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CXCL8 hyper-signaling in the aortic abdominal aneurysm☆

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ABSTRACT

There are indications for elevated CXCL8 levels in abdominal aortic aneurysm disease (AAA).

CXCL8 is concurrently involved in neutrophil-mediated inflammation and angiogenesis, two prominent and distinctive characteristics of AAA. As such we considered an evaluation of a role for CXCL8 in AAA progression relevant.

ELISA's, real time PCR and array analysis were used to explore CXCL8 signaling in AAA wall samples. A role for CXCL8 in AAA disease was tested through the oral CXCR1/2 antagonist DF2156A in the elastase model of AAA disease.

There is an extreme disparity in aortic wall CXCL8 content between AAA and aortic atherosclerotic disease (median [IQR] aortic wall CXCL8 content: 425 [141–1261] (AAA) vs. 23 [2.8–89] (atherosclerotic aorta) μg/g protein ($P < 1 \cdot 10^{-14}$)), and abundant expression of the CXCR1 and 2 receptors in AAA. Array analysis followed by pathway analysis showed that CXCL8 hyper-expression in AAA is followed increased by IL-8 signaling (Z-score for AAA vs. atherosclerotic control: 2.97, $p < 0.0001$).

Interference with CXCL8 signaling through DF2156A fully abrogated AAA formation and prevented matrix degradation in the murine elastase model of AAA disease ($p < 0.001$).

CXCL8-signaling is a prominent and distinctive feature of AAA, interference with the pathway constitutes a promising target for medical stabilization of AAA.

1. Introduction

An Abdominal Aortic Aneurysm (AAA) is a common pathology and a major cause of death due to rupture [1]. Most AAAs are asymptomatic and remain undetected until rupture [1]. Hence, some countries instigated nationwide screening programs for the identification of AAA. These programs resulted in a major increase in patients with an identified AAA, most of them small in size.

In accordance to prevailing guidelines these patients with smaller AAAs are kept under surveillance until the AAA reach the threshold for repair at 55 mm. It is estimated that up to 70% of the patients in the watch and follow up program will eventually reach the 55 mm intervention threshold [2]. Accordingly, it has been pointed out that pharmaceutical intervention reducing or inhibiting progression of small AAA, and thus reducing the need for surgical repair could have major advantages; both from a patients' as from a socio-economical perspective [3]. Despite clear preclinical successes, no pharmaceutical intervention has been proven to be effective so far [4].

The pathology of growing AAAs is thought to be a localized chronic inflammatory response that is accompanied and perpetuated by exaggerated angiogenesis and a proteolytic imbalance; the latter is being held responsible for a progressive weakening of the aortic wall [1]. The actual molecular basis has not been identified.

We previously documented CXCL8 hyper-expression as a clear distinctive and unique feature of AAA with 300-fold higher CXCL8 protein levels in the aneurysm wall than in advanced aortic atherosclerotic wall samples [5,6]. CXCL8 has comprehensive chemotactic effects on a wide-variety of immune cells, in particular *but* not-exclusively on

Abbreviations: AAA, abdominal aortic aneurysm

☆ The DF2156A was a generous gift from Dompé S.p.A., l'Aquila, Italy.

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neutrophils; a cell type that is explicitly implicated in AAA disease [5,7,8]. Moreover, CXCL8 stimulates protease expression and inflammation [9], and exerts strong pro-angiogenic effects by promoting chemotaxis and proliferation of endothelial cells [10–12].

In this context, we considered further examination of a putative role for CXCL8 signaling as a potential therapeutic target in AAA disease relevant. The present study confirms the CXCL8 hyper-expression and exaggerated activation of the CXCL8 downstream pathways in human aneurysms, and shows that interference with CXCL8 signaling through the oral CXCR1/2 antagonist DF2156A fully abrogates aneurysm formation in an accepted model of AAA disease (the murine elastase model).

2. Methods

2.1. Human samples

Collection and handling of the aneurysm and control aortic wall samples was performed in accord with the guidelines of the Medical and Ethical Committee Leiden University Medical Center, Leiden, The Netherlands, and the "code of conduct for responsible use" by the Dutch Federation of Biomedical Scientific Societies (https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf) [13]. Plasma samples used were from the Aneurysm-Express biobank [14]. This study is approved by the Medical Ethics Committees of the participating hospitals, and all participants provided written informed consent.

