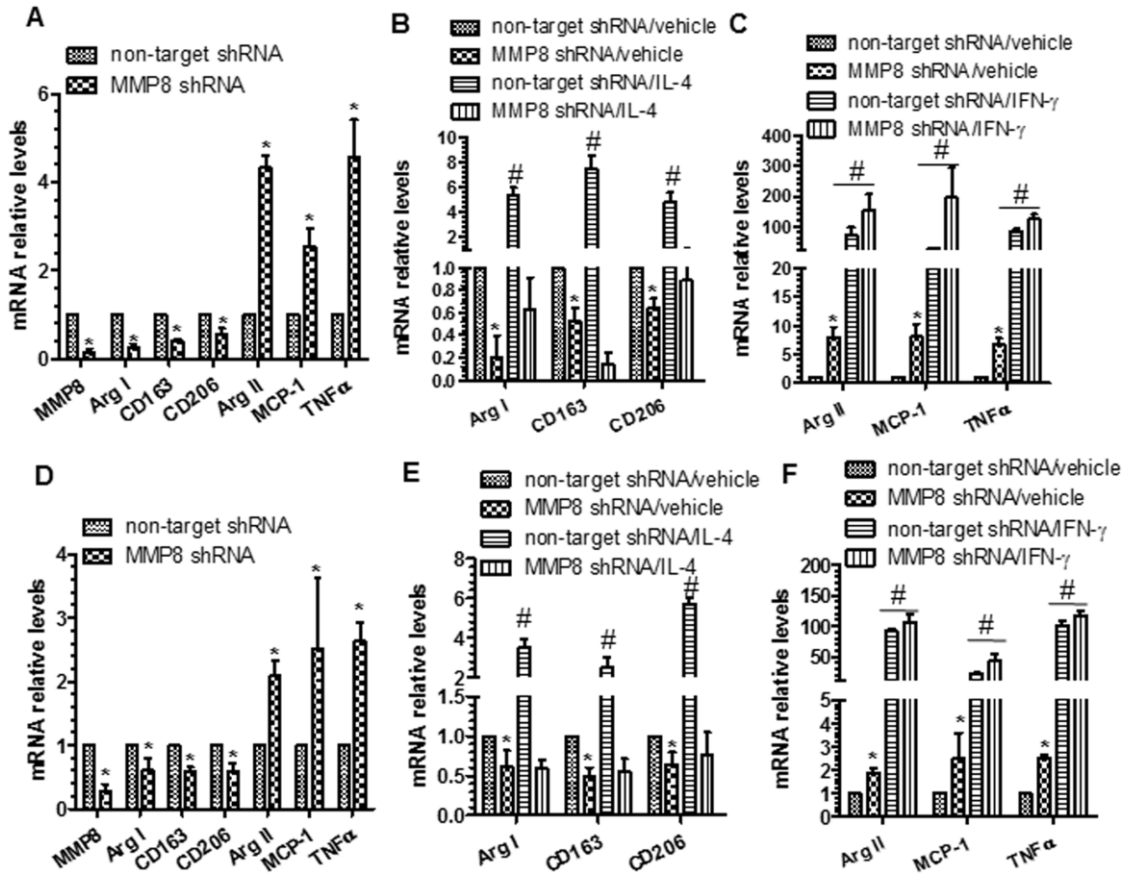


Figure 5

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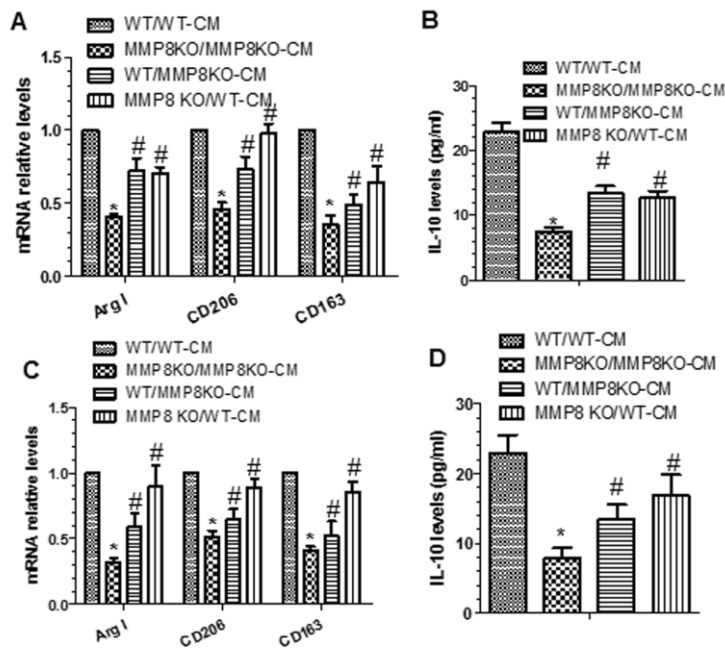




