Activin A and CCR2 regulate macrophage function in testicular fibrosis caused by experimental autoimmune orchitis

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Abstract

Experimental autoimmune-orchitis (EAO), a rodent model of chronic testicular inflammation and fibrosis, replicates pathogenic changes seen in some cases of human spermatogenic disturbances. During EAO, increased levels of pro-inflammatory and pro-fibrotic mediators such as TNF, CCL2, and activin A are accompanied by infiltration of leukocytes into the testicular parenchyma. Activin A levels correlate with EAO severity, while elevated CCL2 acting through its receptor CCR2 mediates leukocyte trafficking and recruits macrophages. CCR2 + CXCR4 + macrophages producing extracellular matrix proteins contribute widely to fibrogenesis. Furthermore, testicular macrophages (TMs) play a critical role in organ homeostasis. Therefore, we aimed to investigate the role of the activin A/CCL2-CCR2/macrophage axis in the development of testicular fibrosis. Following EAO induction, we observed lower levels of organ damage, collagen deposition, and leukocyte infiltration (including fibronectin+, collagen I+ and CXCR4+ TMs) in Ccr2−/− mice than in WT mice. Furthermore, levels of Il-10, Ccl2, and the activin A subunit Inhba mRNAs were lower in Ccr2−/− EAO testes. Notably, fibronectin+ TMs were also present in biopsies from patients with impaired spermatogenesis and fibrotic alterations. Overexpression of the activin A antagonist follistatin reduced tissue damage and collagen I+ TM accumulation in WT EAO testes, while treating macrophages with activin A in vitro increased the expression of Ccr2, Fn1, Cxcr4, and Mmp2 and enhanced migration along a CCL2 gradient; these effects were abolished by follistatin. Taken together, our data indicate that CCR2 and activin A promote fibrosis during testicular inflammation by regulating macrophage function. Inhibition of CCR2 or activin A protects against damage progression, offering a promising avenue for therapeutic intervention.

Keywords Testicular inflammation · Fibrosis · EAO · Macrophages · CCR2 · Activin A · CXCR4 · MMP2
Abbreviations

αSMA α-Smooth muscle actin
BMDM Bone marrow-derived macrophage
CCL2 C–C motif chemokine ligand 2
CCR2 C–C motif chemokine receptor type 2
DEGs Differentially expressed genes
EAO Experimental autoimmune orchitis
ECM Extracellular matrix
FST Follistatin
IL Interleukin
MMP Matrix metalloproteinase
PDGF Platelet-derived growth factor
PTC Peritubular cell
SC Sertoli cell
SCCM Sertoli cell-conditioned medium
TGF-β Transforming growth factor-β
TIMP Tissue inhibitor of metalloproteinase
TM Testicular macrophages
TNF Tumor necrosis factor
WT Wild type

Introduction

Approximately 8–12% of couples worldwide suffer from infertility. Male factors account for ~ 50% of cases, while testicular inflammation induced by bacteria, viruses, or sterile inflammation (including autoimmune diseases) is a significant cause of male infertility [1–4]. Experimental autoimmune orchitis (EAO), a rodent model of chronic testicular inflammation, mimics the pathological changes observed in human testicular biopsies from infertile patients [5–7]. In the mouse, EAO leads to fibrosis and subsequent infertility [6, 8]. Pathological features of EAO include the destruction of the testicular structure, infiltration of the interstitium by leukocytes, elevated levels of pro-inflammatory cytokines (including C–C motif chemokine ligand 2 [CCL2], tumor necrosis factor [TNF], and activin A), as well as the loss of germ cells. Furthermore, levels of C–C motif chemokine receptor type 2 (CCR2), the receptor for CCL2, are significantly increased in interstitial mononuclear cells in EAO testis [9]. Testicular macrophages (TMs) constitute the major immune cell population in the testis and play a critical role in supporting steroidogenesis, promoting spermatogenesis, and possibly also maintaining immune privilege [10, 11]. During testicular inflammation, numbers of infiltrating monocytes and macrophages are increased, while a reduction in TMs correlates with decreased incidence and severity of testicular damage [12–14].

Fibrosis is a hallmark of progressive and severe EAO, characterized by the accumulation of extracellular matrix (ECM) proteins such as collagen and fibronectin and thickening of the α-smooth muscle actin (αSMA)-positive peritubular cell (PTC) layer of the seminiferous tubules [13]. Studies in other organs have indicated that ECM-producing cells may originate not only from resident mesenchymal fibroblasts but also from circulating hematopoietic cells or cells undergoing epithelial/endothelial-to-mesenchymal transition [15]. Monocytes and macrophages that are resident or recruited to injury sites produce a variety of factors, including transforming growth factor-β (TGF-β), platelet-derived growth factors (PDGFs), and matrix metalloproteinases (MMPs). These proteins stimulate the proliferation, differentiation, and activation of fibroblasts, thereby promoting fibrotic remodelling [16, 17]. Tissue inhibitors of metalloproteinases (TIMPs) also play a role in fibrosis by inhibiting MMPs, thereby suppressing ECM proteolysis, TGF-β release, and neutrophil chemotaxis [18].

Activin A, a homodimeric member of the TGF-β superfamily of cytokines, is produced mainly by Sertoli cells (SCs) in the normal postnatal testes and plays multiple biological roles in inflammation, immunity, and fibrosis [19, 20]. In EAO, serum and testicular activin A levels are increased [13, 21]. Activin A exerts both pro- and anti-inflammatory effects and stimulates monocytes and macrophages to produce many inflammatory mediators, including interleukin (IL)-1β, IL-6, and TNF [22, 23]. Furthermore, activin A promotes pro-fibrotic gene expression in many cell types, including PTCs and NIH 3T3 fibroblasts [24]. Activin A also drives the chemotactic migration and adhesion of L929 fibroblasts in vitro [25]. Activin activity is inhibited by follistatin (FST), an endogenous high-affinity activin A binding antagonist [26].

CCL2 and CCR2 play a critical role in the trafficking of lymphocytes, monocytes, macrophages, and bone marrow-derived fibroblasts to sites of injury [27, 28]. In addition to mediating chemotaxis, CCL2 stimulates the production of collagens by fibroblasts and TGF-β by macrophages [29]. CCR2+ cells also contribute to fibrogenesis by producing TIMP1, which inhibits collagen degradation [30]. In the EAO model, increased levels of CCL2 protein were observed in mononuclear and endothelial cells, Leydig cells, and PTCs as well as in testicular fluid and conditioned medium from cultured TMs; furthermore, elevated expression of CCR2 was identified in mononuclear cells [9]. Of note, stimulation of Sertoli cells with TNF led to upregulated levels of Ccl2 and activin A (Inhba) mRNA expression [24, 31].

At present, the role of the activin A/CCL2-CCR2/macrophage axis in this inflammatory and fibrotic response in the testis is unknown. We hypothesized that the interaction of activin A and CCR2 may influence the development of testicular fibrosis by regulating the properties of macrophages, a potentially important source of pro-fibrotic factors during orchitis. To verify our hypothesis, we compared the progress of EAO in wild type (WT) and Ccr2−/− mutant...
mice, which lack peripheral blood monocytes, as well as in mice overexpressing follistatin, which display reduced activin bioactivity. Furthermore, we investigated the influence of activin A on CCR2 and fibrotic mediator expression in macrophages, using bone marrow-derived macrophages (BMDMs) matured in the presence of macrophage-colony stimulating factor (M-CSF) as a surrogate for TMs.

Materials and methods

Animals

In this study, 10- to 12-week-old WT C57BL/6J (Charles River Laboratories, Sulzfeld, Germany) and B6.129P2-Ccr2tm1Mae/tm1Mae (Ccr2−/−) mice were housed in specific pathogen-free conditions (12 h light/dark cycle, 20–22 °C) at the animal facility of Justus Liebig University, Giessen, Germany [32]. The mice had access to water and food ad libitum. Femurs and tibias were collected from male adult WT and Ccr2−/− mice for the isolation of bone marrow progenitor cells, while testes were obtained from 21-day-old immature WT mice for the isolation of SCs.

Animal experiments were approved by the responsible local ethics committees on animal care (Regierungspräsidium Giessen GI 58/2014—Nr. 735-GP and the Monash Medical Centre Animal Experimentation Committee). All experiments involving animals were carried out in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the German animal welfare law and the Australian Code for the Care and Use of Animals for Scientific Purposes.

Induction of EAO in WT and Ccr2−/− mice

Peri-procedural analgesia in mice was provided by supplying tramadol (STADApharm GmbH, Bad Vilbel, Germany) in drinking water (2.5 mg/ml), commencing 24 h before each immunization and continuing for the following 3 days.