We obtained tissue from anterior-lateral aneurysm wall during elective surgery for asymptomatic AAA (> 5.5 cm or larger). Aortic tissue samples removed along with the renal artery during kidney explantation from brain-dead, heart-beating, adult organ donor, were used as control samples. Aortic wall samples were divided in two parts. One half was immediately snap-frozen in CO2-cooled isopentane or liquid N2 and stored at −80 °C for later analysis. The other half was fixed in 4% formalin for 12 h and decalcified. The latter segments were paraffin embedded and 4 μm sections were processed into slices.

For immunohistochemistry, sections (n = 10 AAA, n = 10 control atherosclerotic aortic wall samples) were deparaffinized, treated for 10 min with H_2O_2 to block endogenous peroxidase activity, and incubated overnight at room temperature with the primary antibody diluted in PBS-1% albumin. The following primary antibodies were used: human myeloperoxidase (A398, DAKO, Amstelveen, The Netherlands), CXCL8 (bs-078012, Bioss, Huissen, The Netherlands), CXCR1 (ab124344, Abcam, Cambridge UK), CXCR2 (bs-1629R, Bioss), pERK1/2 (1481-1 Epitomics, Leiden, The Netherlands) and phospho-PKCΔ/ϕ (CST 9376S, Cell Signaling, Leiden, The Netherlands).

CXCL8 mRNA expression was quantified by semi quantitative RT, to that end a total RNA extraction was performed according to manufacturer's instructions. cDNA was prepared by using a Promega kit (Promega, Leiden, the Netherlands) for RT-PCR. For the determination of mRNA expression we used an established CXCL8 primer/probe set (Thermo Fisher Scientific, Bleiswijk, The Netherlands), the mastermix (Eurogentec, Maastricht, the Netherlands) and the ABI-7700 system (Thermo Fisher Scientific) as previously described [13]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Thermo Fisher Scientific) was used for normalization.

Aortic wall CXCL8 protein content was determined using the Aneurysm-Express Biobank [14] (n = 238 AAA samples and n = 26 control atherosclerotic samples) via ELISA, employing Luminex multi-analyte profiling technology [15], using a bio-plex system (Bio-Rad, Veenendaal, the Netherlands). Total protein concentration of every sample was quantified via a BCA protein measurement method (Thermo Fisher Scientific). All measured concentrations were related to the protein concentrate of every sample. Inter-assay coefficient of variation was < 10%.

Microarrays: RNA extraction was performed from full thickness aortic wall samples from 31 AAA patients (mean age 69.5 yrs. mean diameter 62.3 ± 12.1 mm) collected during elective aneurysm repair and 9 control samples (infra renal aorta obtained during kidney procurement for donation).

RNA from aneurysm wall was labeled and hybridized to Illumina HumanHT-12 v4 BeadChips (Illumina, Eindhoven, the Netherlands). Arrays were scanned with an Illumina iScan microarray scanner. Bead level data preprocessing was done in Illumina GenomeStudio.

Analysis of array data: Quantile normalization and background reduction were performed according to standard procedures in the Illumina GenomeStudio software. Gene expression data have been deposited at Gene Expression Omnibus under the GEO Accession number GSE98278.

Association of genome-wide expression data with AAA phenotype revealed 11,486 transcripts with $P < 0.05$. These differentially expressed transcripts were used as an input for pathway analysis through Ingenuity Pathway Analysis suite (http://www.ingenuity.com, accessed 2016). Levels of significance were determined using Fisher's exact tests implemented in the software.[16]

2.2. Elastase model

All murine investigations were approved by the Leiden University Medical Center animal welfare committee and were in compliance with the Dutch government guidelines.

Eight-to-ten weeks old, male, C57BL/6 mice were obtained from Charles River, France. The aneurysms were created via porcine pancreatic elastase (PPE) infusion as previously described [17-20]. After the elastase infusion 0.05–0.1 mg/kg/12 h buprenorfine was given and the mice recovered with free access to food and water. The oral CXCR1/2 antagonist DF2156A (6 mg/kg), a generous gift from Dompé Pharma, Milan, Italy [21] was given (n = 10) daily via oral gavage in 100 μl of 0.25% carboxymethylcellulose in PBS. Treatment was started the day before the elastase infusion and the mice were sacrificed 14 days after the infusion. Control animals (n = 10) received daily oral gavage of 100 μl of 0.25% carboxymethylcellulose in PBS for 15 days.