Figure 6

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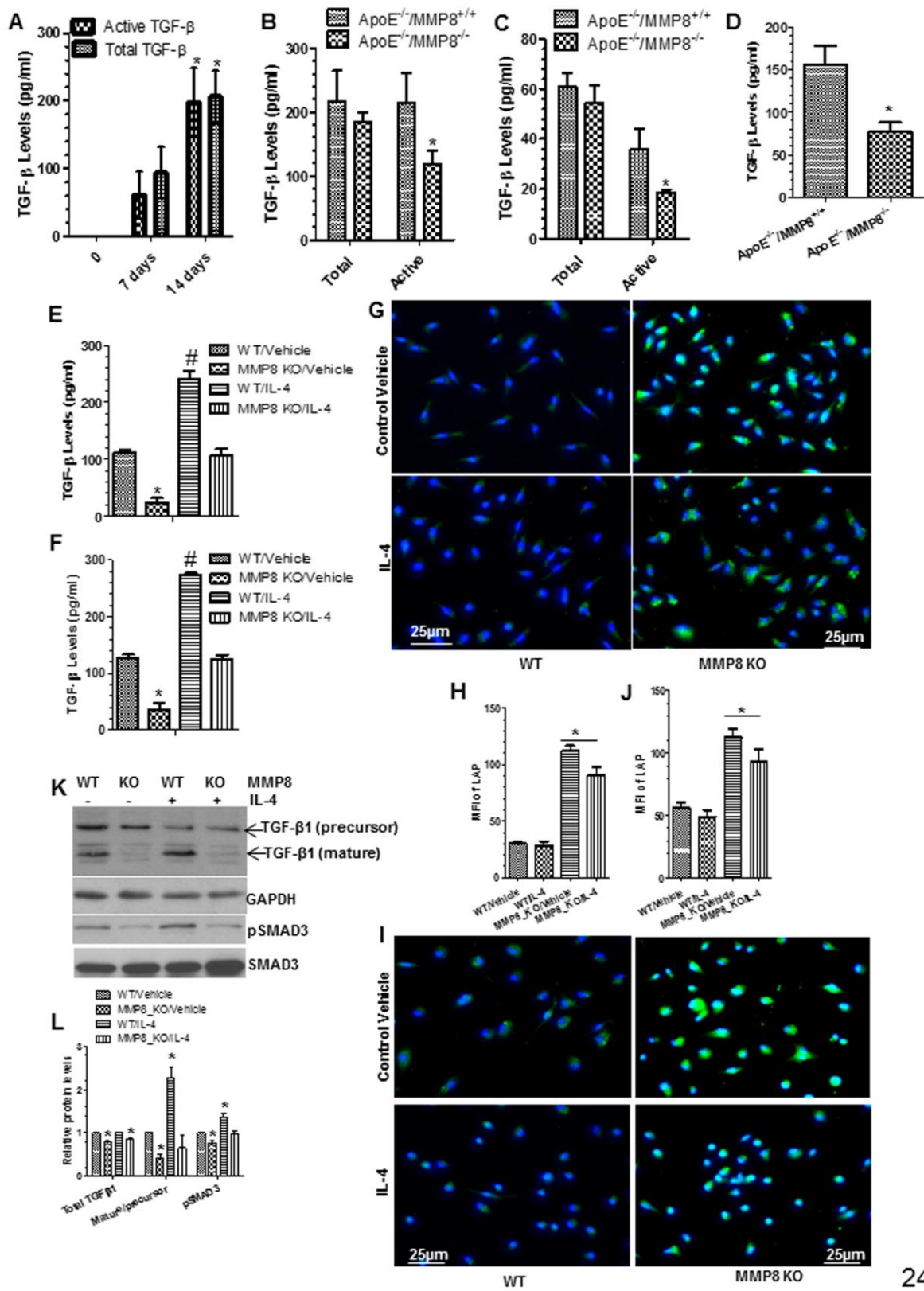