For the induction of EAO, adult male C57BL/6 J and Ccr2−/− mice (n = 29) were first anesthetized by administration of 3–5% isoflurane and then actively immunized with testicular homogenate (TH) in complete Freund’s adjuvant (CFA; Sigma-Aldrich, St. Louis, USA), as previously described [13]. The homogenate was prepared from decapsulated testes collected from adult WT mice and homogenized in sterile 0.9% NaCl at a ratio of 1:1. Animals were immunized dorsally three times every 14 days with a mixture of TH in CFA; a total volume of 200 μl was delivered as four subcutaneous injections (50 μl per injection site). This was followed by intraperitoneal injection of 100 ng Bordetella pertussis toxin (Calbiochem, Darmstadt, Germany) in 100 μl Munoz Buffer (25 mM Tris, 0.5 M NaCl, 0.017% Triton X-100, pH 7.6) [33].

Adjuvant control animals (n = 22) received CFA mixed with 0.9% NaCl instead of TH, following the same injection regimen. Age-matched untreated mice (n = 21) were also included in the study. Animals were sacrificed by cervical dislocation 50 days after the first immunization (following deep anesthesia induced with 5% isoflurane). A schematic diagram illustrating the time course of immunizations and time points for organ collection is shown in Fig. 1A. The testes were removed, weighed, and either snap-frozen in liquid nitrogen or fixed in Bouin’s solution for embedding in paraffin. Freshly collected testes were used for flow cytometric analysis.

Induction of EAO in mice with elevated follistatin levels

Thirty days before the first immunization with TH, C57BL/6J mice were injected intramuscularly with non-replicative recombinant adeno-associated viral (rAAV) vector serotype 6 carrying a gene cassette of the circulating form of follistatin (rAAV-FST315; n = 13) or an empty gene cassette (empty vector; EV; n = 18), as previously described [21]. Adjuvant-only and non-immunized controls were included. The immunization protocol was modified according to Monash University Animal Ethics committee requirements regarding the use of adjuvants: mice were immunized once with TH in CFA, followed by two further immunizations with TH in incomplete Freund’s adjuvant with B. pertussis toxin. Approximately 75% of mice immunized by this protocol developed EAO, ranging from mild to severe, within 50 days. Treatment with rAAV-FST315 resulted in a five-fold increase in serum follistatin levels by the time of the first immunization [21]. After collection, testes were either snap-frozen in liquid nitrogen for RNA analysis or fixed in Bouin’s solution for paraffin embedding and subsequent immunofluorescence [21]. A schematic diagram illustrating the time course of vector injection, immunizations, and time points for organ collection is shown in Fig. 4A.

Human testicular biopsies

Bouin’s-fixed paraffin-embedded testicular tissue samples obtained by routine open testicular biopsy (using the testicular sperm extraction [TESE] protocol) were provided by the University Hospital Zagreb, (Department of Urology, in cooperation with the School of Medicine) at the University of Zagreb. According to current guidelines, three pieces of tissue were obtained through corresponding incisions of the tunica albuginea (upper and lower testis pole and equatorial plane). Biopsied tissues were divided into two parts to provide an accurate diagnosis: one half for cryopreservation.
and the other half for histological analysis [34, 35]. This study was performed in line with the principles of the Declaration of Helsinki. Each patient provided written consent for the TESE procedure as well as the histological analysis in the current study. Approval was granted by the Ethics Committee of the School of Medicine at the University of Zagreb (380-59-10106-20-111/171). The specimens (selected from infertile men with non-obstructive azoospermia) showed histological diagnoses of focal inflammatory lesions associated with disturbed spermatogenesis (mixed atrophy or Sertoli cell-only phenotype) and fibrotic remodeling (thickening of the lamina propria, interstitial accumulation of collagen;

Fig. 1 Tissue damage and collagen deposition caused by EAO is reduced in Ccr2−/− mouse testes. Schematic diagram representing the time points for EAO induction and organ collection in C57BL/6J (WT) and B6.129P2-Ccr2tm1Mae/tm1Mae (Ccr2−/−) mice. Mice were immunized three times every other week starting at day 0, and testes were collected at day 50 (A). Paired testis weight (B; n=20–30) and total collagen content determined by hydroxyproline assay (C; n=5–6) in untreated, adjuvant control, and EAO testes from WT and Ccr2−/− mice. Representative photomicrographs of picro-sirius red-stained paraffin sections (D) of untreated (I, IV), adjuvant control (II, V), and EAO (III, VI) testes from WT (I–III) and Ccr2−/− mice (IV–VI); asterisk in (VI) indicates seminiferous tubule damage. Values are mean ± SEM; statistical analyses used the Kruskal–Wallis test followed by Dunn’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001
tubular fibrosis; \( n = 5 \)). The control group comprised three patients suffering from obstructive azoospermia with fully preserved testicular parenchyma, revealing normal intact spermatogenesis without inflammatory signs.

**Picro-sirius red staining**

Testicular histology and collagen distribution were assessed in paraffin sections by picro-sirius red staining [36].

**Hydroxyproline assay**

Total testicular collagen content was quantified by chromogenic determination of hydroxyproline concentration using the QuickZyme Total Collagen Assay Kit (QuickZyme Biosciences, Leiden, Netherlands) according to the manufacturer’s instructions. Total testicular collagen content was normalized to total testicular protein using the QuickZyme Total Protein Assay Kit (QuickZyme Biosciences).

**Isolation and treatment of Sertoli cells**

SCs were isolated from 21-day-old \( C57BL/6 J \) mice (8 animals/isolation) according to an established protocol [24]. Two days after isolation, SCs were treated with 20 mM hypotonic Tris-hydrochloride solution (Sigma-Aldrich) for 2 min to remove germ cell contamination. One day after hypotonic shock, SCs were treated with 50 ng/ml recombinant mouse TNF (PromoCell, Heidelberg, Germany), 250 ng/ml human recombinant follistatin 288 (FST288; purified from HEK-293 cells transfected with a human FST transgene), or a combination of both [37]. After 24 h in culture, Sertoli cell-conditioned medium (SCCM) was collected. The purity of SCs (> 85%) was determined by double staining for SOX9 (SC marker) and \( \alpha \)SMA (PTC marker) [24].

**Generation and treatment of BMDMs**

Bone marrow progenitor cells were isolated from adult \( C57BL/6 J \) and \( Ccr2^{-/-} \) mice (\( n = 28 \)) according to a published protocol [38] and cultured in a complete RPMI-1640 medium (Gibco). BMDMs were generated by treating the progenitor cells with 50 ng/ml mouse recombinant (M-CSF (Miltenyi Biotec) and then stimulating with 25 or 50 ng/ml human recombinant activin A (Miltenyi Biotec), 250 ng/ml FST288, or a combination of both for 6 days.

For gelatin zymography, after 6 days in culture, BMDMs were treated with 50 ng/ml M-CSF or 50 ng/ml activin A or 250 ng/ml FST288 or a combination of both activin A and FST288 in serum-free RPMI-1640 medium. BMDM-conditioned medium was collected 24 h later.

For SC-conditioned medium (SSCM) treatment, BMDMs were generated as above (50 ng/ml M-CSF for 3 days) and subsequently incubated with SCCM for an additional 3 days.

**Flow cytometry**

Testes were decapsulated and digested in phosphate-buffered saline (PBS; Gibco, Bleiswijk, Netherlands) containing 1.2 mg/ml collagenase A (Roche Diagnostic, Mannheim, Germany) and 15 U/ml DNase I (Roche Diagnostic) in a 34 °C water bath for 15 min, with agitation. After the sedimentation of the seminiferous tubules, supernatants containing interstitial cells were collected. BMDMs growing in Nunc UpCell 6-well Multidishes (Thermo Fisher Scientific, Roskilde, Denmark) were detached at room temperature (RT) for 30 min. All incubation steps were performed at 4 °C. A total of 1 × 10⁶ cells were blocked with mouse FcR blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 10 min, followed by incubation with the appropriate antibodies (see Table 1 for details) for 15 min. Afterwards, cells were permeabilized using the Fix/Permeabilization Staining Buffer Set (Miltenyi Biotec) for 30 min and blocked again. Subsequently, the cells were incubated with anti-fibronectin or anti-collagen I antibody for 45 min and the respective secondary antibodies for 30 min at 4 °C. Data were collected using the MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec) and analyzed using FlowJo V10 software (FlowJo LLC, Oregon, USA). The flow cytometry gating strategy is shown in Supplementary Fig. S1.

**Immunofluorescence**

Frozen testicular sections (10 μm) or fresh BMDMs were fixed in ice-cold methanol for 10 min. BMDMs were permeabilized using 0.5 M glycine for 30 min at RT. Subsequently, specimens were blocked in 5% bovine serum albumin (BSA; Sigma-Aldrich, Steinheim, Germany) and 10% goat serum (BioLegend, San Diego, USA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) at RT for 2 h. After incubating with the appropriate primary antibodies overnight (see Table 2 for details), the sections were washed with TBST and incubated with the corresponding secondary antibodies at RT for 1 h. Nuclei were counterstained with Topro 3 (Life Technologies, Darmstadt, Germany). Finally, the slides were mounted in ProLong™ Gold Antifade Mountant containing DAPI (Invitrogen, Oregon, USA). Fluorescence images were captured on a confocal laser scanning microscope 710 (Carl Zeiss, Göttingen, Germany).