To compare the aortic growth rates of the different groups we measured the maximum axial diameter of the aorta by means of ultrasound one day prior to elastase infusion, after one week and two weeks after infusion by means of the Vevo 770 Imaging system using RMV 704 microvisualization scan head (Visualsonics, CA).

At day 14 after the elastase infusion, the mice were sacrificed and the aorta was removed, and embedded in paraffin for later analysis. Immunohistochemical sections were deparaffinized and incubated overnight at room temperature with the primary antibody diluted in PBS -1% albumin. The sections were incubated with CD45 (BD Pharmingen, Breda, The Netherlands), MAC3 (BD Pharmingen), MMP9 (Santa Cruz Biotechnology) and MPO (Abcam). Additional sections were stained with Sirius Red for collagen and Weigert's elastin stain to visualize elastic laminae. Six slides per animal were used per staining for analysis and only moderate or strongly reactive cells were counted as positive. The slides were blindly evaluated. The mean value for positive staining cells on six slices was calculated for each animal.

2.3. Statistical analysis

All values are shown as mean (SD) and probability values of $P < 0.05$ were considered statistically significant. After performing an ANOVA test to explore the difference between human AAA and human atherosclerotic samples, an unpaired *t*-test was performed.

The Mann-Whitney *U* test was used to detect significant difference in aortic diameter and in cell count between the two groups of mice.

All analysis were performed using SPSS 23.0 (IBM, Amsterdam, The Netherlands).

