

TGF- β , but not IL-4, polarized macrophages induce fibrotic responses in lung fibroblasts

Qian Tian

Amsterdam UMC Location University of Amsterdam

Alexandra Drakaki

Amsterdam UMC Location University of Amsterdam

Hella Aberson

Amsterdam UMC Location University of Amsterdam

Sami Lone

Amsterdam UMC Location University of Amsterdam

Tom van der Poll

Amsterdam UMC Location University of Amsterdam

Marten A. Hoeksema

Amsterdam UMC Location University of Amsterdam

C. Arnold Spek

Amsterdam UMC Location University of Amsterdam

Jan Willem Duitman

`j.w.duitman@amsterdamumc.nl`

Amsterdam UMC Location University of Amsterdam

Research Article

Keywords: TGF- β polarized macrophages, IL-4 polarized macrophages, lung fibroblasts, pulmonary fibrosis

Posted Date: August 11th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10592512/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Introduction

Macrophages are implicated in the initiation and regulation of pulmonary fibrosis, where anti-inflammatory macrophages promote fibrosis. Interleukin-4 (IL-4) and Transforming Growth Factor (TGF)- β stimulation of naïve macrophages results in the polarization to anti-inflammatory macrophages which are often used for mechanistic *in vitro* studies of fibrotic properties of these cells.

Aims

To assess the effect of IL-4- and TGF- β -polarized macrophages on fibrotic responses of normal human lung fibroblasts (NHLFs) in order to identify the most appropriate anti-inflammatory macrophage subset for use in *in vitro* model systems.

Methods

Peripheral blood mononuclear cells, human THP-1 monocytes and B-cell precursor leukemia cells were stimulated with IL-4 or TGF- β to induce anti-inflammatory macrophage polarization. Naïve macrophages were used as control. Whole-genome transcriptomes were analysed using RNA-sequencing. NHLFs were cocultured with polarized macrophages derived from THP-1, and conditioned medium transfer experiments were performed. Fibrotic responses were assessed by immunofluorescence. Size exclusion columns were used to separate the conditioned medium on molecular weight. TGF- β receptor kinase inhibitor RepSox was used to block TGF- β pathway signaling in NHLFs.

Results

Coculture with TGF- β -, but not IL-4-polarized macrophages, induced α -smooth muscle actin (α SMA) levels in NHLFs. Conditioned medium from TGF- β -, but not IL-4-, polarized macrophages induced α SMA, collagen and fibronectin protein levels in NHLFs. Size exclusion experiments revealed that the profibrotic effect remained intact in the conditioned medium fraction containing proteins larger than 100kD. Subsequent RNA-sequencing analysis showed increased expression of several potential high molecular weight profibrotic proteins related to TGF- β signaling pathway activation in TGF- β polarized macrophages. Pretreatment of NHLFs with RepSox blocked the profibrotic response of NHLFs induced by conditioned medium from TGF- β -polarized macrophages.

Conclusion

TGF- β -polarized macrophages promoted fibrotic responses of NHLFs, whereas IL-4-polarized macrophages did not; the profibrotic activity of TGF- β -polarized macrophages is mediated through activation of TGF- β signaling pathway in NHLFs.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive disease characterized by disruption of normal lung architecture, excessive deposition of extracellular matrix (ECM) proteins and subsequent impairment of respiratory function. Despite available anti-fibrotic drugs, which are only able to slow down disease progression, median survival remains poor, varying between 2.8 and 4.5 years after diagnosis (1–3). The current paradigm about the mechanism underlying the pathophysiology of IPF is repetitive injury of the alveolar epithelial cells lining the alveoli in an aging environment (4). Repetitive microinjuries of the epithelium lead to a derailed wound repair response leading to the accumulation of ECM proteins and scarring of the interstitium. Following infection, inhalation of environmental pollutants/toxic compounds or mechanical injury to the lung, a complex inflammatory response is triggered, involving recruitment, proliferation and activation of multiple cell types that coordinate tissue repair. When this wound healing response becomes uncontrolled or dysregulated, pathological and aberrant fibrosis may develop, ultimately leading to impaired lung function (5). Among the diverse cell populations involved in the repair process, macrophages seem to play a central regulatory role throughout all stages of tissue remodeling and fibrosis (6–8).

Macrophages exhibit substantial phenotypic plasticity, but are generally broadly categorized into two major subtypes; pro-inflammatory versus anti-inflammatory macrophages. Pro-inflammatory macrophages contribute to pathogen clearance, but also can cause tissue damage and are often considered anti-fibrotic. In contrast, anti-inflammatory macrophages are associated with wound healing, tissue repair and the promotion of fibrosis (5, 9, 10). Macrophage phenotypes are dictated by sensing signaling molecules in the surrounding environment. *In vitro*, pro-inflammatory macrophages are typically generated by stimulation with interferon (IFN)- γ and/or lipopolysaccharide (LPS), and anti-inflammatory macrophages by stimulation with Interleukin (IL)-4, IL-13, IL-10 or transforming growth factor β (TGF- β) or a combination of different cytokines/growth factors. The different stimuli each lead to distinct gene expression profiles and functional characteristics that have been extensively characterized (11). More recently, four anti-inflammatory subsets –previously denoted as M2a, M2b, M2c and M2d– have been identified, each induced by different environmental cues (12, 13). In human monocytes, IL-4 stimulation induces anti-inflammatory macrophage differentiation, characterized by high expression of CD206, CD209, early growth response 2 (EGR2) and Dectin-1 (12–14). Stimulation with IL-10 or TGF- β induces anti-inflammatory macrophage differentiation, characterized by elevated CD163, Mer tyrosine kinase (MerTK) expression, along with low to intermediated CD206 levels (12, 13). Currently, to polarize distinct subsets, macrophages are named after the stimulus used for polarization (e.g., M(IL-4), M(IL-10) or M(TGF- β)).

Persistent activation and recruitment of anti-inflammatory macrophages have been proposed to drive pathological fibrosis. Global depletion of lung macrophages during the progressive fibrotic phase of bleomycin-induced lung fibrosis in mice markedly reduced fibrotic severity, accompanied by decreased Ym1 protein levels and reduced arginase1 activity –two hallmark markers of murine M(IL-4) (15). It was further reported that CD163, a human M(TGF- β) marker, was expressed on more than 90% of lung macrophages from IPF patients, but was absent on lung macrophages from age-matched healthy individuals, underscoring the importance of anti-inflammatory polarization in fibrotic disease (15). In

addition, myeloid-specific deletion of IL-4Ra attenuated progressive parenchymal liver fibrosis, yet inhibited fibrosis resolution during the reversal phase, owing to a reduction in fibrolytic matrix metalloproteinase 12 (MMP12) levels (16). Moreover, anti-inflammatory macrophages were shown to be the predominant macrophage population recruited in the lung during bleomycin-induced fibrosis, and M(IL-4) induced myofibroblast differentiation of lung-resident mesenchymal stem cells through Wnt/ β -catenin signaling. Consistent with these findings, lung tissue from IPF patients exhibited increased infiltration of CD206⁺ macrophages and activation of Wnt/ β -catenin signaling pathway (17). Collectively, these studies suggest that IL-4 polarized anti-inflammatory macrophages may play a prominent profibrotic role in the development of fibrosis, although they also may contribute to fibrosis resolution.

TGF- β is widely recognized as a central mediator in the pathogenesis of fibrosis. Although its profibrotic effects are classically attributed to direct actions on fibroblasts, several studies indicate that TGF- β may activate other cell types, including macrophages (18). In a mouse model of acute kidney injury, deletion of TGF- β receptor II (TGF- β RII) specifically in monocytes/macrophages reduced macrophage infiltration and significantly attenuated subsequent tubulointerstitial fibrosis, demonstrating that TGF- β signaling within macrophages enhances their profibrotic activity (19). Several mechanisms through which M(TGF- β) may promote fibrogenesis have been proposed: Increasing macrophage accumulation in injured tissues through chemotaxis, upregulating profibrotic cytokine production, stimulating synthesis of extracellular matrix proteins, enhancing TGF- β activation via secretion of TGF- β activating factors, and undergoing phenotypic conversion towards fibroblast-like cells (18). However, despite these insights, the specific role of M(TGF- β) in pulmonary fibrosis and the underlying mechanisms remain to be fully elucidated.

In this study, we aimed to identify the most appropriate anti-inflammatory macrophage model for studying macrophage-driven fibrosis *in vitro* and to determine how M(IL-4) and M(TGF- β) influence the fibrotic responses of normal human lung fibroblasts (NHLFs). In addition, we performed whole-genome transcriptome profiling by RNA sequencing to compare M(IL-4) and M(TGF- β) phenotypes to elucidate the molecular mechanisms underlying the profibrotic properties induced in lung fibroblasts.

Materials and Methods

2.1 Cells and Culture Conditions

Human leukemia monocytic cell line THP-1 (obtained from ATCC, #TIB-202), human embryonic kidney (HEK) 293T cells (obtained from ATCC, #CRL-3216) and NHLFs (obtained from Lonza Bioscience, #CC-2512) were cultured in RPMI (THP-1, #31870-025, Gibco) or DMEM (HEK293T and NHLFs, #11995-073, Gibco) supplemented with 10% fetal bovine serum (FBS, #F9665, Sigma-Aldrich), 2 mM L-glutamine (#25030-024, Gibco), 100 U/mL penicillin, and 100 μ g/mL streptomycin (#15140-122, Gibco). All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Human B cell leukemia C/EBPalphaER clone 1 (BLaER1) GFP^{-/-} cells were kindly provided by Prof. Holger Heine (Research Centre Borstel, Leibniz Lung Centre). BLaER1 cells were cultured at 37°C, 5% CO₂, in RPMI 1640 without phenol red (#11835-030, Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate (#11360-070, Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS (#A5256801, Gibco).

Buffy coats of healthy anonymous blood donors were obtained from Sanquin blood bank in Amsterdam, the Netherlands. These experiments were approved by the ethical board of the Amsterdam UMC under contract number NVT0639.02 and all subjects provided written informed consent. All procedures were conform the Declaration of Helsinki and Dutch regulations. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll density gradient separation with Lymphoprep (#AXI-114545, Axis Shield supplied by Lucron) and washed with PBS without calcium and magnesium. CD14⁺ monocytes were subsequently isolated from the PBMCs by immunomagnetic separation using MACS CD14 beads (#130-050-201; Miltenyi Biotec) in MACS buffer (filtered and supplemented with 1% FBS, 2mM EDTA in PBS). Isolated CD14⁺ monocytes were resuspended and cultured in IMDM (#21056023, Gibco) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS.

2.2 Macrophage polarization and conditioned medium collection

2.2.1 Trans-differentiation and stimulation of THP-1 cells

3×10^5 THP-1 cells were seeded into 6-well plates and treated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA, #P8139, Sigma-Aldrich) for 24 hours (h) to differentiate them into macrophage-like cells (naïve). After 24h, cells were washed once with PBS and incubated in complete RPMI medium without PMA for an additional 24h. For polarization, cells were stimulated with either 20 ng/ml IL-4 (#200-04, PeproTech) or 10 ng/ml recombinant human TGF-β (#100 – 21, Thermo Fisher scientific) for 72h.