Paraffin-embedded testicular sections (8 μm) were deparaffinized in xylene twice for 10 min and rehydrated by immersing in a series of ethanol solutions of gradually decreasing concentrations. Afterwards, sections were treated with pre-warmed proteinase K solution (20 μg/ml;
### Table 1
List of antibodies and isotype control antibodies used for flow cytometric analysis

| Antibodies                                      | Companies                                      | Catalog No    | Clones       | Dilution |
|------------------------------------------------|-----------------------------------------------|---------------|--------------|----------|
| Recombinant monoclonal anti-CD45 VioGreen REAfinity™ | Miltenyi Biotec, Bergisch-Gladbach, Germany   | 130-110-803   | REA737       | 1:50     |
| Rat monoclonal anti-CD45 PE                    | BioLegend, San Diego, USA                     | 103106        | 30-F11       | 1:100    |
| Rabbit polyclonal anti-fibronectin             | Abcam, Cambridge, UK                          | ab2413        | –            | 1:100    |
| Rabbit polyclonal anti-collagen I, biotin      | Rockland, Limerick, USA                       | 600-406-103   | –            | 1:200    |
| Recombinant monoclonal anti-CXCR4 PE REAfinity™ | Miltenyi Biotec                              | 130-118-682   | REA107       | 1:50     |
| Rat monoclonal anti-Ly6G APC                   | BioLegend                                    | 127614        | IA8          | 1:100    |
| Rat monoclonal anti-Ly6C PerCP                 | BioLegend                                    | 128027        | HK1.4        | 1:80     |
| Rat monoclonal anti-Ly6C VioGreen              | BioLegend                                    | 130-102-207   | G7.10        | 1:50     |
| Recombinant monoclonal anti-CXCR4 PE-Vio770 REAfinity™ | Miltenyi Biotec                            | 130-110-840   | REA754       | 1:50     |
| Recombinant monoclonal anti-F4/80 APC REAfinity™ | Miltenyi Biotec                             | 130-116-547   | REA126       | 1:50     |
| Recombinant monoclonal anti-CXCR4 PE-Vio770 REAfinity™ | Miltenyi Biotec                          | 130-113-803   | REA592       | 1:50     |
| Rat monoclonal anti-CD11b PerCP/Cy5.5          | BioLegend                                    | 101228        | M1/70        | 1:80     |
| Rat monoclonal anti-CD64 PerCP/Cy7             | BioLegend                                    | 139314        | X54-5/7.1    | 1:40     |
| Rat monoclonal anti-CCR2 FITC                  | BioLegend                                    | 150608        | SA203G11     | 1:50     |
| Goat anti-rabbit Alexa Fluor 488               | Invitrogen, Oregon, USA                      | A-11034       | –            | 1:500    |
| Streptavidin                                    | BioLegend                                    | 405235        | –            | 1:1000   |
| Recombinant monoclonal REA control VioGreen    | Miltenyi Biotec                              | 130-104-624   | REA293       | 1:50     |
| Recombinant monoclonal REA control PE          | Miltenyi Biotec                              | 130-104-628   | REA293       | 1:50     |
| Recombinant monoclonal REA control PE-Vio770   | Miltenyi Biotec                              | 130-104-632   | REA293       | 1:50     |
| Recombinant monoclonal REA control APC         | Miltenyi Biotec                              | 130-104-630   | REA293       | 1:50     |
| Recombinant monoclonal REA control APC-Vio770  | Miltenyi Biotec                              | 130-104-634   | REA293       | 1:50     |

### Table 2
List of primary and secondary antibodies used in immunofluorescence staining (IF) and western blotting (WB)

| Antibodies                                      | Company                                      | Catalog No    | Dilution          |
|------------------------------------------------|----------------------------------------------|---------------|-------------------|
| **Primary antibodies**                         |                                              |               |                   |
| Rabbit polyclonal anti-fibronectin; IF         | Abcam                                        | ab2413        | 1:300 (Testis)    |
|                                                 |                                               |               | 1:200 (BMDM)      |
| Rabbit monoclonal anti-collagen I; IF          | Abcam                                        | ab21286       | 1:300 (Frozen)    |
|                                                 |                                               |               | 1:100 (Paraffin)  |
| Rat monoclonal anti-CXCR4 Alexa Fluor 647; IF | BioLegend                                    | 146,503       | 1:100             |
| Rat monoclonal anti-F4/80; IF                  | Bio-Rad, Munich, Germany                     | MCA497G       | 1:200 (Frozen)    |
| Rat monoclonal anti-F4/80; IF                  | BioLegend                                    | 123,101       | 1:50 (Paraffin)   |
| Mouse monoclonal anti-CD68; IF                 | BioLegend                                    | 375,602       | 1:20              |
| Rabbit monoclonal anti-CCR2; WB                | Abcam                                        | ab203128      | 1:1000            |
| Mouse monoclonal anti-β-Actin; WB              | Sigma-Aldrich, Darmstadt, Germany            | A5441         | 1:5000            |
| Rabbit monoclonal anti-phospho-SMAD2 (Ser465/467); WB | Cell Signaling Technology, Danvers, USA   | 3108          | 1:1000            |
| Rabbit monoclonal anti-SMAD2/3 (D7G7) XP; WB   | Cell Signaling Technology                    | 8685          | 1:1000            |
| **Secondary Antibodies**                       |                                              |               |                   |
| Goat anti-rat IgG (H+L) Alexa Fluor 546; IF    | Invitrogen                                   | A-11081       | 1:1500            |
| Goat anti-rabbit IgG (H+L) Alexa Fluor 488; IF | Invitrogen                                   | A-11034       | 1:1500            |
| Goat anti-mouse IgG F(ab)2 Alexa Fluor 488; IF | Jackson ImmunoResearch Labs, West Grove, USA | 115-546-072   | 1:800             |
| HRP-conjugated sheep anti-mouse IgG (H+L); WB  | Sigma-Aldrich, Eschwege, Germany             | A5906         | 1:5000            |
| HRP-conjugated goat anti-rabbit IgG (H+L); WB  | MP Biomedicals, Eschwege, Germany            | 0855676       | 1:1000            |
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RNA extraction and qRT-PCR

Total RNA was isolated from frozen testes using the RNaseasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) and from cells using the RNaseasy Mini Kit (Qiagen), according to the manufacturer’s instructions. Contaminating DNA was removed using the RNase-Free DNase Set (Qiagen). Total RNA was reverse transcribed as described previously [21, 24]. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Munich, Germany) for self-designed primers or QuantiTect SYBR Green PCR Master Mix (Qiagen) for QuantiTect primer assays (Qiagen) in a CFX96 Touch thermal cycler (Bio-Rad). Primer sequences and annealing temperatures are listed in Table 3. Hypoxanthine–guanine phosphoribosyltransferase (Hprt) and 18S rRNA were selected as stably expressed housekeeping genes. All samples were run as duplicates. Relative mRNA expression was calculated using the 2−ΔΔCt method [39].

Bulk RNA sequencing

Total RNA was isolated from BMDMs using the RNaseasy Mini Kit (Qiagen) combined with on-column DNase digestion (RNase-Free DNase Set, Qiagen) to avoid contamination by genomic DNA. RNA and library preparation integrity were verified with the LabChip Gx Touch 24 (Perkin Elmer, Waltham, USA). Library preparation was carried out with 4 µg of total RNA as input using the VAHTS Stranded mRNA-seq Library Prep kit according to the manufacturer’s protocol (Vazyme, Nanjing, China). Sequencing was performed on the NextSeq500 System (Illumina, San Diego, USA) using v2 chemistry with a 1 × 75 bp single-end setup. The resulting raw reads were assessed for quality, adapter content, and duplication rates using FastQC [40]. Trimmomatic version 0.39 was employed to trim reads after a quality drop below a mean of Q20 in a window of 5 nucleotides [41]. Only reads between 30 and 150 nucleotides were cleared for further analysis. Trimmed and filtered reads were aligned against Ensembl mouse genome version mm10 (ensemble release 101) using STAR 2.7.9a with the parameter “--outFilterMismatchNoverLmax 0.1” to increase the maximum ratio of mismatches to mapped length to 10% [42]. The number of reads aligning to genes was counted using the featureCounts 2.0.2 tool of the Subread package [43]. Only reads mapping at least partially inside exons were admitted and aggregated per gene; reads overlapping multiple genes or aligning to multiple regions were excluded. Differentially expressed genes (DEGs) were identified using DESeq2 version 1.30.0 [44]. Only genes with a minimum fold change of ±2 (log2 = ±1), a maximum Benjamini-Hochberg-corrected p-value of 0.05, and a minimum combined mean of five reads were deemed to be significantly differentially expressed. Ensemble annotation was enriched using UniProt data (release 06.06.2014) based on Ensembl gene identifiers [45].