Figure 7

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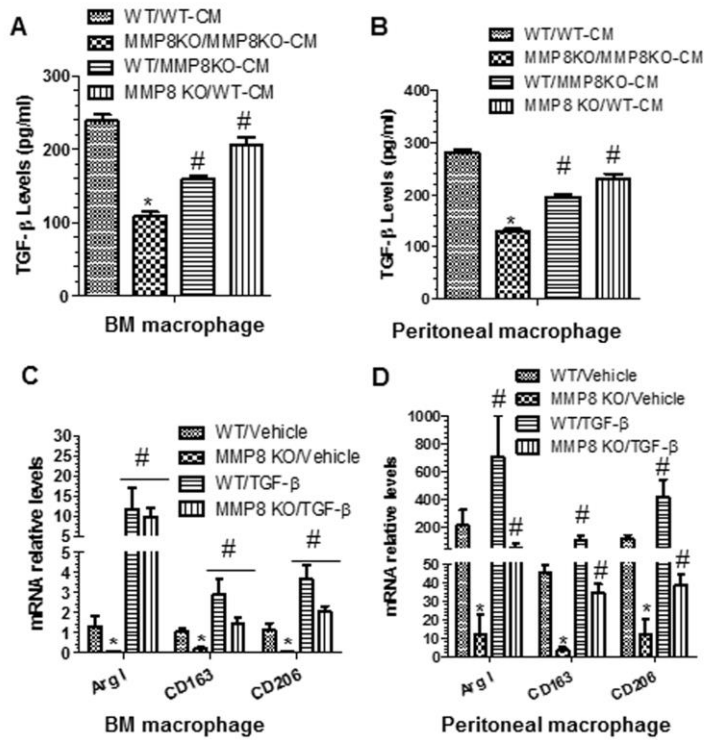