2.2.2 Trans-differentiation and stimulation of BLaER1 cells

BLaER1 cells were seeded in 10 cm cell culture-dishes at a density of 4×10^6 cells per 12 ml of trans-differentiation medium, consisting of complete RPMI medium supplemented with 100 nM β-estradiol (#E2758, Sigma-Aldrich), 10 ng/ml human IL-3 (#AF-200- 03, PeproTech), and 10 ng/ml human M-CSF (#130-096-493, Miltenyi Biotec). The cells were incubated for 5 days at 37°C and 5% CO₂ to induce trans-differentiation into macrophages. On day 3, the trans-differentiation medium was doubled to 24 ml considering the cells have typically divided twice by the end of day 5. On day 5 of differentiation, cells were detached using TrypleE Express without phenol red (#12604039, Gibco) at 37°C for 2–3 minutes (mins), counted and reseeded in trans-differentiation medium at densities appropriate for downstream experiments. After allowing the cells to attach for at least 3h, macrophages were polarized by stimulation with 50 ng/mL IL-4 or 10 ng/ml recombinant human TGF-β for 24h.

2.2.3 Trans-differentiation and stimulation of CD14 + monocytes

Monocytes were seeded at approximately 3×10^5 /mL in medium containing 50 ng/mL human M-CSF (#130-096-493, Miltenyi Biotec). On day 6, differentiated cells were harvested using TrypleE Express and seeded in IMDM with 10% FBS at a concentration of 1×10^6 /mL and incubated for at least 3h at 37°C, 5% CO₂. For polarization, adherent macrophages were stimulated with 50 ng/mL IL-4 or 10ng/ml recombinant human TGF- β , for 24h.

2.2.4 Collection of polarized macrophages or conditioned medium

After differentiation, macrophages derived from THP-1 cells were washed using PBS, collected and counted using a CASY Cell Counter and Analyzer System (Schärfe System GmbH, Reutlingen, Germany). Naïve, M(IL-4) and M(TGF- β) were transferred to culture plates containing NHLFs (cell number ratio: 1 to 1) for coculture.

For all macrophages derived from different precursor cells, after polarization, the stimulus-containing medium was washed off from the cells using PBS, and fresh DMEM supplemented with 1% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin was added. After 24h, conditioned medium was collected and stored at – 80°C for subsequent fibroblast stimulation experiments.

2.3 Coculture of polarized macrophages and NHLFs or conditioned medium transfer experiments

NHLFs were seeded at a density of 7,500 cells/well into a 96-well plate. On the following day, cells were washed once with PBS and serum-starved for 6h in serum-free medium. For coculture experiments, 7,500 polarized macrophages derived from THP-1 cells were added to NHLFs. For conditioned medium transfer experiments, conditioned medium (50 μ l) derived from M(IL-4) or M(TGF- β), or fractionated conditioned media obtained using size exclusion filters (see below), were added to NHLFs with 50 μ l serum-free medium (1:2 dilution). For experiments involving inhibition of TGF- β signaling, NHLFs were pre-incubated with the TGF- β receptor kinase inhibitor RepSox (1 μ M; #616452, Sigma Aldrich) for 1h prior to conditioned media stimulation.

After 48h of coculture or stimulation, NHLFs were fixed with ice cold 100% methanol for 2 mins at room temperature, and subsequently blocked with 3% BSA for 1h. Following two PBS washes, cells were incubated overnight at 4°C with primary antibodies against α -smooth muscle actin (α SMA; #sc-32251, Santa Cruz), Collagen type I (Col1; #1310-01, Southern Biotech) and Fibronectin (FN; #sc-8422, Santa Cruz). The following day, cells were incubated with the appropriate Alexa Fluor™ 647(#A21447, Thermo fisher scientific) or Alexa Fluor™ 488 (#A11029, Thermo Fisher scientific) secondary antibodies for 1h at

room temperature in the dark. After washing with PBS, cells were imaged with an IncuCyte imaging system (Sartorius).

2.4 Conditioned medium fractionation by size-exclusion centrifugal filters

Conditioned medium from M(TGF- β) was collected and processed using Amicon Ultra centrifugal filters with molecular-weight cutoffs of 3, 10, 50, and 100 kD respectively (#UFC200324, #UFC201024, #UFC205024, #UFC210024, Merck Millipore), following the manufacturer's instructions. Samples were centrifuged at $1200 \times g$ for 25 mins, resulting in separation into an upper retention fraction (containing proteins > 3 , > 10 , > 50 or > 100 kD, respectively) and a lower filtrate fraction (proteins < 3 , < 10 , < 50 , < 100 kD respectively). Each separated fraction was collected and adjusted back to the original sample volume using serum-free DMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

2.5 Sample and library preparation for RNA-sequencing

Total RNA from M(IL-4) or M(TGF- β) was isolated using a NucleoSpin RNA kit (#740955.250, Macherey-Nagel) and quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA). PolyA-enriched cDNA libraries were generated using the KAPA mRNA HyperPrep Kit (#8098123702, Roche). Each sample was labeled using a unique IDT adapter and subsequently pooled. Paired-end sequencing (2 x 150 bp) was performed on the Illumina NovaSeq X Plus platform by the Core Facility Genomics (CFG) at Amsterdam UMC.

2.6 RNA-sequencing analysis

The fastq files of the sequenced libraries were obtained from the CFG and quality control was performed on the raw sequencing data using the FastQC tool (<https://github.com/s-andrews/fastqc>). After confirming sample quality, reads were trimmed and Illumina universal adapter sequences were removed using Trimmomatic v0.39 in paired-end mode using the following parameters: ILLUMINACLIP:TruSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 (<https://doi.org/10.1093/bioinformatics/btu170>). Unpaired reads were discarded from subsequent analysis, while successful read pairs were aligned to the human genome assembly hg38 with the alignment program HISAT2 v2.1.0 using the parameters “--mm --add-chrname --new-summary --no-spliced-alignment” (20).. Output sam files were converted to bam format, and secondary, supplementary and reads with a mapping quality (MAPQ) lower than 20 were removed using samtools v1.17. The number of read pairs mapping to genes were quantified with featureCounts v2.0.2 using GENCODE's gtf annotation file (GRCh38 gencode.v46.annotation) (21). The output raw count table was loaded into RStudio (R version 4.3.0) and differential gene expression analysis was performed with DESeq2 package. Differentially expressed genes were chosen based on the following cutoffs: fold change > 2 & Benjamini-Hochberg corrected p-value (False Discovery Rate; FDR) < 0.01 . KEGG pathway and gene ontology analyses were performed using clusterProfiler (22).

2.7 Statistical analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA. A p -value < 0.05 was considered statistically significant. All statistical analysis were performed using GraphPad Prism version 10.6.0.

Results

3.1 Coculture with M(TGF- β), but not M(IL-4), induces α SMA levels in NHLFs

In order to assess the profibrotic potential M(IL-4) and M(TGF- β) were cocultured with NHLFs. Macrophages were polarized by IL-4 and TGF- β stimulation for 3 days, washed and transferred to NHLFs without additional stimulations. As a positive control, NHLFs were stimulated with TGF- β directly (Fig. 1). M(IL-4) did not induce detectable changes in α SMA protein levels compared to naïve macrophages (Fig. 1). In contrast, M(TGF- β) significantly induced α SMA protein level in NHLFs ($p < 0.0001$). These results indicate that M(TGF- β) macrophages, but not M(IL-4) macrophages, promote differentiation of NHLFs into myofibroblasts.

3.2 Conditioned medium from M(TGF- β), but not M(IL-4), induces α SMA, collagen type I and fibronectin levels in NHLFs

In order to assess whether the profibrotic potential of M(TGF- β) is dependent on paracrine signaling, NHLFs were stimulated with conditioned medium derived from macrophages differentiated from THP-1 cells. In line with the coculture experiments, conditioned medium from M(IL-4) derived from THP-1 did not induce detectable changes in α SMA, Col1 or FN protein levels compared to conditioned medium obtained from naïve macrophages. In contrast, conditioned medium from M(TGF- β) significantly induced profibrotic proteins, including α SMA ($p < 0.0001$), Col1 ($p < 0.001$), and FN ($p < 0.001$) levels, in NHLFs (Fig. 2A-B). Overall, these results demonstrate that M(TGF- β), but not M(IL-4), secrete factors that drive profibrotic fibroblast activation.

3.3 Confirmation of proper polarization of M(TGF- β) and M(IL-4) and extensive analysis of transcriptional differences between M(TGF- β) and M(IL-4)

In order to gain insight in the factors driving the profibrotic response in NHLFs, we performed whole-genome transcriptome profiling of naïve, M(IL-4) and M(TGF- β). First, to confirm appropriate polarization of THP-1 cells into anti-inflammatory M(IL-4) and M(TGF- β), the expression of marker genes that characterize these functional states was assessed. M(IL-4) showed a markedly increased expression of *CD209* and *EGR2* (an indispensable regulator of the late IL-4 response), compared to naïve and their TGF- β -polarized counterparts. Conversely, M(TGF- β) were characterized by high expression of *CD163* and *MERTK* (encoding Mer Tyrosine Kinase) (Fig. 3A). These expression patterns suggest successful

polarized of THP-1 cells into M(IL-4) and M(TGF- β) subsets, previously established in other model cell lines.

We then went on to systematically examine transcriptional differences between these subsets in order to identify the potential drivers of the profibrotic effects in NHLFs. As shown in Fig. 3B, IL-4 and TGF- β stimulations had profound effects on the transcriptome of naïve macrophages, with 2106 and 2432 genes being differentially expressed, respectively (Fold change > 2; corrected p-value < 0.01). However, the most pronounced difference was noted when comparing M(IL-4) and M(TGF- β), with 2518 genes significantly different between these two conditions (Fig. 3B), out of which 1239 genes were more highly expressed in M(TGF- β), while 1279 were preferentially induced following IL-4 treatment (Fig. 3C).

Among the top 5 Gene Ontology (GO) terms related to biological pathways enriched in the genes preferentially induced by TGF- β (over IL-4) were the regulation of neuron projection development, and the regulation of synapse activity or structure (Figure S1A). These pathways are reminiscent of processes involving cytoskeleton remodeling, vesicular transport and cell adhesion, confirmed by the genes that make up the enriched terms, including *SPP1*, *DNM3*, *SPARCL1*, *ITGA6*, and *VEGFA*. It should also be noted that the phenotype of the primary tissue-resident macrophages of the central nervous system, the microglia, is shaped by pervasive TGF- β signaling in the brain. It is likely that supplementation with TGF- β leads to preferential expression of microglia-associated genes, which would explain the nervous system-associated hits in our GO term enrichment analysis (23). Instead, IL-4-biased genes are enriched in leukocyte activation, proliferation and cell-cell adhesion (Figure S1A). Enriched KEGG pathways for IL-4-biased genes included cytokine-cytokine receptor interaction and phagocytosis, while TGF- β -biased genes were enriched for Mitogen-activated protein kinase (MAPK) and Ras signaling pathways (Figure S1B).

The top 30 upregulated genes and top 30 downregulated genes in M(TGF- β) compared to M(IL-4) are shown as a heatmap in Fig. 3D. Of note, the top 30 downregulated genes in M(TGF- β) include macrophage phenotypic markers or regulators like *MRC1* and *FABP4*, previously shown to be downregulated in M(TGF- β) (24, 25).