Protein extraction and Western blot

BMDMs were lysed on ice in RIPA buffer (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris–HCl pH 8.0, 2 mM EDTA) supplemented with protease inhibitor cocktail (1:100; Sigma-Aldrich) and centrifuged at 13,000 g for 30 min at 4 °C. The RC DC Protein Assay (Bio-Rad, Hercules, USA) was used to measure total protein concentration. Protein samples diluted in Laemmli buffer (30 µg/lane) were resolved by 10% (SMADs only) or 12.5% SDS–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane (GE Healthcare, Darmstadt, Germany), blocked with 5% non-fat milk in TBST for 1 h at RT, and subsequently washed in TBST. Incubation with primary antibodies was performed overnight at 4 °C (Table 2). Afterwards, membranes were washed three times for 10 min at RT in TBST before incubation with the appropriate secondary antibodies for 1 h at RT (Table 2). Finally, the membranes were probed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and subsequently developed using the Fusion FX Western Blot and chemiluminescence imaging system ( Vilber Lourmat, Eberhardzell, Germany). The results were quantified using FusionCapt Advance Solo 4 16.07 software (Vilber Lourmat).

BMDM migration assay

BMDM generation and treatment with activin A and/or FST288 were performed as described above, and cells were cultivated in a Nunc UpCell 6-well dish before harvesting. For the migration assay, cells were gently washed with PBS and incubated on ice for 15 min before collection. After seeding the BMDMs (3 × 10^5) in Transwell inserts (8 µm pore size; Corning Life Sciences, Tewksbury, USA) in serum-free RPMI-1640 medium, 750 µl serum-free
| Gene      | Sequence (5’–3’) | Amplicon size (bp) | Annealing temperature (°C) |
|-----------|-----------------|--------------------|---------------------------|
| 18S rRNA  | F: TACCACATCCAAAGGAAGGCGAGCA R: TGGAATTACCCTGGGCTGCTGAGCA | 180                | 55                        |
| Acvr1b    | F: CCAACTGGGTGGCAGAGTTAT R: CTTGGACAGAGTCTTCCTTGAG | 119                | 55                        |
| Acvr2b    | F: ATGAGTACATGCTGCCCTTC R: CTTAATCTGGGGCCCTCATT | 101                | 55                        |
| Adgre1 (F4/80) | F: TCTGACAGTTCAGCTCAGAA R: GAAGTCGGAATGGGAGCT | 277                | 60                        |
| Ccl2      | QuantiTect Primer assay (QT00167832, Qiagen) | 118                | 55                        |
| Cxcl12    | F: CAGTGACGTAACACCAGTCACGC R: TGGGCATGTGGCCTCTCG | 68                | 64                        |
| Cxcr4     | F: GACTTGCCAGTGCAGCAGTGAAGG | 131                | 60                        |
| Fn1 (fibronectin) | F: AGAAGGCGAGTACAGCAGA R: TCTCCCTCAAGGCTAGTAG | 110                | 55                        |
| Fst       | F: AGAGAGATTGGAGACAGCAATAC R: CAGTGTCTCAGCTCAG | 95                | 55                        |
| Hprt      | F: CTGTTGAAAGGAGCTC R: CTGAAAGTACTCTTATTAGTACAGA | 110                | 55                        |
| Il-1a     | F: CGAAGACTACAGTCTGACATT R: GACGTTCAGGAGGTTCTGAG | 126                | 60                        |
| Il-1b     | F: CAACCAACAGTGATATTCCTCCAGT R: GATGCCAAGCTCCAGCAGCTCA | 152                | 60                        |
| Il-6      | QuantiTect Primer assay (QT00098875, Qiagen) | 128                | 55                        |
| Il-10     | QuantiTect Primer assay (QT00106169, Qiagen) | 109                | 55                        |
| Inha      | F: GCCAAGGTGAAAGCTCATT | 126                | 55                        |
| Inhba     | F: AGAAGGCGGATGTGGAGATA GAGA R: GACTCGGCAAAGGTTGATGAT | 97                | 55                        |
| Inhbb     | F: CTGCCAGTCGGCGAGGTATAA R: CCTCACTCCAGCTCATT | 110                | 55                        |
| Itgav     | F: AAGGGCGAGATACAGAGGGA R: CCAGGCTTCTCATGGTTCCT | 182                | 60                        |
| Itgb3     | F: TGTTGCTAGTGAGCTAGTCTGTC R: GACTCGGAACAGATTTGCTTCA | 86                | 60                        |
| Itgb5     | F: TGTTGCTAGTGAGCTAGTCTGTC R: GACTCGGAACAGATTTGCTTCA | 198                | 55                        |
| Mmp2      | F: CAGGGGAAATGACTCGGCTCTATT R: ACTCCAGTAAAAAGGCAGCATCTAC | 119                | 60                        |
| Mmp9      | F: AAGGACGCGCTTCTGGCAGACGTCT GTGAGACAGCGCTTCTTC | 874                | 60                        |
| Mmp14     | F: TTACAAGTGACAGGCAAGG R: GCTTCTCTCCAAGCTATTG | 112                | 60                        |
| Pdgfa     | F: CAAGACAGGCGTCATTT R: CCTACTGGGACCTCTTC | 223                | 55                        |
| Pdgfb     | F: CCCACAGTGCTTCTCTTACTT R: GTGAAAGGGAAGTGGAGAAGG | 137                | 55                        |
| Pdgfrb    | F: GAACGACATTGCCAGTAGA R: GCATCGGATAAGGCTCAACA | 146                | 60                        |
RPMI-1640 medium containing 500 ng/ml CCL2 was added to the lower chamber. Cells were allowed to migrate for 3 h at 37 °C. Migrated cells were collected from the lower chamber and counted using the MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec).

**Gelatin zymography**

The enzymatic activity levels of MMP2 and MMP9 were measured in BMDM-conditioned medium (generated as described above); gelatin zymography was performed as reported [46]. BMDM-conditioned medium was loaded and gels were stained with Coomassie Brilliant blue solution. Band intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, USA). Relative enzymatic activity was calculated by normalizing the band intensity of the untreated group to 1.0.

**Statistical analysis**

Statistical data analysis was performed using GraphPad Prism 7 software (GraphPad Software, San Diego, USA). Data are presented as mean ± standard error of the mean (SEM) and were tested for normal distribution using the Kolmogorov–Smirnov test. The statistical significances of differences in multiple groups were calculated using one-way or two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test (Gaussian distribution) or the Kruskal–Wallis test followed by Dunn’s multiple comparisons test (non-normal distribution). Correlation significance between two groups was analyzed using the Pearson coefficient (normal distribution) or Spearman coefficient (non-normal distribution). Statistical significance was set at \( P < 0.05 \).

**Results**

**Ccr2 deficiency protects the testis from damage caused by EAO**

To determine the role of CCR2 in EAO-induced fibrosis, we compared EAO in WT and Ccr2<sup>−/−</sup> mice. Measurement of testicular weight 50 days after the first immunization showed that EAO caused a reduction in mean testis weight in both WT and Ccr2<sup>−/−</sup> mouse strains, compared with untreated and adjuvant control groups (Fig. 1B). However, mean testis weight was significantly higher in the Ccr2<sup>−/−</sup> EAO mice (0.089 ± 0.004 g) than in the WT EAO mice (0.045 ± 0.004 g) (Fig. 1B), indicating lower levels of testicular damage in the Ccr2 null mice. To quantify the fibrotic response, testicular collagen content was analyzed by hydroxyproline assay and picro-sirius red staining and found to be significantly elevated in WT EAO testes, compared with WT untreated testes (Fig. 1C). In contrast, EAO did not result in significantly increased levels of collagen in Ccr2<sup>−/−</sup> mice, and total collagen content was 2.7-fold lower in EAO testes than in WT testes (Fig. 1C). Histological examination of testicular sections revealed that normal testicular architecture was destroyed in 94% of WT EAO testes, with germ cell sloughing, atrophy of seminiferous tubules, thickening of the lamina propria of seminiferous tubules, and an increase in the density of collagen fibers (Fig. 1D). In contrast, although a few disrupted seminiferous tubules were visible in 3 of 15 mice (20%), testicular structure was largely intact in Ccr2<sup>−/−</sup> EAO testes, indicating a crucial role for CCR2 in inflammation and damage following EAO (Fig. 1D).