Figure 8

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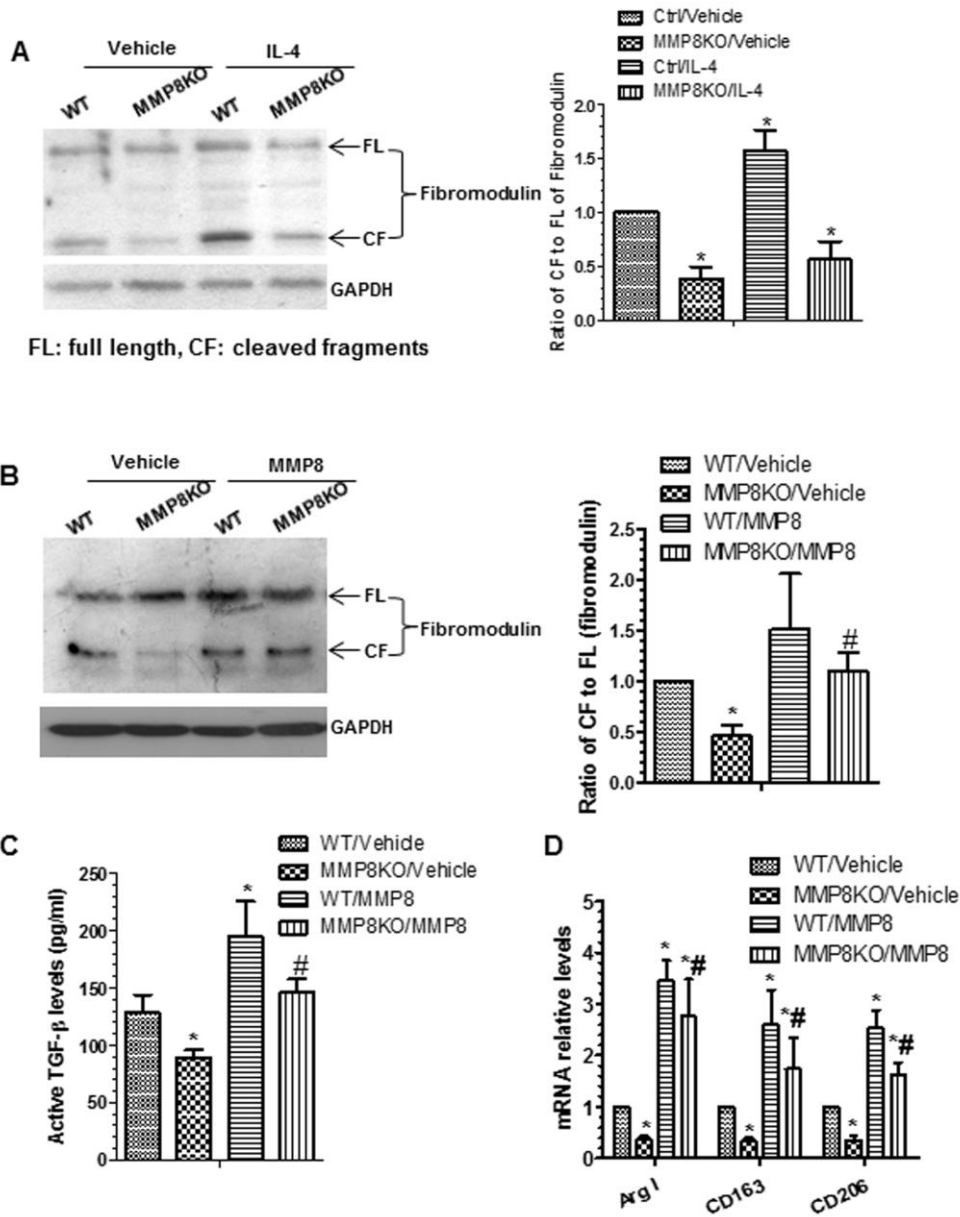
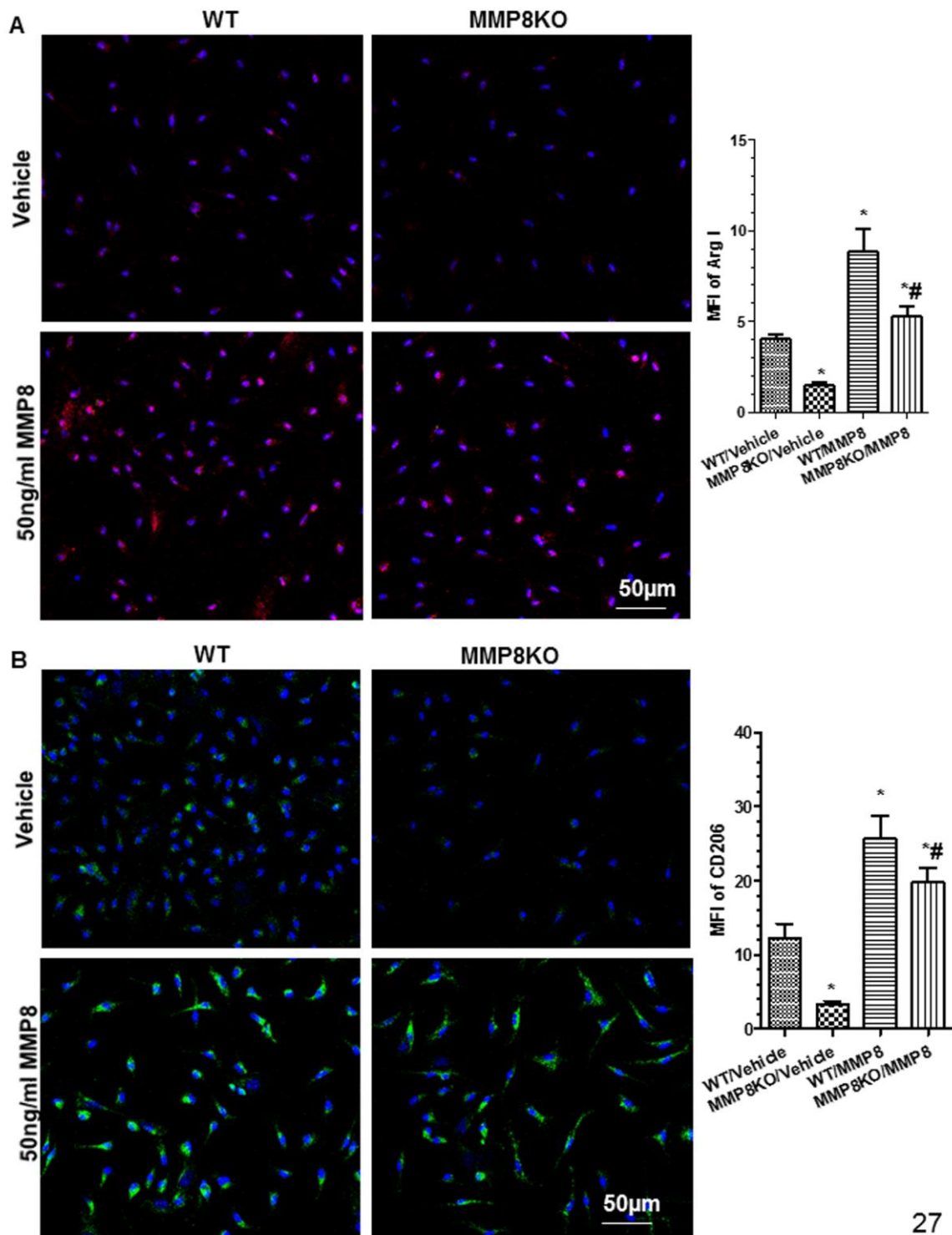


Figure 9

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Figure 10

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