Since conditioned medium from M(TGF- β) and not M(IL-4) promoted a profibrotic response in NHLFs, we reasoned that genes specifically induced following TGF- β stimulation would encode for secreted proteins that facilitate profibrotic responses. We therefore focused on the 859 genes regulated by TGF- β when comparing both to naïve and M(IL-4) (Fig. 3B).

3.4 Gene expression levels of secreted factors, collagen, ECM-affiliated proteins, ECM regulators and glycoproteins from Matrisome database in M(TGF- β) or M(IL-4)

Because the factors driving profibrotic responses in NHLFs are secreted by the M(TGF- β) (evident from the medium transfer experiments described above), genes annotated as secreted factors, collagen, ECM-affiliated proteins, ECM regulators and glycoproteins in the Matrisome database were identified.

Genes belonging to these matrisome categories were extracted from both the comparison of M(TGF β) vs naïve and M(TGF β)-vs M(IL-4) and listed in Table S1.

Across these analyses, a total of 55 genes were found to be upregulated in M(TGF- β) relative to both M(IL-4) and naïve macrophages. These genes represent candidate mediators that may contribute to the enhanced profibrotic activity observed in M(TGF- β)-derived conditioned medium.

3.5 Several high-molecular-weight proteins were identified as potential profibrotic factors secreted by M(TGF- β), but not M(IL-4)

To estimate the approximate molecular size of the profibrotic factors secreted by M(TGF- β), conditioned medium was fractionated using centrifugal size exclusion filters with molecular weight cutoffs of 3, 10, 50 or 100 kD respectively. Each fraction –containing proteins either smaller or larger than the respective cutoff– was subsequently used to stimulate NHLFs, and fibrotic responses were determined. Increased α SMA and collagen levels were retained in the conditioned medium fractions containing proteins larger than 3 kD (Fig. 4), 10 kD (Figure S2), 50 kD (Figure S3), and 100 kD (Fig. 5). These results indicate that the molecular weight of potentially key profibrotic factors secreted by M(TGF- β) are likely larger than 100 kD.

To identify proteins of more than 100 kD that might be secreted by M(TGF- β), the molecular weight of DEGs listed in Table S1 was assessed (Table 1). Strikingly, several candidates from this list have a strong link with the induction of the TGF- β pathway or the regulation of the availability of active TGF- β , including A Disintegrin And Metalloproteinase with ThromboSpondin Motif 6 and 10 (ADAMTS6 and ADAMTS10), COL22A1, latent transforming growth factor- β binding protein 1 and 2 (LTBP1 and LTBP2), multiple EGF like domain 6 (MEGF6) and sulfatase 2 (SULF2) (26–37). Our findings suggest that M(TGF- β) may secrete factors that potentially affect profibrotic TGF- β signaling in NHLFs.

Table 1

Proteins with a molecular weight of > 100 kD of DEGs associated with M(TGF- β), but not M(IL-4)

Candidate gene	Molecular weight	Association with activation of TGF-β pathway in lung described?	References
<i>ADAMTS6</i>	115 kD	Yes	(27, 28)
<i>ADAMTS10</i>	140 kD	Yes	(28, 29)
<i>COL7A1</i>	290–300 kD	Limit the availability of latent TGF- β	(38, 39)
<i>COL15A1</i>	180–200 kD	Unknown	-
<i>COL19A1</i>	115 kD	Unknown	-
<i>COL22A1</i>	161 kD	Yes	(33)
<i>COL24A1</i>	175 kD	Primarily osteogenic	(40)
<i>CPAMD8</i>	206–215 kD	May antagonize	(41)
<i>KCP</i>	166 kD	Yes, but as suppressor	(42, 43)
<i>LTBP1</i>	150–210 kD	Yes	(26, 30, 31)
<i>LTBP2</i>	195–240 kD	Yes	(26, 32)
<i>MEGF6</i>	161 kD	Yes	(34)
<i>NRG1</i>	Precursor: 90–135 kd	No evidence, but involved in fibrosis	(44, 45)
<i>PAPPA2</i>	170–250 kD	No, but regulates IGF availability	(46, 47)
<i>PLXNB3</i>	206 kD	No evidence	-
<i>PLXNC1</i>	176 kD	Yes, but as suppressor	(48)
<i>SEMA6D</i>	120 kD	Unknown, but downstream of BMP signaling	(49)
<i>SERPING1</i>	Full-length glycoprotein 100–110 kD	No direct evidence	-
<i>SULF2</i>	130–150 kD	Yes	(35–37)
<i>THSD4</i>	112–120 kD	No evidence	-

ADAMTS, A Disintegrin And Metalloproteinase with ThromboSpondin Motifs; CPAMD8, complement component 3- and pregnancy zone protein-like alpha-2-macroglobulin domain-containing protein 8; KCP, kielin/chordin-like protein; LTBP, latent transforming growth factor β binding protein; MEGF6, multiple epidermal growth factor-like domains protein 6; NRG1, Neuregulin-1; PAPPA2, pregnancy-associated plasma protein A2; IGF, insulin-like growth factor; PLXNB3, plexin-B3; PLXNC1, plexin C1; SEMA6D, Semaphorin 6D; BMP, bone morphogenetic protein; SERPING1, C1 inhibitor (C1-INH); SULF2, sulfatase 2; THSD4, Thrombospondin type 1 domain containing 4, also known as ADAMTSL6.

3.6 The profibrotic effect of M(TGF- β) is mediated through activation of TGF- β signaling pathway in NHLFs

To prove or refute our hypothesis that the profibrotic activity of M(TGF- β) conditioned medium was mediated through activation of the TGF- β signaling pathway in NHLFs, NHLFs were pre-treated with RepSox, a TGF- β receptor kinase inhibitor, prior to stimulation with conditioned medium. Of note, RepSox treatment reversed the induction of α SMA and collagen expression triggered by conditioned medium from M(TGF- β) (Fig. 6), indicating that M(TGF- β) indeed promote profibrotic responses in NHLF primarily by engaging in the TGF- β signaling pathway.

3.7 Conditioned medium from M(TGF- β) derived from primary monocytes and BLaER1 cells also induces profibrotic responses through activation of the TGF- β pathway in NHLFs

In order to evaluate and ensure the profibrotic potential of M(TGF- β), but not M(IL-4), is a general phenomenon, primary monocytes and BLaER1 cells were used to polarized with TGF- β or IL-4 and to subsequently assess the fibrotic response in NHLFs of conditioned medium obtained from those macrophages. Stimulation of NHLFs with conditioned medium derived from these macrophages showed that M(IL-4) —whether derived from primary monocytes (Fig. 7) or BLaER1 cells (Fig. 8)— did not induce detectable changes in α SMA or Col1 protein levels compared to conditioned medium obtained from naïve macrophages. In line with previous findings, conditioned medium from M(TGF- β) induced profibrotic protein expression in NHLFs. Indeed, conditioned medium from TGF- β -polarized primary monocyte-derived macrophages significantly increased α SMA ($p < 0.0001$), and Col1 levels, although the latter did not reach statistical significance (Fig. 7). Similarly, conditioned medium from TGF- β -polarized BLaER1 macrophages induced a significant increase in α -SMA ($p < 0.001$) and Col1 ($p < 0.0001$) (Fig. 8). Moreover, the increased level of α SMA and Col1 in NHLFs induced by conditioned medium from M(TGF- β) was reversed with RepSox pretreatment. Overall, these results show that M(TGF- β), but not M(IL-4), secrete factors that drive profibrotic responses through activation of the TGF- β pathway in NHLFs.

Discussion

Anti-inflammatory macrophages are broadly implicated in the orchestration of profibrotic responses across diverse fibrotic diseases. However, the selection of an appropriate *in vitro* model to interrogate anti-inflammatory macrophage-mediated mechanisms has remained a matter of considerable uncertainty. In the present study, we demonstrate that macrophages polarized with TGF- β , but not those polarized with IL-4, elicit robust fibrotic activation in normal human lung fibroblasts (NHLFs). Transcriptomic profiling revealed that M(TGF- β) express a distinct repertoire of secreted mediators, collagen, ECM-affiliated proteins, ECM regulators, and glycoproteins that are not induced in M(IL-4). Furthermore, our data indicate that the profibrotic influence of M(TGF- β) on NHLFs is mediated primarily through activation of the canonical TGF- β signaling pathway within fibroblasts. Collectively, these

findings identify M(TGF- β), rather than M(IL-4), as the most physiologically relevant *in vitro* model for mechanistic studies of macrophage-driven fibrotic responses of lung fibroblasts.

Several studies have proposed that M(IL-4) exert profibrotic effects via the release of profibrotic factors in the setting of pulmonary fibrosis (50). However, there are also conflicting studies suggesting that M(IL-4) may play a fibrosis-resolving role by activating MMP-12 (16). Moreover, deletion of macrophage specific IL-4Ra did not affect fibrosis formation in a model for acute schistosomiasis (51). The effect of M(IL-4) on fibrosis may be context dependent and evidence that M(IL-4) directly induce fibrotic responses in lung fibroblasts is lacking. In the current study we aimed to establish an *in vitro* model to study the fibrotic effects of macrophages on fibroblasts and we show that M(IL-4) do not induce fibrotic responses of lung fibroblasts in a direct manner. This is in line with a study performed by Sheng et al. who generated IL-4-polarized THP-1-derived macrophages and co-cultured them with different types of prostate fibroblasts (52). In fibroblasts obtained from healthy prostate, M(IL-4)-fibroblast co-cultures did not induce α SMA and Col1 mRNA and protein expression, although increased α SMA and Col1 mRNA and protein expression was observed in primary prostate fibroblasts from benign prostatic hyperplasia patients (52). Together, these results underscore the context-dependent nature of macrophage-fibroblast interactions and similar experiments using lung fibroblasts obtained from IPF patients should elucidate whether M(IL-4) affect fibrotic responses in diseased fibroblasts. Given these divergent observations, it remains challenging to conclude whether fibrosis is dependent on M(IL-4). One could even argue against since several mechanisms have been proposed through which M(IL-4) may inhibit ECM synthesis or promote ECM degradation (53, 54). These contrasting perspectives highlight the complexity of macrophage biology in the context of fibrosis and moreover suggest that M(IL-4) may not constitute a reliable *in vitro* model for mechanistic studies on the role of macrophages in fibrosis. In light of these uncertainties, alternative macrophage models –such as the use of M(TGF- β)– are needed to more accurately delineate direct macrophage contributions to fibrotic remodeling.

A key finding of our study is that M(TGF- β), derived from different cell types including THP-1, primary monocytes and BLaER1 cells, showed a strong and consistent capacity to induce fibrotic responses of lung fibroblasts. Recent studies further support a profibrotic role for M(TGF- β). Conditioned medium of M(TGF- β) derived from THP-1 cells or peripheral blood monocytes, induced a secretome that promoted migration, proliferation, and profibrogenic activity in hepatic stellate cells –the principal fibrogenic cell type in the liver– in a MerTK dependent manner (55). Interestingly, MerTK expression is a hallmark of the M(TGF- β) (Fig. 3A) and these findings underscore the importance of MerTK in anti-inflammatory macrophage polarization in hepatic fibrosis and parallel the profibrotic behavior we observed in M(TGF- β). Moreover, THP-1-derived macrophages polarized with IL-10 –an alternative stimulus for anti-inflammatory macrophage polarization expressing similar phenotypic markers compared to TGF- β polarization (12)– showed a significantly stronger induction of epithelial-mesenchymal transition in peritoneal mesothelial cells compared to IL-4 polarized THP-1 cells when co-cultured (56). Together these studies support the findings that M(TGF- β) bear a higher pro-fibrotic potential compared to M(IL-4).