**Ccr2 deficiency reduces inflammatory responses in EAO testes**

Increased numbers of CD45<sup>+</sup> leukocytes and elevation of inflammatory cytokines such as CCL2, TNF, IL-1 and IL-10, and activin A are important hallmarks of the inflamed testis in EAO [6, 13]. To investigate whether depletion of Ccr2 affects the testicular inflammatory response, the presence and number of immune cells and expression of cytokines and activins in testes were analyzed. Flow cytometry demonstrated significantly lower numbers of total CD45<sup>+</sup> leukocytes (Fig. 2A and B), CD45<sup>+</sup>Ly6G<sup>+</sup> neutrophils (Fig. 2C), and CD45<sup>+</sup>Ly6C<sup>−</sup>CD11b<sup>+</sup>CD64<sup>+</sup> macrophages (Fig. 2E), as well as an approximately 14-fold reduced population of CD45<sup>+</sup>Ly6C<sup>+</sup> monocytes (Fig. 2D) in Ccr2-deficient EAO testes, compared with EAO WT testes. These results imply that the depletion of CCR2 inhibits the recruitment of infiltrating immune cells in the EAO testis.
Quantitative RT-PCR showed that Ccr2 deficiency led to significant suppression of testicular Ccl2 (Fig. 2F), Il-10 (Fig. 2G), and Il-1α (Supplementary Fig. S2A) mRNAs during EAO, compared with the levels observed in WT mice undergoing EAO. Moreover, levels of Ccl2 and Il-10 mRNA were not significantly changed in any Ccr2−/− experimental group (Fig. 2F and G). Absence of Ccr2 also inhibited the EAO-mediated increase in expression of the activin A

Fig. 2 Immune cell infiltration and expression of inflammatory mediators caused by EAO are reduced in Ccr2−/− mouse testes. Representative density plots for CD45+ leukocytes in untreated, adjuvant control, and EAO testicular single-cell suspensions from WT and Ccr2−/− mice (A). After gating out doublets and nonviable cells, numbers of testicular CD45+ leukocytes (B), CD45+Ly6G+ neutrophils (C), CD45+Ly6C+ monocytes (D), and CD45+Ly6C−CD11b+CD64+ macrophages (E), were determined in untreated, adjuvant control, and EAO testicular single-cell suspensions from WT and Ccr2−/− mice by flow cytometry (n = 5–6). Relative expression (RE) of Ccl2 (F), Il-10 (G), Inhba (H), and Acvr2b (I) was determined in untreated, adjuvant control, and EAO testes from WT and Ccr2−/− mice by qRT-PCR analysis and normalization to 18S rRNA and Hprt (n = 4–6). Values are mean ± SEM; statistical analyses used the Kruskal–Wallis test followed by Dunn’s multiple comparison test or two-way ANOVA followed by Bonferroni’s multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
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subunit Inhba (Fig. 2H). Expression of follistatin (Fst) was significantly increased in EAO testes (Supplementary Fig. S2E), while expression of the activin A receptor subunit Acvr1b was reduced (Supplementary Fig. S2F). In addition, following EAO, expression of the Acvr2b subunit was significantly reduced in WT testes but remained in the normal range in Ccr2−/− testes (Fig. 2I). However, other inflammation-related genes, including Il-1b and other activin-related genes (Inhbb and Inhba), were not significantly altered in untreated, adjuvant control, or EAO testes from WT or Ccr2−/− mice (Supplementary Fig. S2B and G-H). Furthermore, expression of Tnf and Il-6 mRNA was significantly increased in EAO WT testes, but unchanged in all Ccr2−/− groups (Supplementary Fig. S2C and D).

Ccr2 deficiency suppresses changes in the numbers of ECM-expressing immune cells and CXCL12/CXCR4 expression induced by EAO

Since the above results indicated that loss of Ccr2 expression reduced immune cell accumulation and inhibited the fibrotic response in EAO testes, we wanted to investigate the involvement of immune cells (particularly macrophages) in the development of testicular fibrotic remodeling. Therefore, the numbers and localization of ECM-expressing immune cells in the testes during EAO were analyzed by flow cytometry and immunofluorescence, respectively; CXCR4+ cells were also quantified as CXCR4 plays a significant role in ECM-producing immune cells [47]. The proportions of CD45+fibronectin+ cells (Fig. 3A) and CD45+fibronectin+CXCR4+ cells (Fig. 3B) within the total CD45+ leukocyte population were significantly increased in WT EAO testes, while Ccr2 deficiency inhibited this effect. Similar results were observed for CD45+collagen I+ cells and CD45+collagen I+CXCR4+ cells (Supplementary Fig. S3A and B). Among immune cells, macrophages are an important source of ECM proteins [48]. The numbers of fibronectin+ and collagen I+ macrophages (identified by F4/80 and CD11b) were increased 117-fold and 70-fold, respectively, in WT EAO testes, compared with testes from untreated and adjuvant controls (Fig. 3C and Supplementary Fig. S3C). These results were confirmed by immunofluorescence (Fig. 3D and Supplementary Fig. S3D). In contrast, the numbers of fibronectin- and collagen I-expressing TMs detected in Ccr2−/− EAO testes were approximately sevenfold lower, compared with the numbers in WT EAO testes (Fig. 3C, D and Supplementary Fig. S3C, D). Furthermore, analysis of testicular biopsies from patients with impaired spermatogenesis, testicular fibrosis, and inflammation showed that fibronectin expression was increased in CD68+ macrophages. This indicated that similar to mouse EAO, macrophages express fibronectin under pathological conditions (Fig. 3E). These results suggest that infiltrating bone marrow CCR2+ cells play a role in modulating the levels of ECM proteins during testicular inflammation and thus also in fibrotic remodeling.

Because CXCR4 and its ligand CXCL12 contribute to the recruitment of circulating progenitors of active fibroblasts [49], we also analyzed the testicular expression of CXCR4 by flow cytometry, qRT-PCR, and immunofluorescence in WT and Ccr2−/− EAO mouse models. While increased numbers of CD45+CXCR4+ cells (within the total CD45+ leukocyte population) were detected in WT testes in response to EAO, this increase was abrogated in the absence of Ccr2 expression (Fig. 3F). Furthermore, reduced levels of Cxcr4 (Fig. 3G) and Ccxl12 (Fig. 3H) mRNA were detected in Ccr2−/− EAO testes, compared with WT EAO testes. Immunofluorescence revealed elevated numbers of CXCR4+ macrophages (as well as total numbers of CXCR4+ cells) in WT EAO testes, while infiltration by CXCR4+ macrophages was not increased in Ccr2−/− EAO testes (Supplementary Fig. S3E). These data suggest that CCR2 signaling is also involved in the recruitment of CXCR4+ cells in EAO testis.

Activin A contributes to the production of collagen I by macrophages and increases MMP expression during EAO

We previously reported a positive correlation between the severity of disease and testicular activin A concentration [13, 21]. In addition, inhibition of activin A through overexpression of follistatin (using a viral vector expressing FST315) significantly reduced the fibrotic response in EAO [21, 24]. In the present study, the activation of Inhba during EAO was suppressed in Ccr2-deficient mice (see Fig. 2H), accompanied by decreased macrophage infiltration. Transfection with FST315 also clearly reduced testicular infiltration by collagen I+ TMs in mice undergoing EAO (compared with empty vector [EV]-treated control mice; Fig. 4A, B). Collagen I was almost undetectable in TMs from untreated and adjuvant controls (Fig. 4B). These data suggest that activin A is involved in regulating the expression of collagen I by TMs in EAO testis. In addition, as MMPs and their inhibitors TIMPs are key regulators of ECM component degradation and fibrotic remodeling [50], we quantified the levels of Mmp and Timp1 mRNAs by qRT-PCR. Subsequently, correlation analysis demonstrated significant associations between Inhba and Mmp2, Mmp9, Mmp14, and Timp1 mRNA levels in testes from mice treated with control EV or FST315 after induction of EAO (Fig. 4C–F). Altogether, these data suggest that activin A influences the expression of MMPs and TIMP1 by macrophages.
AB C
D
E
F
G
H

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Figure A: CD45^+fibronectin^+ cells in WT and Ccr2^−/− mice.

Figure B: CD45^+fibronectin^+CXCR4^+ cells in WT and Ccr2^−/− mice.

Figure C: CD45^+fibronectin^+F4/80^+CD11b^+ cells in WT and Ccr2^−/− mice.

Figure D: Immunofluorescence images of WT and Ccr2^−/− mice in Untreated, Adjuvant, and EAO conditions.

Figure E: Normal spermatogenesis (I, II) and Impaired spermatogenesis (III, IV).

Figure F: CD45^+CXCR4^+ cells in WT and Ccr2^−/− mice.

Figure G: Cxcr4 mRNA RE in WT and Ccr2^−/− mice.

Figure H: Cxcl12 mRNA RE in WT and Ccr2^−/− mice.
Activin A affects BMDMs and their transcriptome

To investigate how regulation of macrophage function by activin A may contribute to inflammatory and fibrotic responses, mouse BMDMs were stimulated with activin A in vitro (Fig. 5A). FST288 was used as an antagonist of activin A. Phosphorylation of SMAD2 was analyzed to confirm the effect of activin A in BMDMs as activin A downstream signaling is induced by phosphorylation of SMAD2/3 and translocation of SMAD2/3-SMAD4 to the nucleus [22, 24]. Western blotting demonstrated that SMAD2 in BMDMs was phosphorylated within 30 min and 60 min of treatment with 25 ng or 50 ng/ml activin A; these effects were abolished by the addition of 250 ng/ml FST288 (Supplementary Fig. S4A and B).