In vivo evidence also points to a profibrotic role for macrophages expressing phenotypic markers of TGF- β /IL-10 polarization in pulmonary fibrosis. In bleomycin-induced pulmonary fibrosis, markers such as YM1/2, Fizz, and Arg1 are elevated (57, 58). Following bleomycin administration, the proportion of inflammatory macrophages decreases, whereas anti-inflammatory populations expand, with the TGF- β phenotypic subset showing the most pronounced increase (59). Interestingly, in bone marrow–derived macrophages obtained 7 days after bleomycin instillation and *ex vivo* TGF- β stimulation to induce polarization—consistent with our approach—, increased profibrotic gene expression was observed (8). Lastly, *in vitro* M(TGF- β) resemble phenotypic marker expression observed in macrophages identified using single-cell sequencing analysis of lung tissue of IPF patients like SPP1 and MerTK (8). Overall, these findings identify M(TGF- β) as key drivers of pulmonary fibrosis and support their use as an *in vitro* model for mechanistic studies over M(IL-4).

Our study has several limitations. First, although we identified several profibrotic factors secreted by M(TGF- β) that may be responsible for the induction of the profibrotic responses, we cannot exclude that high-molecular-weight protein complexes or larger particles may contribute to the observed effects. Protein complexes or particles, like micro vesicles, with a molecular weight of > 100 kD will be present in the retention fractions used to stimulate NHLFs, although it remains unknown whether those complexes or particles should also be responsible for the activation of the TGF- β pathway. Second, while we demonstrated activation of the TGF- β pathway in NHLFs by macrophage-derived secreted factors and highlighted several candidate mediators, we did not explore which specific factors are responsible for the fibrotic response induction. These limitations underscore the need for future studies employing alternative strategies to dissect the molecular drivers of M(TGF- β)-mediated fibroblast activation.

In conclusion, our study demonstrates that M(TGF- β) exert potent profibrotic effects on NHLFs, whereas M(IL-4) do not. We further show that the profibrotic activity of M(TGF- β) is mediated through the activation of the TGF- β signaling pathway. Together, these findings establish M(TGF- β) as a more relevant and mechanistically informative *in vitro* model for studying macrophage–fibroblast interactions in the context of pulmonary fibrosis. This model provides a valuable platform for identifying macrophage-derived profibrotic mediators and may facilitate the development of targeted therapeutic strategies against macrophages for fibrotic disease.

Abbreviations

IL-4
Interleukin-4
TGF- β
Transforming growth factor
NHLFs
Normal human lung fibroblasts
 α SMA
 α -smooth muscle actin

IPF
Idiopathic pulmonary fibrosis
ECM
Extracellular matrix
IFN- γ
Interferon- γ
LPS
Lipopolysaccharide
EGR2
Early growth response 2
MerTK
Mer tyrosine kinase
M(IL-4)
IL-4 polarized macrophages
M(IL-10)
IL-10 polarized macrophages
M(TGF- β)
TGF- β polarized macrophages
MMP12
Matrix metalloproteinase 12
TGF- β R
TGF- β receptor
HEK
Human embryonic kidney
FBS
Fetal bovine serum
BLaER1
Human B cell leukemia C/EBP α ER clone 1
PBMCs
Peripheral blood mononuclear cells
PMA
Phorbol 12-myristate 13-acetate
Mins
Minutes
Col1
Collagen type I
FN
Fibronectin
SEM
Standard error of mean

GO
Gene Ontology
MAPK
Mitogen activated protein kinase
ADAMTS
A Disintegrin And Metalloproteinase with ThromboSpondin Motifs
CPAMD8
Complement component 3- and pregnancy zone protein-like alpha-2-macroglobulin domain-containing protein 8
KCP
Kielin/chordin-like protein
LTBP
Latent transforming growth factor β binding protein
MEGF6
Multiple epidermal growth factor-like domains protein 6
NRG1
Neuregulin-1
PAPPA2
Pregnancy-associated plasma protein A2
IGF
Insulin-like growth factor
PLXNB3
Plexin-B3
PLXNC1
Plexin C1
SEMA6D
Semaphorin 6D
BMP
Bone morphogenetic protein
SULF2
Sulfatase 2
THSD4
Thrombospondin type 1 domain containing 4

Declarations

Availability of data and materials

The data supported this article/Supplementary Material are available from the corresponding author upon request.

Acknowledgements

BLaER1 GFP-/- cells were kindly provided by Prof. Holger Heine (Research Centre Borstel, Leibniz Lung Centre).

Funding

This work was supported by a collaboration grant from the Amsterdam institute for Immunology and Infectious diseases to JWD and MAH. M.A.H. was supported by the European Union's Horizon 2020 research and innovation programme through a Marie Skłodowska-Curie Individual Fellowship (grant agreement No. 895411, MacrophageEGR) and by the European Research Council (ERC) under the European Union's Horizon Europe research and innovation programme (grant agreement No. 101076170, CYTOMAC). has received funding from the European Union (ERC, CytoMAC, 101076170). Funded by the European Union.

Author information

Authors and Affiliations

Department of Experimental Immunology, Amsterdam UMC, location University of Amsterdam, Amsterdam, The Netherlands

Qian Tian, Jan Willem Duitman

Amsterdam Institute for Immunology & Infectious disease, Amsterdam UMC, location University of Amsterdam, Amsterdam, The Netherlands

Qian Tian, Alexandra Drakaki, Marten A. Hoeksema, Jan Willem Duitman

Department of Medical Biochemistry, Amsterdam UMC, location University of Amsterdam, Amsterdam, The Netherlands

Alexandra Drakaki, Marten A. Hoeksema

Center for Infection and Molecular Medicine, Amsterdam UMC, location University of Amsterdam, Amsterdam, The Netherlands

Hella Aberson, Sami Lone, Tom van der Poll, C. Arnold Spek

Division of Infectious Disease, Amsterdam UMC, location University of Amsterdam, Amsterdam, The Netherlands

Tom van der Poll

Laboratory of Experimental Oncology and Radiobiology, Amsterdam UMC, location University of Amsterdam, Amsterdam, the Netherlands

C. Arnold Spek

Department of Pulmonary Medicine, Amsterdam UMC, location University of Amsterdam, Amsterdam, The Netherlands

Jan Willem Duitman

Contributions

Study concept and design: J.W.D., C.A.S. and M.A.H.; drafting of the manuscript: Q.T. and A.D.; implement of the study: Q.T., A.D., H.A., S.L.; data analysis and interpretation of data: Q.T., A.D., J.W.D., C.A.S. and M.A.H.; revision of the manuscript: J.W.D., C.A.S., M.A.H. and T.v.d.P.; study supervision: J.W.D., C.A.S. and M.A.H. All authors have read and approved the final manuscript.

Corresponding authors

Correspondence to Qian Tian, Alexandra Drakaki, Jan Willem Duitman.

Ethics Declarations

Ethics approval and consent to participate

Buffy coats of healthy anonymous blood donors were obtained from Sanquin blood bank in Amsterdam, the Netherlands. These experiments were approved by the ethical board of the Amsterdam UMC under contract number NVT0639.02 and all subjects provided written informed consent. All procedures were conform the Declaration of Helsinki and Dutch regulations.

Consent for publication

Informed consent was obtained from all donors of the primary cells involved in the study.

Competing interests

The authors declare no competing interests. Views and opinions expressed are however, those of the authors only and do not necessarily reflect those of the European Union or the European Research Council Executive Agency. Neither the European Union nor the granting authority can be held responsible for them.

Additional information

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information

Supplementary Material (download zip)

References

1. Herberts MB, et al. Idiopathic pulmonary fibrosis in the United States: time to diagnosis and treatment. *BMC Pulm Med*. 2023;23:281.
2. Kaunisto J, et al. Demographics and survival of patients with idiopathic pulmonary fibrosis in the FinnishIPF registry. *ERJ Open Res*. 2019;5:00170–2018.
3. Strongman H, Kausar I, Maher TM. Incidence, Prevalence, and Survival of Patients with Idiopathic Pulmonary Fibrosis in the UK. *Adv Ther*. 2018;35:724–36.
4. May J, Jenkins G. Idiopathic Pulmonary Fibrosis: An Overview. *Br J Hosp Med (Lond)*. 2026;87:50844.
5. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med*. 2012;18:1028–40.
6. Wynn TA, Barron L. Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis*. 2010;30:245–57.
7. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature*. 2013;496:445–55.
8. Morse C, et al. Proliferating SPP1/MERTK-expressing macrophages in idiopathic pulmonary fibrosis. *Eur Respir J*. 2019;54:1802441.
9. Hams E, Bermingham R, Fallon PG. Macrophage and Innate Lymphoid Cell Interplay in the Genesis of Fibrosis. *Front Immunol*. 2015;6:597.
10. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8:958–69.
11. Murray PJ, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. 2014;41:14–20.
12. Kulakova K, Lawal TR, McCarthy E, Floudas A. The Contribution of Macrophage Plasticity to Inflammatory Arthritis and Their Potential as Therapeutic Targets. *Cells*. 2024;13:1586.
13. Zhang Q, Sioud M. Tumor-Associated Macrophage Subsets: Shaping Polarization and Targeting. *Int J Mol Sci*. 2023;24:7493.
14. Daniel B, et al. The transcription factor EGR2 is the molecular linchpin connecting STAT6 activation to the late, stable epigenomic program of alternative macrophage polarization. *Genes Dev*. 2020;34:1474–92.

15. Gibbons MA, et al. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. *Am J Respir Crit Care Med*. 2011;184:569–81.
16. Weng SY, et al. IL-4 Receptor Alpha Signaling through Macrophages Differentially Regulates Liver Fibrosis Progression and Reversal. *EBioMedicine*. 2018;29:92–103.
17. Hou J, et al. M2 macrophages promote myofibroblast differentiation of LR-MSCs and are associated with pulmonary fibrogenesis. *Cell Commun Signal*. 2018;16:89.
18. Frangogiannis N. Transforming growth factor-beta in tissue fibrosis. *J Exp Med*. 2020;217:e20190103.
19. Chung S, et al. TGF-beta promotes fibrosis after severe acute kidney injury by enhancing renal macrophage infiltration. *JCI Insight*. 2018;3:e123563.
20. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol*. 2019;37:907–15.
21. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2014;30:923–30.
22. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics*. 2012;16:284–7.
23. Gosselin D, et al. Environment Drives Selection and Function of Enhancers Controlling Tissue-Specific Macrophage Identities. *Cell*. 2014;159:1327–40.
24. Batty MJ, Chabrier G, Sheridan A, Gage MC. Metabolic Hormones Modulate Macrophage Inflammatory Responses. *Cancers (Basel)*. 2021;13:4661.
25. Liu S, et al. The regulatory role of FABP4 in macrophage polarization and lipid accumulation in silicosis. *Cell Signal*. 2025;135:112041.
26. Robertson IB, et al. Latent TGF-beta-binding proteins. *Matrix Biol*. 2015;47:44–53.
27. Cain SA, Woods S, Singh M, Kimber SJ, Baldock C. ADAMTS6 cleaves the large latent TGFbeta complex and increases the mechanotension of cells to activate TGFbeta. *Matrix Biol*. 2022;114:18–34.
28. Cain SA, Mularczyk EJ, Singh M, Massam-Wu T, Kielty CM. ADAMTS-10 and – 6 differentially regulate cell-cell junctions and focal adhesions. *Sci Rep*. 2016;6:35956.
29. Wareham LK, et al. Adamts10 controls transforming growth factor beta family signaling that contributes to retinal ganglion cell development. *Front Mol Biosci*. 2022;9:989851.
30. Lin R, et al. Suppression of latent transforming growth factor-beta (TGF-beta)-binding protein 1 (LTBP1) inhibits natural killer/ T cell lymphoma progression by inactivating the TGF-beta/Smad and p38(MAPK) pathways. *Exp Cell Res*. 2021;407:112790.
31. Lepparanta O, et al. Regulation of TGF-beta storage and activation in the human idiopathic pulmonary fibrosis lung. *Cell Tissue Res*. 2012;348:491–503.
32. Bodmer NK et al. (2026) Latent transforming growth factor binding protein-2 (LTBP2), an IPF biomarker of clinical decline, promotes TGF-beta signaling and lung fibrosis in mice. *Am J Respir*

33. Watanabe T, et al. A Human Skin Model Recapitulates Systemic Sclerosis Dermal Fibrosis and Identifies COL22A1 as a TGFbeta Early Response Gene that Mediates Fibroblast to Myofibroblast Transition. *Genes (Basel)*. 2019;10:75.
34. Hu H, et al. MEGF6 Promotes the Epithelial-to-Mesenchymal Transition via the TGFbeta/SMAD Signaling Pathway in Colorectal Cancer Metastasis. *Cell Physiol Biochem*. 2018;46:1895–906.
35. Nakamura I, et al. Sulfatase-2 Regulates Liver Fibrosis through the TGF-beta Signaling Pathway. *Cancers (Basel)*. 2021;13:5279.
36. Kim HS, Kim HY. Hypertensive effects of transforming growth factor-beta1 in vascular smooth muscles cells from spontaneously hypertensive rats are mediated by sulfatase 2. *Cytokine*. 2022;150:155754.
37. Yue X, Lu J, Auduong L, Sides MD, Lasky JA. Overexpression of Sulf2 in idiopathic pulmonary fibrosis. *Glycobiology*. 2013;23:709–19.
38. Akasaka E, Kleiser S, Sengle G, Bruckner-Tuderman L, Nystrom A. Diversity of Mechanisms Underlying Latent TGF-beta Activation in Recessive Dystrophic Epidermolysis Bullosa. *J Invest Dermatol*. 2021;141:1450–60.
39. Nystrom A, et al. Collagen VII plays a dual role in wound healing. *J Clin Invest*. 2013;123:3498–509.
40. Wang W, et al. Collagen XXIV (Col24alpha1) promotes osteoblastic differentiation and mineralization through TGF-beta/Smads signaling pathway. *Int J Biol Sci*. 2012;8:1310–22.
41. Wiggs JL. CPAMD8, a New Gene for Anterior Segment Dysgenesis and Childhood Glaucoma. *Ophthalmology*. 2020;127:767–8.
42. Lin J, Patel SR, Wang M, Dressler GR. The cysteine-rich domain protein KCP is a suppressor of transforming growth factor beta/activin signaling in renal epithelia. *Mol Cell Biol*. 2006;26:4577–85.
43. Soofi A, et al. The kielin/chordin-like protein (KCP) attenuates high-fat diet-induced obesity and metabolic syndrome in mice. *J Biol Chem*. 2017;292:9051–62.
44. Bossers GPL, et al. Neuregulin-1 enhances cell-cycle activity, delays cardiac fibrosis, and improves cardiac performance in rat pups with right ventricular pressure load. *J Thorac Cardiovasc Surg*. 2022;164:e493–510.
45. Takada YK, Takada Y. Neuregulin-1 (NRG1) Binds to the Allosteric Binding Site (Site 2) and Suppresses Allosteric Integrin Activation by Inflammatory Cytokines: A Potential Mechanism of Anti-Inflammatory and Anti-Fibrosis Action of NRG1. *Cells*. 2025;14:617.
46. Andrew M, et al. PAPP2 as a Therapeutic Modulator of IGF-I Bioavailability: in Vivo and in Vitro Evidence. *J Endocr Soc*. 2018;2:646–56.
47. Oxvig C, Conover CA. The Stanniocalcin-PAPP-A-IGFBP-IGF Axis. *J Clin Endocrinol Metab*. 2023;108:1624–33.
48. Peng X, et al. Plexin C1 deficiency permits synaptotagmin 7-mediated macrophage migration and enhances mammalian lung fibrosis. *FASEB J*. 2016;30:4056–70.

49. Peng Y, et al. Sema6D acts downstream of bone morphogenetic protein signalling to promote atrioventricular cushion development in mice. *Cardiovasc Res*. 2016;112:532–42.
50. Zhang L, et al. Macrophages: friend or foe in idiopathic pulmonary fibrosis? *Respir Res*. 2018;19:170.
51. Herbert DR, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity*. 2004;20:623–35.
52. Sheng J, et al. M2 macrophage-mediated interleukin-4 signalling induces myofibroblast phenotype during the progression of benign prostatic hyperplasia. *Cell Death Dis*. 2018;9:755.
53. Gieseck RL 3rd, Wilson MS, Wynn TA. Type 2 immunity in tissue repair and fibrosis. *Nat Rev Immunol*. 2018;18:62–76.
54. Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*. 2016;44:450–62.
55. Pastore M, et al. Macrophage MerTK promotes profibrogenic cross-talk with hepatic stellate cells via soluble mediators. *JHEP Rep*. 2022;4:100444.
56. Tian L, et al. Epithelial-mesenchymal Transition of Peritoneal Mesothelial Cells Is Enhanced by M2c Macrophage Polarization. *Immunol Invest*. 2022;51:301–15.
57. Ishida Y, Kuninaka Y, Mukaida N, Kondo T. (2023) Immune Mechanisms of Pulmonary Fibrosis with Bleomycin. *Int J Mol Sci* 24.
58. Kishore A, Petrek M. Roles of Macrophage Polarization and Macrophage-Derived miRNAs in Pulmonary Fibrosis. *Front Immunol*. 2021;12:678457.
59. Hong SH, et al. Loss of ATF3 exacerbates pulmonary fibrosis via enhanced neutrophil recruitment and profibrotic macrophage polarization. *Clin Sci (Lond)*. 2026;140:179–99.

Figures

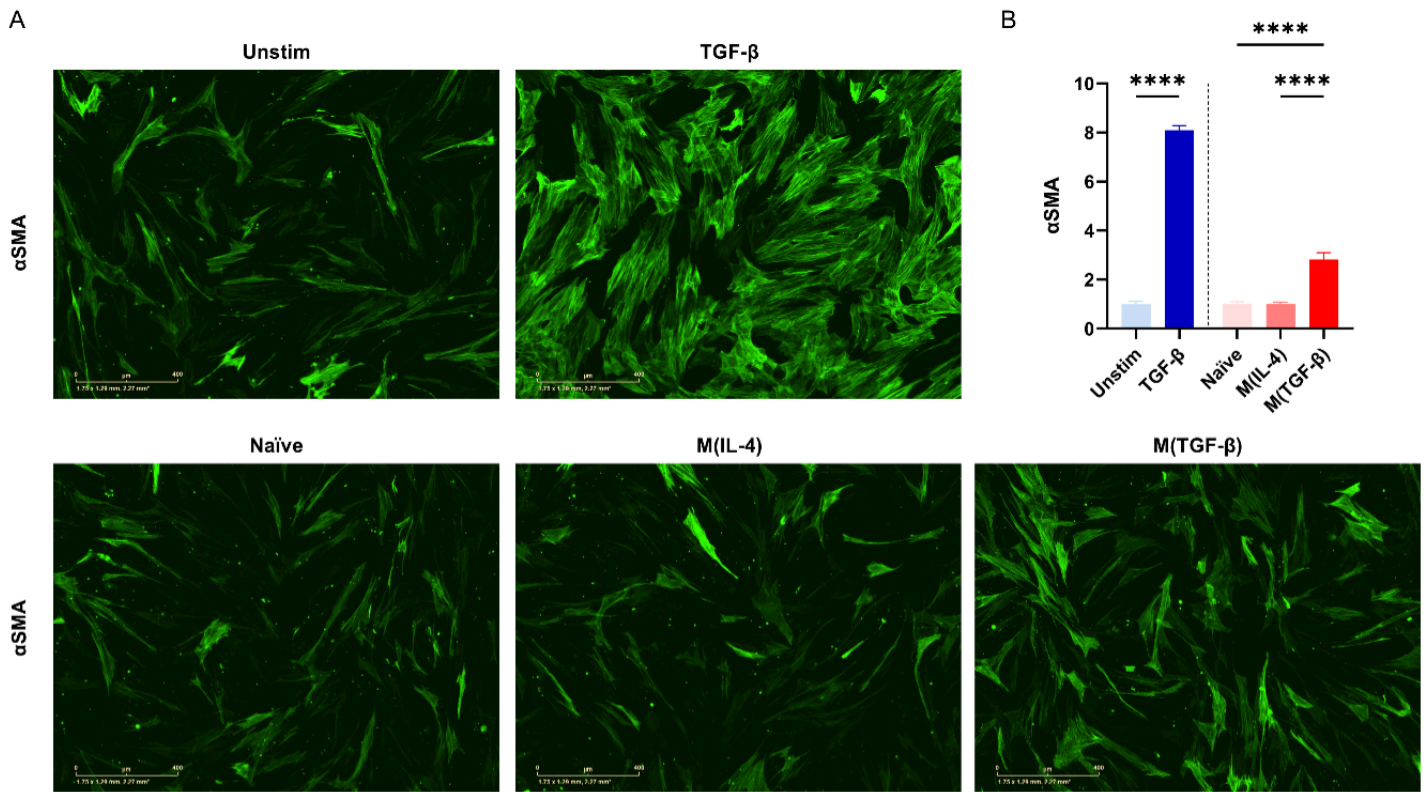


Figure 1

Coculture with TGF- β , but not IL-4 polarized macrophages derived from THP-1 cells increased α SMA levels in NHLFs. (A) Representative images of immunofluorescence staining for α SMA in NHLFs cocultured for 48h with THP-1-derived macrophages. (B) Quantification of α SMA expression in NHLFs cocultured with THP-1-derived from macrophages. Data is expressed as mean $\pm$ standard error of the mean (SEM). Quantification of the fluorescent signal is corrected for confluence. Direct TGF- β stimulation of NHLFs, which served as positive control, is shown relative to unstimulated fibroblasts. M(IL-4) and M(TGF- β) stimulations are shown relative to naïve. n=6 for all conditions. Statistical significance was tested using one-way ANOVA. p-values: ** <0.01, *** <0.001 and **** <0.0001.