Next, a whole transcriptomic analysis of BMDMs was performed after treatment with activin A, FST288, or a combination of both (Fig. 5A). Overall, the samples from untreated (CTRL), FST288-alone or FST288 + activin A-treated BMDMs showed similar gene expression patterns, which differed from those identified in activin A-treated BMDMs (Fig. 5B–D). Comparison of the activin A-stimulated BMDMs and the CTRL group revealed 936 upregulated and 799 downregulated DEGs (Fig. 5B and C). Further analysis of the top 50 DEGs identified by the term “inflammation mediated by chemokine and cytokine signaling pathway” from the Panther database demonstrated that several cytokine and chemokine signaling-related genes were regulated by activin A. For example, Ccr2 and Cxcr4 were upregulated, while Ccl2 was downregulated following activin A treatment (Fig. 5E), pointing to dual pro- and anti-inflammatory effects mediated by activin A. In addition, the selection of the top 50 significantly altered DEGs enriched for the term “extracellular matrix organization” from the Reactome database indicated that many upregulated genes, including Tgfb1, Fn1, Mmp2, Mmp14, Pdgfa, and Itgav, were related to TGF-β signaling and ECM organization (Fig. 5F). Therefore, we focused on the selected genes (Ccr2, Cxcr4, Fn1, Mmp2, Mmp14, Pdgfa, Itgav, and Itgb3) in the subsequent experiments.

Activin A upregulates CCR2 in BMDMs and induces the migration of macrophages along a CCL2 gradient

The above RNAseq data analysis indicated that treating BMDMs with 50 ng/ml activin A-induced elevated Ccr2 expression. This was confirmed by Western blotting (Fig. 6A), while the addition of FST288 abolished this increase at both mRNA and protein levels (Figs. 5E and 6A). To investigate these findings at a functional level, we analyzed the ability of BMDMs (isolated from WT and Ccr2−/− mice in the absence/presence of activin A and/or FST288) to migrate along a CCL2 gradient. Given that CCL2 stimulates macrophage migration through binding to CCR2 [51], increasing the levels of CCR2 was anticipated to increase migratory capacity. Accordingly, the results of transwell migration assays revealed that activin A significantly increased migration by WT BMDMs, while this effect was abolished by the addition of FST288 (Fig. 6B). In contrast, activin A did not influence the migratory capability of Ccr2−/− BMDMs (Fig. 6B).

Activin A increases fibronectin and CXCR4 expression in BMDMs

In vivo analyses demonstrated that numbers of both fibronectin+ and CXCR4+ macrophages were elevated in fibrotic lesions during EAO (Fig. 3 and Supplementary Fig. S3). Moreover, RNAseq confirmed that fibronectin (Fn1) and Cxcr4 were upregulated upon activin A stimulation of BMDMs (Fig. 5E and F). Consistent with these findings, qRT-PCR and immunofluorescence showed that fibronectin mRNA and protein levels were increased in BMDMs after activin A treatment (Fig. 6C and D); these effects were inhibited by FST288. Activin A treatment also induced changes in the morphology of BMDMs from round, oval, or irregular to more elongated in shape (Fig. 6D); this was accompanied by a decrease in the mean fluorescence intensity of the macrophage-specific marker F4/80 (Supplementary Fig. S4C and D). In addition, as TNF stimulates activin A secretion by mouse SCs in vitro, culture medium from TNF-stimulated SCs (SCCM) was used to evaluate the effects on macrophages of activin A originating directly
A

C57BL/6J (WT) mice

Injection of adeno-associated viral vector carrying the gene cassette of FST315 or an empty cassette

-30 d 0 d 14 d 28 d 50 d

Immunization  Collection of organs

B

Untreated  Adjuvant  EAO

rAAV-EV

I  II  III

rAAV-FST315

IV  V  VI

Collagen I, F4/80 and DAPI

C

\[ R^2 = 0.4651 \]

\[ r = 0.6819 \]

\[^{**}p<0.01\]

Mmp2 mRNA RE

Inhba mRNA RE

D

\[ R^2 = 0.7642 \]

\[ r = 0.8742 \]

\[^{***}p<0.0001\]

Mmp9 mRNA RE

Inhba mRNA RE

E

\[ R^2 = 0.4308 \]

\[ r = 0.6563 \]

\[^{*}p<0.05\]

Mmp14 mRNA RE

Inhba mRNA RE

F

\[ R^2 = 0.4329 \]

\[ r = 0.6580 \]

\[^{*}p<0.05\]

Timp1 mRNA RE

Inhba mRNA RE
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from testicular cells [24]. Analysis by qRT-PCR confirmed that TNF-stimulated SCCM induced a significant increase in Fbn1 mRNA expression in BMDMs (Supplementary Fig. S5A), while non-TNF-SCCM had no effect. FST288 inhibited the increase in Fbn1 mRNA, confirming that the effect of SCCM was due to activin A (Supplementary Fig. S5A). Moreover, flow cytometry showed that activin A-treatment of BMDMs significantly increased the percentage of fibronectin+ macrophages (Fig. 6E), while the percentage of fibronectin+ monocytes was significantly decreased in a dose-dependent manner (Supplementary Fig. S4E).

Similarly, activin A treatment caused a fivefold increase in Cxcr4 mRNA expression in cultured BMDMs; increased percentages of CCR4+ macrophages (approximately 22-fold) and monocytes (approximately fourfold) were also observed (Fig. 6F–H and Supplementary Fig. S4F). In contrast, the mRNA expression of the CCR4 ligand Cxcl12 was reduced in activin A-treated BMDMs (Fig. 6I). Moreover, although TNF-stimulated SCCM did not induce increased Cxcr4 expression in BMDMs, the inclusion of FST288 resulted in reduced levels of Cxcr4 mRNA (Supplementary Fig. S5B). These data clearly demonstrate the pro-fibrotic function of activin A in BMDMs.

The expression of MMPs that are dysregulated during EAO is affected by activin A in BMDM culture

As shown above, increased Inhba expression correlated with elevated levels of Mmp and Timp mRNAs in EAO testes in vivo (Fig. 4C–F). Moreover, RNAseq indicated that expression of Mmp2 and Mmp14 was increased in activin A-stimulated BMDMs (Fig. 5F). Therefore, we investigated the effects of activin A stimulation and Ccr2 absence on MMPs in vitro and in vivo. Analysis by qRT-PCR showed that activin A significantly upregulated the expression of Mmp2 and Mmp14 in BMDMs, while Mmp9 and Timp1 mRNAs were downregulated (Fig. 7A); these effects were blocked by FST288. Treatment with TNF-stimulated SCCM also led to a 50-fold increase in Mmp2 expression (Supplementary Fig. S5C). Notably, treatment of BMDMs with TNF-stimulated SCCM also upregulated the expression of Mmp14 (46-fold), Mmp9 (16-fold), and Timp1 (1.5-fold); however, these increases were not abolished by FST288, pointing to regulation by SC-derived factors other than activin A (Supplementary Fig. S5D–F). In agreement with the qRT-PCR results, gelatin zymography showed that activin A-induced significant upregulation of MMP2 and downregulation of MMP9 enzymatic activity (Fig. 7B and C). However, in contrast, induction of EAO resulted in increased expression of Mmp9 and Timp1 in addition to Mmp2 and Mmp14 in WT testes (Fig. 7D–G). Moreover, the EAO-induced increases in Mmp2 and Mmp14 mRNA were significantly reduced in Ccr2−/− testes (Fig. 7D and E). Although Mmp9 (Fig. 7F) was also downregulated, this decrease was not statistically significant due to the variable levels of Mmp9 expression in Ccr2−/− EAO testes. Our results also indicated that Timp1 expression was not influenced by Ccr2 deficiency (Fig. 7G). Interestingly, Spearman correlation analysis of mRNA data showed that Adgre1 (the gene encoding F4/80) was significantly positively correlated with Fbn1 and increased, while upregulation of these genes was inhibited (Supplementary Fig. S7A and B). In EAO testes, expression of Ccr2, Mmp2, Mmp14, Mmp9, and Timp1 in WT and Ccr2−/− EAO testes indicating a connection between macrophages and MMP expression (Fig. 7H and Supplementary Fig. S6A–C).