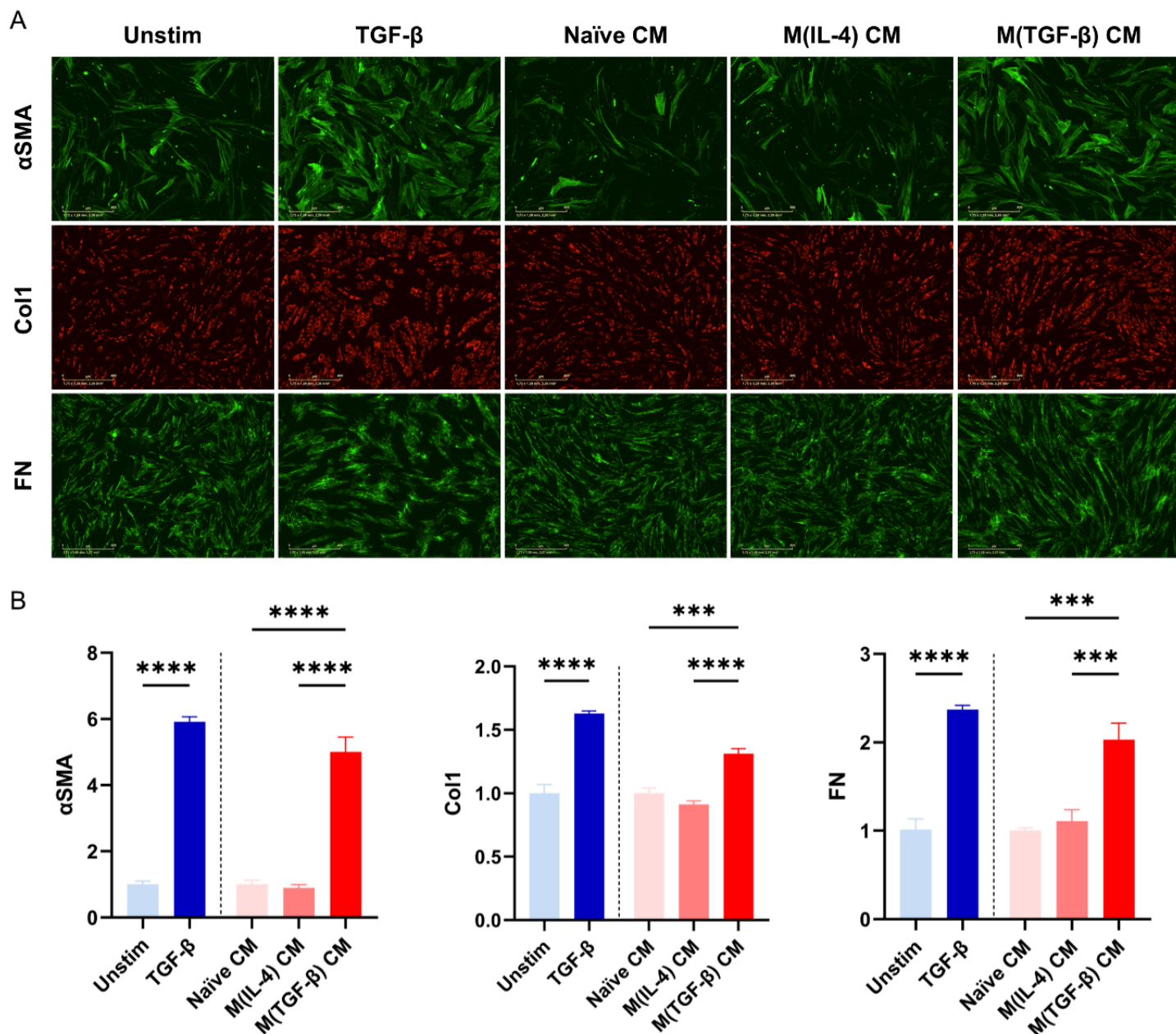


Figure 2

Conditioned medium from TGF- β , but not IL-4 polarized macrophages derived from THP-1 cells increased α SMA, Col1 or FN levels in NHLFs. (A) Representative images of immunofluorescence staining for α SMA, Col1 and FN in NHLFs stimulated with conditioned medium (CM) from THP-1 derived macrophages. (B) Quantification of α SMA, Col1 and FN expression in NHLFs stimulated with conditioned medium from THP-1 derived macrophages. Data is expressed as mean $\pm$ standard error of the mean (SEM). Quantification of the fluorescent signal is corrected for confluence. Direct TGF- β stimulation of NHLFs, which served as positive control, is shown relative to unstimulated fibroblasts. M(IL-4) CM and M(TGF- β) CM stimulations are shown relative to naïve CM stimulation. $n=3$ for all conditions. Statistical significance was tested using one-way ANOVA. p -values: *** <0.001 and **** <0.0001 .

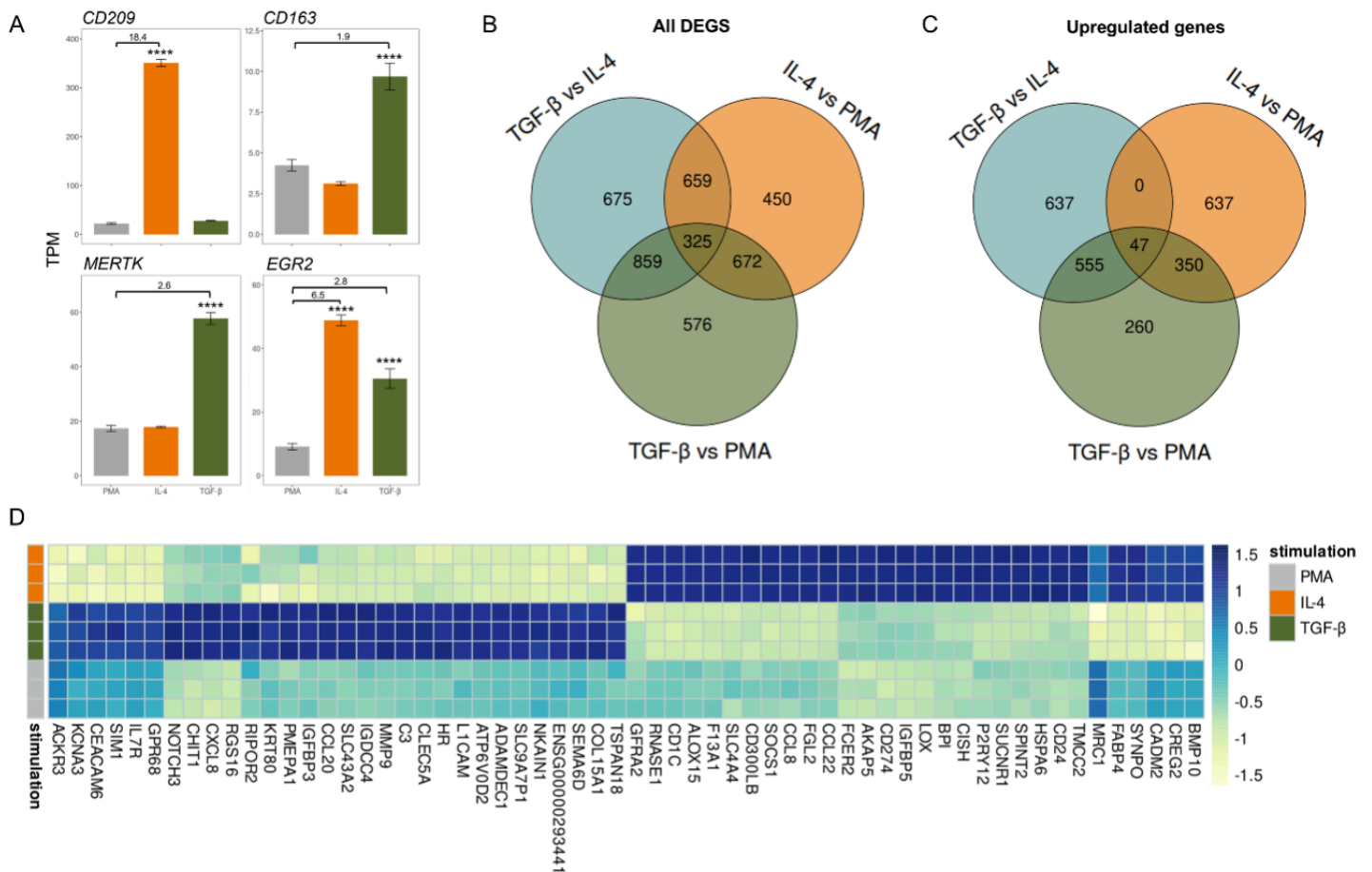


Figure 3

Overview of RNA sequencing results comparing TGF- β , IL-4 and PMA differentiated macrophages. (A) Transcripts per million (TPM) values of *CD163* and *MERTK* (phenotypic markers of TGF- β polarized macrophages), *CD209* and *EGR2* (phenotypic markers of IL-4 polarized macrophages) in the different macrophage subtypes. Numbers indicate fold change in the stimulated condition compared to PMA (Wald test). ****FDR<0.000001 (Benjamini-Hochberg correction). (B,C) Number of all differentially expressed genes (DEGs) (B) or upregulated genes (C) identified in the three different comparisons. (D) Heatmap showing the top 30 upregulated and top 30 downregulated genes in TGF- β differentiated macrophages compared to IL-4 differentiated macrophages. n=3 for all conditions. For panels B, C and D, DEGs: $\text{abs}(\log_2\text{FC}) \geq 1$ and $p_{\text{adjust}} \leq 0.05$.