Integrin subunits and PDGFs expression are dysregulated in activin A-treated BMDMs and WT EAO testes

Integrins (receptors for ECM proteins) and PDGFs produced by immune cells contribute to ECM deposition and fibrosis development [52, 53]. Our RNAseq results suggested that activin A treatment regulated the expression of Pdgfa and integrin subunits Itgb3 and Itgav (Fig. 5F). Therefore, we went on to investigate the expression of integrin and PDGF genes in activin A-treated BMDMs and EAO testes by qRT-PCR. In BMDMs, activin A-induced significant dose-dependent upregulation of Itgav, Itgb5, Pdgfa, Pdgfb, and Pdgfrb expression, whereas Itgb3 mRNA was downregulated (Supplementary Fig. S7A and B). In WT EAO testes, expression of Itgav, Itgb3, Pdgfb, and Pdgfrb (Supplementary Fig. S7C, D and G, H) was also significantly increased, while upregulation of these genes was inhibited in the absence of Ccr2. Furthermore, expression of Itgav and Pdgfrb was positively associated with Adgre1 levels in EAO testis (Supplementary Fig. S6D and I). These two genes showed similar changes in vitro and in vivo, indicating that activin A may regulate the production of the α5 integrin subunit and PDGFRβ in macrophages, facilitating...
Fig. 5 Whole transcriptome analysis of BMDMs treated with activin A in vitro. Experimental set-up for transcriptomic analysis in BMDMs. Cells were left untreated (CTRL) or stimulated with 50 ng/ml activin A (Act), 250 ng/ml FST288 (FST), or a combination of both (Act + FST) (A; n = 3). Venn diagram of up- (B) and downregulated (C) DEGs in BMDMs treated with Act, Act + FST, or FST versus CTRL cells. Heat map (D) showing expression of the top 50 DEGs in CTRL, Act, Act + FST, and FST groups (based on false discovery rate > 0.05 and minimal count number > 5). Heat maps showing significant DEGs enriched for the terms “Inflammation mediated by chemokine and cytokine signaling pathway” (E) and “Extracellular matrix organization” (F) from the Panther and Reactome databases, respectively in CTRL, Act, Act + FST, and FST groups of BMDMs, presented with Z-score normalization. Arrows indicate the investigated genes.
the development of testicular fibrosis. However, the levels of *Itgb5* and *Pdgfa* mRNAs were unchanged during EAO, and *Itgb5* was expressed at lower levels in Ccr2<sup>−/−</sup> EAO testes, compared with WT EAO testes (Supplementary Fig. S7E and F).

**Discussion**

In this study, we aimed to investigate the role of the activin A/CCL2-CCR2/macrohage axis in the development of testicular fibrosis. We hypothesized that activin A may be involved in the regulation of inflammatory and fibrotic responses during EAO by regulating the potential pro-fibrotic properties of newly-recruited CCR2<sup>+</sup> macrophages. To examine these possibilities, we compared the progression of EAO in WT and Ccr2<sup>−/−</sup> mice as well as in mice overexpressing follistatin to reduce activin bioactivity. Our results demonstrated that CCR2 and activin A, acting through macrophages, are crucial for the development of testicular inflammation and fibrosis in EAO. In short, testicular inflammation and fibrosis were reduced in the absence of Ccr2, as indicated by lower numbers of immune cells expressing ECM proteins (fibronectin, collagen I) and decreased production of collagen, MMPs, as well as inflammatory mediators (indicated by changes in *Il-10*, *Ccl2*, and *Inhba* expression). Furthermore, activin A acted as an inducer of CCR2 and fibrosis-related mediators (fibronectin, CXCR4, MMP2, and PDGF) in macrophages. Inhibition of activin A in vivo by overexpression of FST during EAO decreased testicular fibrosis and expression of collagen I by TMs.

EAO is a well-established mouse model of autoimmune-based male infertility featuring marked testicular deposition of ECM proteins (including collagens and fibronectin), increased numbers of TMs, and elevated levels of inflammatory mediators such as TNF, CCL2, and activin A. These pathological alterations combine to cause tissue destruction, inhibition of spermatogenesis, and subsequent infertility [8, 13, 21, 24]. Overall, our study revealed CCR2 and activin A to be important players in the progression of fibrosis in testicular inflammation. Furthermore, our findings underlined the pro-fibrotic role of monocyte-derived macrophages in the development of testicular fibrosis.

CCR2 is a receptor for CCL2, an inflammatory chemokine that mediates the chemotaxis of leukocytes to injury sites [54]. In this study, the absence of CCR2 during testicular inflammation not only preserved the tissue from the deposition of ECM and production of activin A, IL-10, and CCL2 but also protected the testis from damage by reducing the accumulation of immune cells. This is in accordance with previous reports demonstrating reduced inflammatory and fibrotic responses in different organs in the absence of CCR2 signaling [14, 28, 30, 55–57]. Here, we showed that in contrast to WT animals, Ccr2<sup>−/−</sup> mice exhibited normal levels of *Il-1a*, *Il-10*, *Ccl2*, and *Inhba* mRNA in the testes following EAO induction, pointing to the essential contribution of CCR2<sup>+</sup> cells to the induction of the inflammatory response.

Since the mechanisms leading to the fibrotic response and the origin of ECM proteins during the progression of testicular inflammation were unknown, we wanted to address these questions. ECM-producing cells originate from a variety of sources, including bone marrow-derived circulating cells known as fibrocytes [15, 58]. Fibrocytes are characterized by the co-expression of hematopoietic markers such as CD45, CD34, or CD11b and mesenchymal markers such as collagen or vimentin [59]. These cells mainly migrate to injury sites by chemotaxis via chemokine ligand/receptor pathways, including CCL2/CCR2 or CXCL12/CXCR4 [59, 60]. Fibrocytes can also originate from a subpopulation of monocytes via monocyte-to-fibroblast transition [61–63]. Our results indicate that during the severe stage of EAO, immune cells (particularly macrophages) act as important sources of fibronectin and collagen I production. Since the CD34 marker was not included in our analysis, we cannot definitely exclude the possibility that a proportion of the ECM-expressing CD45<sup>+</sup> cells may have been fibrocytes. The significant reduction in the accumulation of CD45<sup>+</sup> cells and TMs expressing fibronectin, collagen I, and CXCR4 observed in EAO Ccr2<sup>−/−</sup> testis indicates the importance of CCR2 and CXCR4 signaling in testicular ECM expression. In addition, the very high levels of *Cxcl12* and *Cxcr4* mRNA detected in WT EAO testis were significantly reduced in the absence of Ccr2, underlining the importance of newly-recruited CCR2<sup>+</sup> cells as a source of these molecules during the testicular fibrotic response. Similarly, the expression of fibronectin in TMs under pathological conditions in the human testis (impaired spermatogenesis and collagen accumulation) points to the possible involvement of macrophages in fibrotic remodeling. These data are in agreement with earlier reports demonstrating CCR2-dependent inhibition of BMDM and fibrocyte infiltration in kidney injury, colon fibrosis, and colitis [28, 30, 64–66]. Moreover, CXCR4<sup>+</sup> macrophages have also been shown to act as a pro-fibrotic cell subpopulation in the kidney and lung [67–69]. Future studies are required to establish whether CCR2<sup>+</sup>CXCR4<sup>+</sup> TMs are the key players involved in the EAO fibrotic response. However, currently, we cannot exclude the possibility that the production of *Cxcl12* by CCR2<sup>+</sup> cells may account for the reduced infiltration of CXCR4<sup>+</sup> cells in Ccr2<sup>−/−</sup> mouse testes.

Our results also showed that expression of *Mmp2*, *Mmp14*, *Mmp9*, and *Timp1* was increased in WT EAO testes, indicating activation of the ECM machinery. The gelatinases MMP2 and MMP9 mainly cleave collagen IV, an important component of the basement membrane, making it easier for leukocytes
to transmigrate [70, 71]. Activation of MMP2 requires the involvement of MMP14 [72]. Aggregated ECM proteins require degradation by MMPs to prevent fibrosis; on the other hand, TIMP1 inhibits the degradative function of MMP proteins, thereby exacerbating the fibrotic process. However, elevated levels of MMPs could also contribute to immune cell migration or activate PTCs/fibroblasts to produce more ECM proteins, thereby promoting tissue fibrosis [73, 74]. Thus, the process of testicular fibrosis appears to depend on the dynamic balance between MMP and TIMP activities. In this study, loss of Ccr2 abrogated the elevated expression of Mmp2 and Mmp14 occurring in EAO testes. Interestingly, the
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Fig. 6 Levels of CCR2, fibronectin, and CXCR4 are elevated in BMDMs following exposure to activin A. Western blotting analysis and quantification of CCR2 protein in BMDMs 6 days after treatment with 25 ng/50 ng/ml activin A, 250 ng/ml FST288, or a combination of both (A); β-actin was used as a loading control (n=6). Migration of BMDMs generated from WT and Ccr2−/− mice in the presence of 50 ng/ml activin A, 250 ng/ml FST288, or both was quantified by incubating within a 500 ng/ml CCL2 gradient in a transwell system (B); migrated cells in the lower chamber were quantified by flow cytometry (n=3). Relative expression (RE) of Fnl (C), Cxcr4 (H), and Cxcl12 (I) in BMDMs 6 days after treatment with 25 ng/50 ng/ml activin A, 250 ng/ml FST288, or a combination of both was determined by qRT-PCR analysis and normalized to Hprt (n=8). Fibronectin (green), F4/80 (red), and DNA (Topro3; blue) immunofluorescence staining in BMDMs left untreated or treated with 50 ng/ml activin A, 250 ng/ml FST288, or a combination of both (D). After gating CD45+CD11c−Ly6C−F4/80−CD11b+ macrophages, the percentages of fibronectin+ (E) and CXCR4+ (F, G) macrophages in single-cell BMDM suspensions following treatment with 25 ng/50 ng/ml activin A, 250 ng/ml FST288, or a combination of both were measured by flow cytometry (n=5). Values are mean±SEM; statistical analyses used the Kruskal–Wallis test followed by Dunn’s multiple comparison test or one-way/two-way ANOVA followed by Bonferroni’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

expression of these genes positively correlated with levels of Adgre1 mRNA, indicating that macrophages are involved in MMP and TIMP1 production and thereby in the promotion of testicular fibrosis at the inflammation stage.