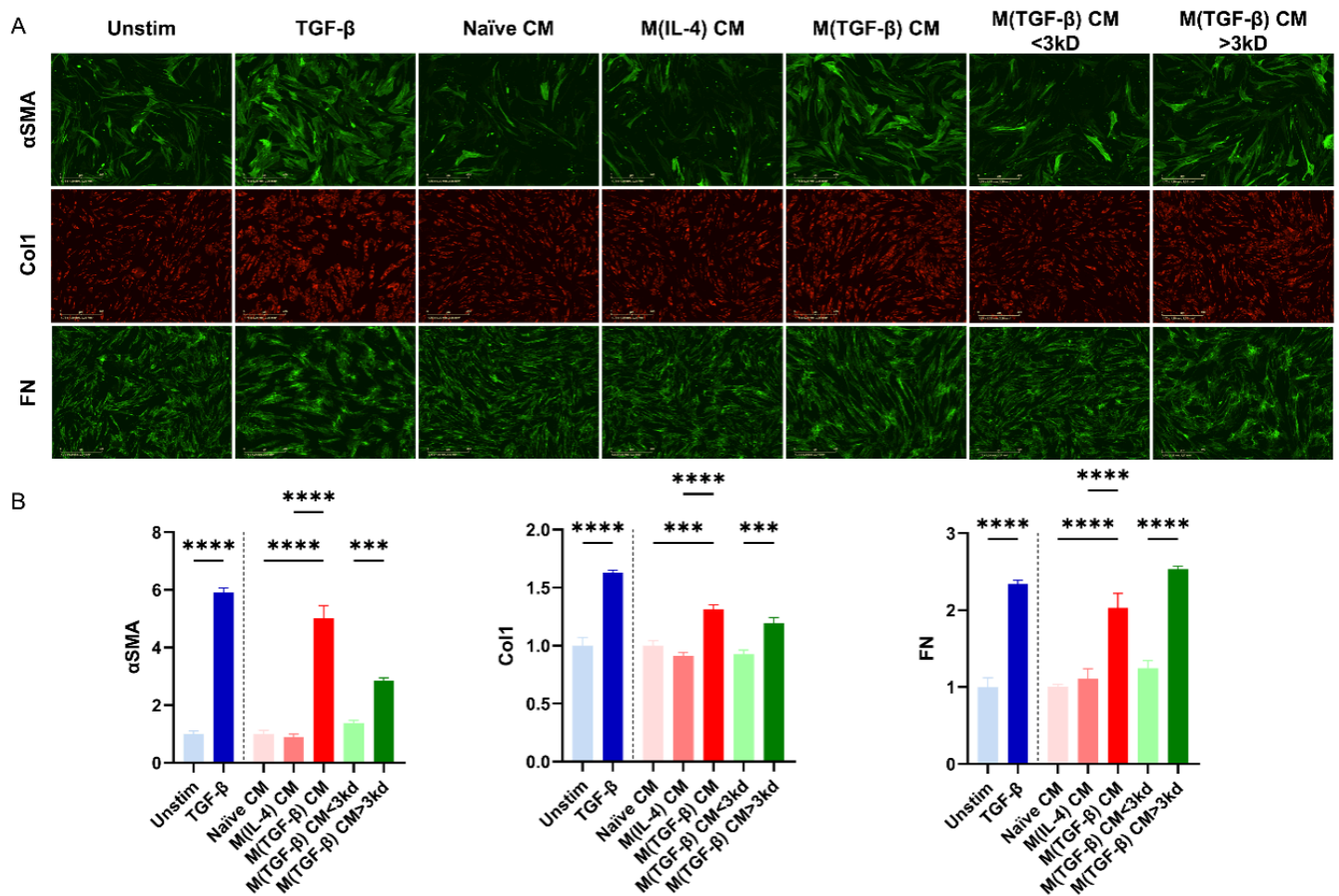


Figure 4

Molecular weight of potential profibrotic proteins secreted by TGF-β, but not IL-4, polarized macrophages is larger than 3 kD. (A) Representative images of immunofluorescence staining for αSMA, Col1 and FN in NHLFs 48h after stimulation with different conditioned medium (CM) fractions containing proteins smaller or larger than 3 kD. (B) Quantification of αSMA, Col1 and FN expression in NHLFs stimulated with separated conditioned medium fractions containing proteins smaller or larger than 3 kD. Quantification of the fluorescent signal is corrected for confluence. Direct TGF-β stimulation of NHLFs, which served as positive control, is shown relative to unstimulated fibroblasts. M(IL-4) CM, M(TGF-β) CM, M(TGF-β) CM <3 kD and M(TGF-β) CM >3 kD stimulations are shown relative to naïve CM stimulation. n=3 for all conditions. Statistical significance was tested using one-way ANOVA. p-values: *** <0.001 and **** <0.0001.

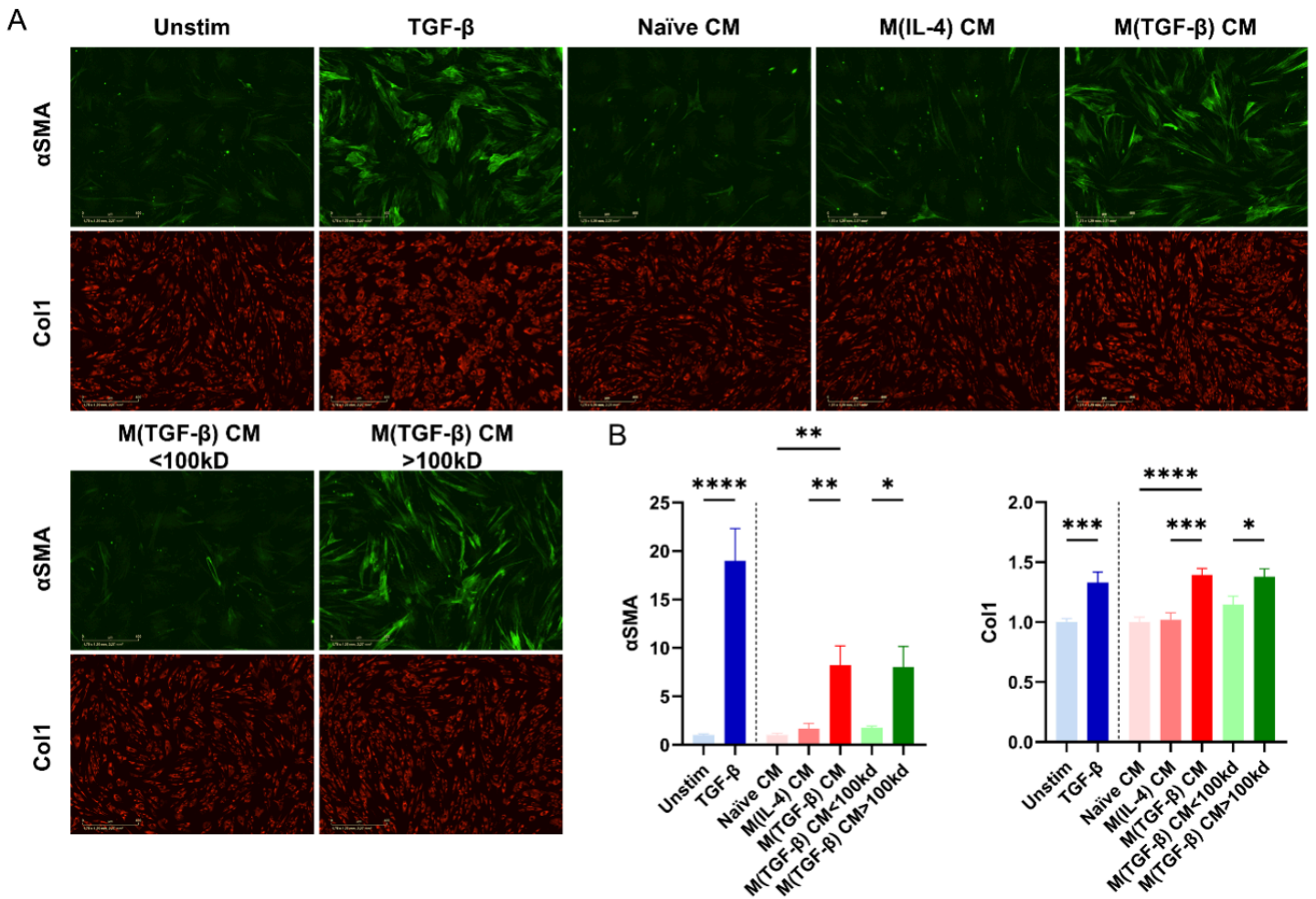


Figure 5

Molecular weight of potential profibrotic proteins secreted by TGF β , but not IL-4, polarized macrophages are larger than 100 kD. (A) Representative images of immunofluorescence staining for α SMA, Col1 and FN in NHLFs 48h after stimulation with different conditioned medium (CM) fractions containing proteins smaller or larger than 100 kD. (B) Quantification of α SMA, Col1 and FN expression in NHLFs stimulated with separated conditioned medium fractions containing proteins smaller or larger than 100 kD. Quantification of the fluorescent signal is corrected for confluence. Direct TGF- β stimulation of NHLFs, which served as positive control, is shown relative to unstimulated fibroblasts. M(IL-4) CM, M(TGF- β) CM, M(TGF- β) CM <100 kD and M(TGF- β) CM >100 kD stimulations are shown relative to naïve CM stimulation. n=6 for all conditions. Statistical significance was tested using one-way ANOVA. p-values: * <0.05, *** <0.001 and **** <0.0001.

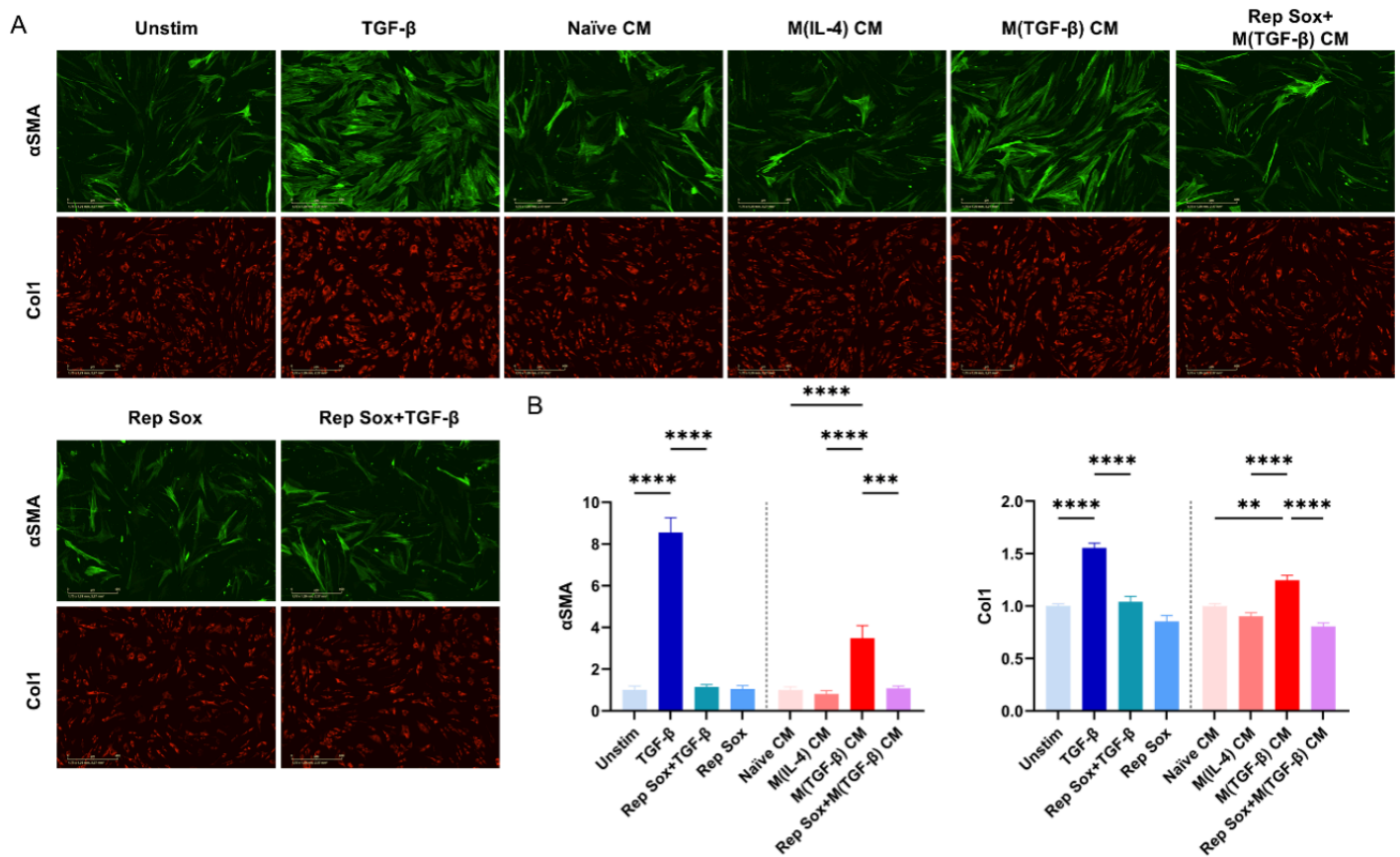


Figure 6

The profibrotic effect of TGF-β polarized macrophage is mediated through activation of the TGF-β signaling pathway in NHLFs. (A) Representative images of immunofluorescence staining for αSMA and Col1 in NHLFs stimulated with conditioned medium (CM) pretreated with or without 1μM RepSox (TGF-β type 1 receptor inhibitor). (B) Quantification of αSMA and Col1 in NHLFs stimulated with conditioned medium (CM) pretreated with or without 1μM RepSox. Data is expressed as mean ± standard error of the mean (SEM). Quantification of the fluorescent signal is corrected for confluence. Direct TGF-β, which served as positive control, RepSox and RepSox + TGF-β (control for RepSox dependent inhibition of TGF-β signaling) stimulations of NHLFs are shown relative to unstimulated fibroblasts. M(IL-4) CM, M(TGF-β) CM, RepSox + M(TGF-β) CM stimulations are shown relative to naïve CM stimulation. n=6 for all conditions. Statistical significance was tested using one-way ANOVA. p-values: *** <0.001 and **** <0.0001.

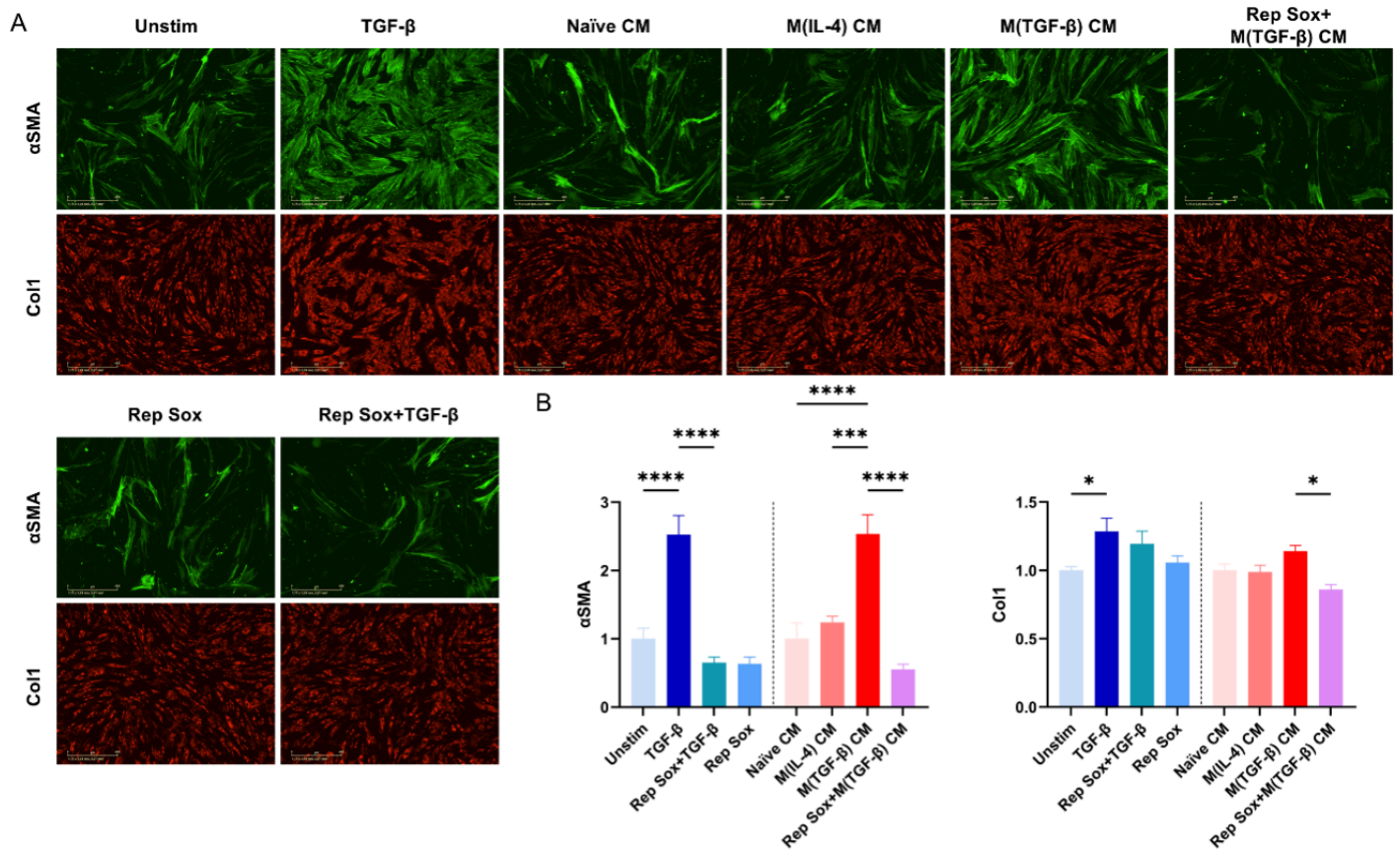


Figure 7

Conditioned medium from TGF- β , but not IL-4, polarized macrophages derived from primary monocytes increased α SMA levels through activation of the TGF- β signaling pathway in NHLFs. (A) Representative images of immunofluorescence staining for α SMA and Col1 in NHLFs stimulated with conditioned medium (CM) from primary monocyte-derived macrophages. (B) Quantification of α SMA and Col1 expression in NHLFs stimulated with conditioned medium (CM) from primary monocyte-derived macrophages. Data is expressed as mean $\pm$ standard error of the mean (SEM). Quantification of the fluorescent signal is corrected for confluence. Direct TGF- β , which served as positive control, RepSox and RepSox + TGF- β (control for RepSox dependent inhibition of TGF- β signaling) stimulations of NHLFs are shown relative to unstimulated fibroblasts. M(IL-4) CM, M(TGF- β) CM and RepSox + M(TGF- β) CM stimulations are shown relative to naïve CM stimulation. $n=4$ for all conditions. Statistical significance was tested using one-way ANOVA. p-values: * <0.05 , *** <0.001 and **** <0.0001 .

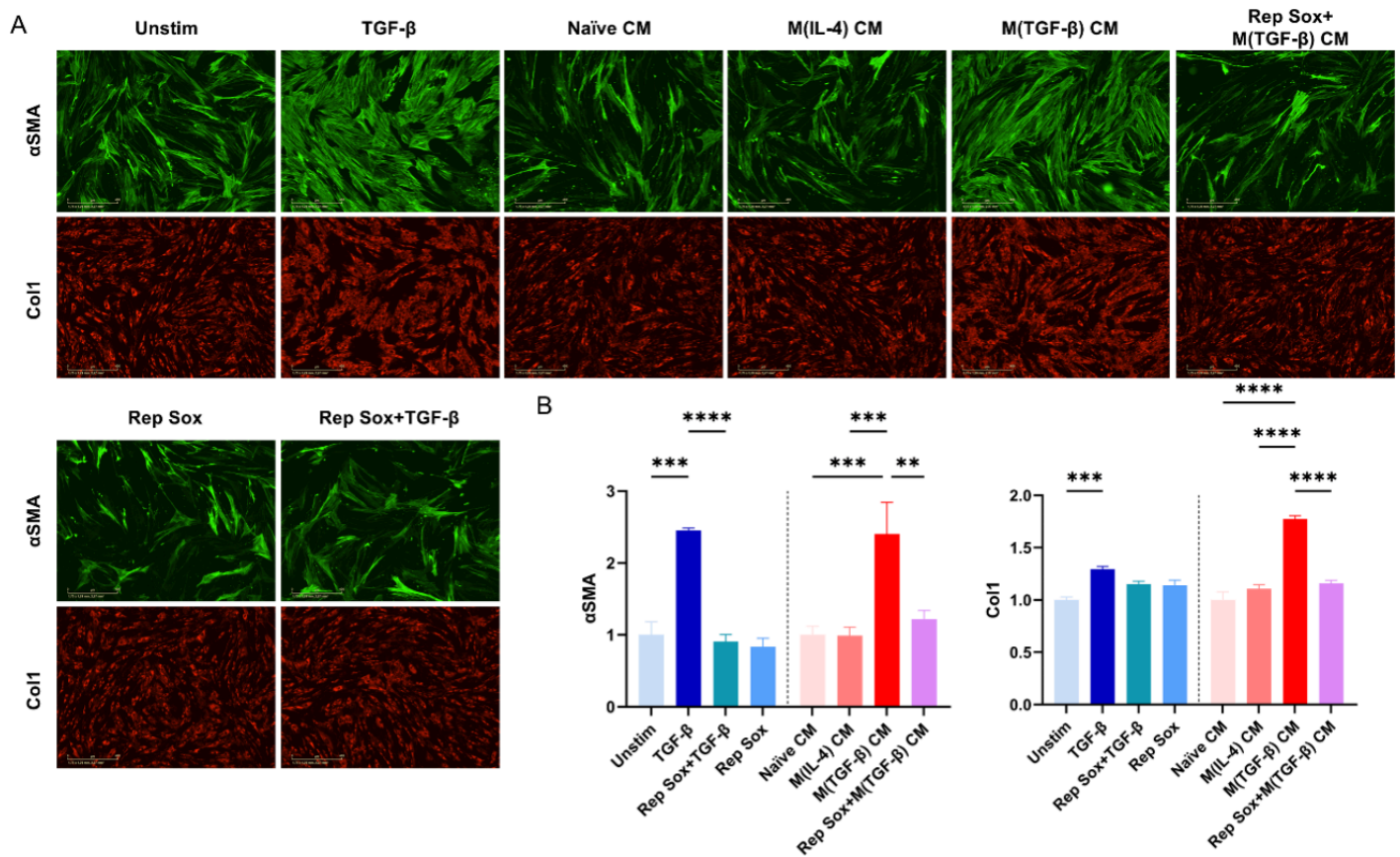


Figure 8

Conditioned medium from TGF- β , but not IL-4, polarized macrophages derived from BlaER1 increased α SMA and Col1 levels through activation of the TGF- β signaling pathway in NHLFs. (A) Representative images of immunofluorescence staining for α SMA and Col1 in NHLFs stimulated with conditioned medium (CM) from BlaER1 derived macrophages. (B) Quantification of α SMA and Col1 expression in NHLFs stimulated with conditioned medium (CM) from BlaER1 derived macrophages. Data is expressed as mean $\pm$ standard error of the mean (SEM). Quantification of the fluorescent signal is corrected for confluence. Direct TGF- β , which served as positive control, RepSox and RepSox + TGF- β (control for RepSox dependent inhibition of TGF- β signaling) stimulations of NHLFs are shown relative to unstimulated fibroblasts. M(IL-4) CM, M(TGF- β) CM and RepSox + M(TGF- β) CM stimulations are shown relative to naïve CM stimulation. n=4 for all conditions. Statistical significance was tested using one-way ANOVA. p-values: ** <0.01, *** <0.001 and **** <0.0001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.zip](#)