The changes in integrins and PDGF gene expression observed in EAO may also contribute to the fibrotic phenotype, as these groups of molecules are known as important promoters of fibrosis [52, 53, 75, 76]. The increased levels of Itgav/Itgb3 (encoding integrin αvβ3) and Pdgfb/Pdgfrb mRNAs detected during EAO (dependent on Ccr2 expression) were likely due to the infiltration of macrophages, as Itgav and Pdgfrb correlated positively with Adgre1 expression. Furthermore, PDGF acts as a strong chemoattractant for fibrocytes in pulmonary fibrosis, while pharmacological blockade of the PDGF/PDGFR axis is considered a promising treatment option [77].

Taken together, these in vivo data indicate that CCR2 contributes to the development of testicular fibrosis during inflammation by mediating leukocyte infiltration, cytokine release, and ECM protein production by immune cells, particularly TMs. In addition, our results suggest that CCR2+ TMs may produce multiple factors, including the CXCL12/CXCR4 axis, MMPs, αv integrin, PDGFB, and PDGFββ, which potentially act as downstream signaling molecules to regulate immune processes during testicular inflammation (summarized in Fig. 8).

In addition to CCR2, activin A is another important fibrotic regulator [6, 23]. In the current study, activin A stimulated CCR2 expression (at the mRNA and protein level) in macrophages and reduced Ccl2 levels. Similarly opposing effects of activin A on Ccr2 and Ccl2 expression have been demonstrated in human monocyte-derived macrophages [78]. Our findings indicated that activin A can increase the responsiveness of inflammatory BMDMs to CCL2 by enhancing the expression of CCR2. Furthermore, according to our results, activin A also induced the expression of CXCR4 in macrophages. As CXCR4 is highly expressed by macrophages during testicular inflammation, reduced infiltration by monocytes/macrophages in Ccr2−/− mice during EAO may explain why the level of testicular Cxcr4 mRNA did not increase in CCR2-depleted mice following induction of EAO (see Fig. 8).

Previously, we showed that activin A-induced the expression of fibrosis-specific genes in mouse PTCs and NIH 3T3 fibroblasts. Furthermore, expression of the activin dimer subunit was increased, not only in inflamed mouse testes but also in human testes with impaired spermatogenesis and focal leukocytic infiltrates [13, 24]. Notably, the severity of the induced inflammation and damage was directly proportional to the level of activin A [13, 24]. These findings, together with the effects of Ccr2 deficiency during EAO (including reduced infiltration of macrophages, lower levels of ECM deposition and Inhba) implicate activin A as a key regulator of testicular inflammation and fibrosis, acting through TMs. In vivo inhibition of activin A by overexpression of follistatin clearly demonstrated the role of activin A in the development of testicular inflammation; for example, suppression of the accumulation of TM-derived collagen I by follistatin indicated that activin A affected the progression of testicular fibrosis by regulating pro-fibrotic properties of macrophages. Moreover, the expression of Inhba correlated positively with testicular Mmp and Timp1 expression during EAO. Therefore, elevated levels of MMPs and TIMP1 in EAO testes were associated with both macrophage infiltration and activin A expression.

Activin A treatment influenced the transcriptome of BMDMs in vitro and increased the expression of Fnl, Ccr2, Cxcr4, Mmp2, Mmp14, Itgav, Pdgfa, Pdgfb, and Pdgfrb genes as well as the levels of fibronectin, CCR2, CXCR4, and MMP2 proteins. Moreover, activin A enhanced the migratory ability of macrophages in response to CCL2 stimulation. Interestingly, in addition to increased expression of fibrosis-related genes, activin A-induced transformation to spindle-shaped morphology and diminished F4/80 fluorescence in BMDMs, indicating possible conversion to a fibroblastic phenotype in vitro. Similarly, activin A reportedly reprogrammed pre-tumorigenic macrophages to a tumor-associated phenotype, promoted conversion of CD4+CD25+ naïve T cells into regulatory T cells, and induced a pro-fibrotic transcriptome in fibroblasts, thereby transforming them into myofibroblasts [79–81]. Furthermore, macrophages from the synovium of active rheumatoid arthritis patients, which exhibited pro-inflammatory, macrophage-polarizing properties, were inhibited by the addition of an anti-activin A neutralizing antibody [82].
A. mRNA RE

|        | Mmp2 | Mmp14 | Mmp9 | Timp1 |
|--------|------|-------|------|-------|
| Untreated |      |       |      |       |
| Act A (25) |      |       |      |       |
| Act A (50) |      |       |      |       |
| Act A (25) + FST288 |      |       |      |       |
| Act A (50) + FST288 |      |       |      |       |
| FST288 |      |       |      |       |

**In vitro**

B. Gel Electrophoresis

- Act A (50)
- FST288
- Marker

C. Relative enzymatic activity

D. Mmp2

|        | Mmp2 mRNA RE |
|--------|--------------|
| WT     |              |
| Ccr2⁻⁻⁻ |              |

E. Mmp14

|        | Mmp14 mRNA RE |
|--------|--------------|
| WT     |              |
| Ccr2⁻⁻⁻ |              |

F. Mmp9

|        | Mmp9 mRNA RE |
|--------|--------------|
| WT     |              |
| Ccr2⁻⁻⁻ |              |

G. Timp1

|        | Timp1 mRNA RE |
|--------|--------------|
| WT     |              |
| Ccr2⁻⁻⁻ |              |

H. Mmp14

|        | Mmp14 mRNA RE |
|--------|--------------|
| Adgre1 mRNA RE |              |

**In vivo**
Activin A and CCR2 regulate macrophage function in testicular fibrosis caused by experimental...

Combining these results, we postulate that activin A promotes the progression of testicular fibrosis by inducing the production of fibrosis-related mediators in macrophages. Furthermore, although activin A treatment reduced the expression of Mmp9, Timp1, and Itgb3 in BMDMs, other factors, such as TNF, may increase the levels of MMP9 and TIMP1 in macrophages or other testicular cells, thereby promoting their expression in EAO testes [83]. In addition, cells such as fibroblasts can produce large amounts of β3 integrin in response to inflammatory stimuli, which could underlie the high levels of Itgb3 mRNA observed in EAO testes [84].

Critically, it should be noted that utilizing BMDMs matured in the presence of M-CSF as a surrogate for TMs may not exactly replicate all functional aspects of the TMs. However, M-CSF is a critical regulator of the maturation and tissue-specific functions of TMs [85]. As TMs make up a very small proportion of the normal mouse testis cell population, in vitro experimentation on isolated TMs would require an unreasonably large number of mice. In accordance with 3R principles, TMs were replaced by BMDMs, drastically reducing the number of animals required.

Taken together, our in vivo and in vitro results illustrate that activin A promotes the differentiation of macrophages to a pro-fibrotic phenotype through the induction of a variety of inflammatory mediators and receptor molecules (summarized in Fig. 8). Activin A-stimulated BMDMs are potential fibroblast precursors, which likely facilitate tissue fibrosis. In addition, by inducing CCR2 expression, activin A potentially affects the recruitment of monocytes/macrophages to injury sites. Although our data indicate that CCR2, activin A, and macrophages are responsible for the testicular fibrotic response, the precise immune mechanisms that regulate this process require...
further investigation. Future studies are needed to address the direct influence of activin A using targeted depletion of activin A receptors (in monocytes/macrophages) to analyze their role in inflammatory responses and fibrotic remodeling.

In conclusion, our data indicate that CCR2 and activin A regulate the development of fibrosis during testicular inflammation and underlie the crucial pro-fibrotic function of macrophages in inflammation-associated fibrotic remodeling in EAO. Future therapeutic targeting of CCR2 and/or activin A may offer a possible strategy to address testicular fibrosis.

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Data availability The data presented in this study are available in the article and supplementary material. Transcriptome data are available online at the GEO repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE210004).

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the School of Medicine at the University of Zagreb (380-59-10106-20-111/171). Animal experiments were approved by the responsible local ethics committees on animal care (Regierungspräsidium Giessen GI 58/2014 — Nr. 735-5P and the Monash Medical Centre Animal Experimentation Committee). All experiments involving animals were carried out in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the German law on animal welfare and the Australian Code for the Care and Use of Animals for Scientific Purposes.

Consent to participate Informed consent was obtained from all men included in the study.

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