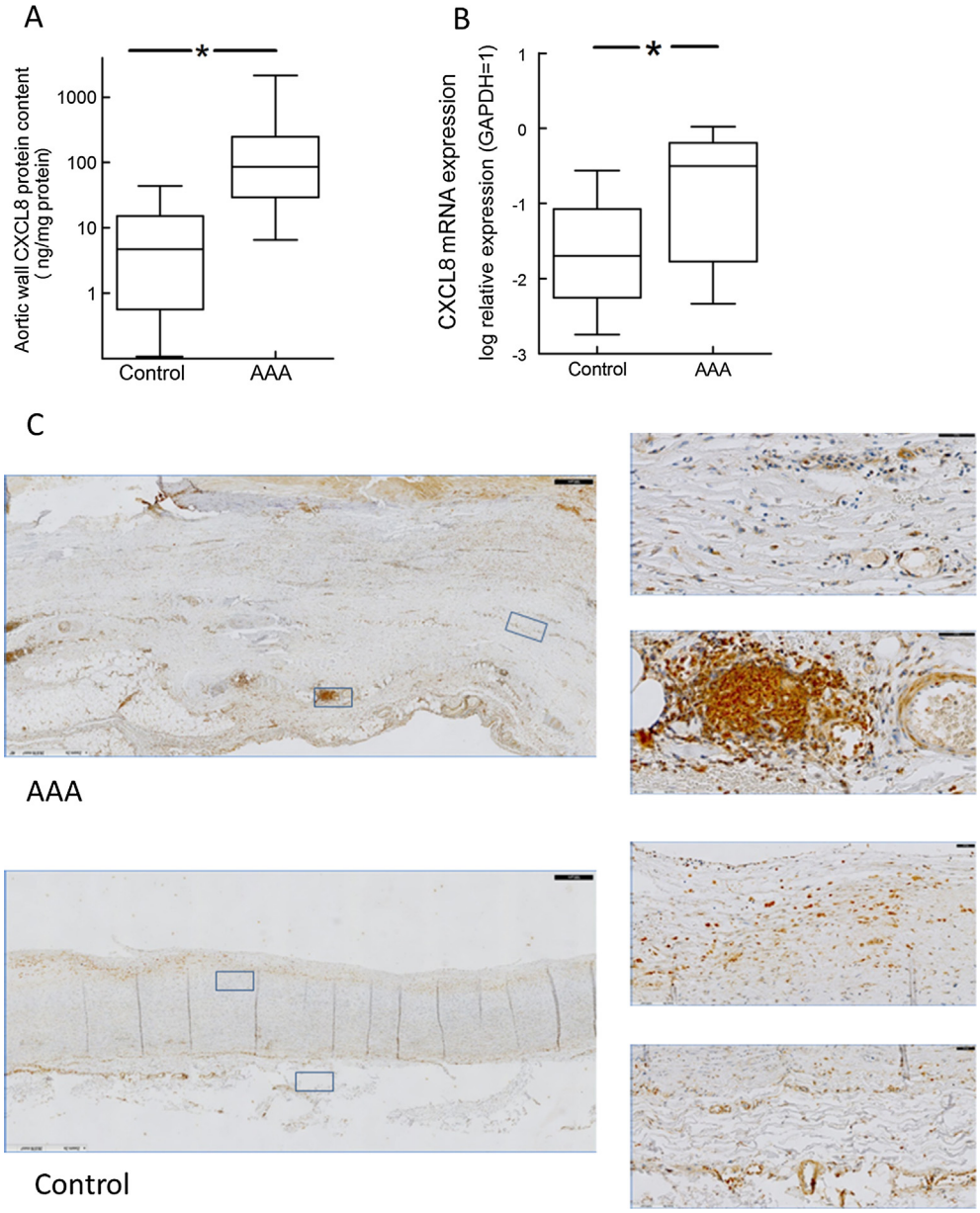

Fig. 1. CXCL expression in human AAA and atherosclerotic ,control aorta wall. (A) Relative aortic wall CXCL8 protein content in AAA and atherosclerotic control aorta (CXCL8 protein expression normalized on basis of protein content). AAA vs controls: $P < 1.5 \cdot 10^{-15}$. (B) Relative aortic wall CXCL8 mRNA content in AAA and atherosclerotic control aorta (mRNA expression normalized on basis of GAPDH expression). AAA vs Control, $P < 0.01$. (C) Representative samples illustrating aortic wall CXCL-8 distribution (immunohistochemistry) in AAA and control aorta. Overview 5X (bar = 500 μm), details 40× (bar = 50 μm).

3. Results

3.1. CXCL8 expression in human abdominal aortic aneurysms

We first performed a validation of our previous observations of CXCL8 hyper-expression in 238 AAA wall samples and control aorta samples from the Aneurysm-Express Biobank[14]. Results confirmed previous observations and showed a several hundred-fold increase CXCL8 protein content in aneurysm wall samples ($P < 0.0001$, Fig. 1A) and an approximately 16-fold higher CXCL8 mRNA expression ($P < 0.01$, Fig. 1B) . Immunohistochemical staining for CXCL-8 in the aneurysm wall shows comprehensively expression in macrophages, neutrophils, and smooth muscle cells; as well as in a subpopulation of lymphocytes, and occasional endothelial cells (Fig. 1C). CXCL-8 expression in advanced aortic atherosclerotic disease on the other hand is essentially confined to foam cells, macrophages, and occasional smooth muscle cells the intimal layer and intimal border zone of the medial layer of the aortic wall, and incidental lymphocytes (Fig. 1C).

3.2. Abundant presence and activation of the CXCL8- pathway in human abdominal aortic aneurysms

CXCL8 signaling in humans is mediated through the chemokine receptors: CXCR1 and 2. CXCL8 binding to these receptors results in phosphorylation of factors such as the ERK (extracellular signal regulated protein kinase) 1/2 and PKCΔ/ϕ. Immunohistochemical analysis shows abundant expression of both the CXCR1 and CXCR2 receptors (Fig. 2), and enhanced ERK and PKCΔ/ϕ phosphorylation in AAA compared to aortic atherosclerotic disease (Fig. 3A and B). CXCL8 signaling was further explored through Ingenuity-based transcriptomics analysis. This analysis identified CXCL8 (IL-8) signaling pathway (Z-score for AAA vs. atherosclerotic control: 2.97, $P < 0.0001$) and granulocyte adhesion and diapedesis ($P < 0.00001$, Z-score not available) among the top enriched pathways in AAA disease (Fig. 3C,

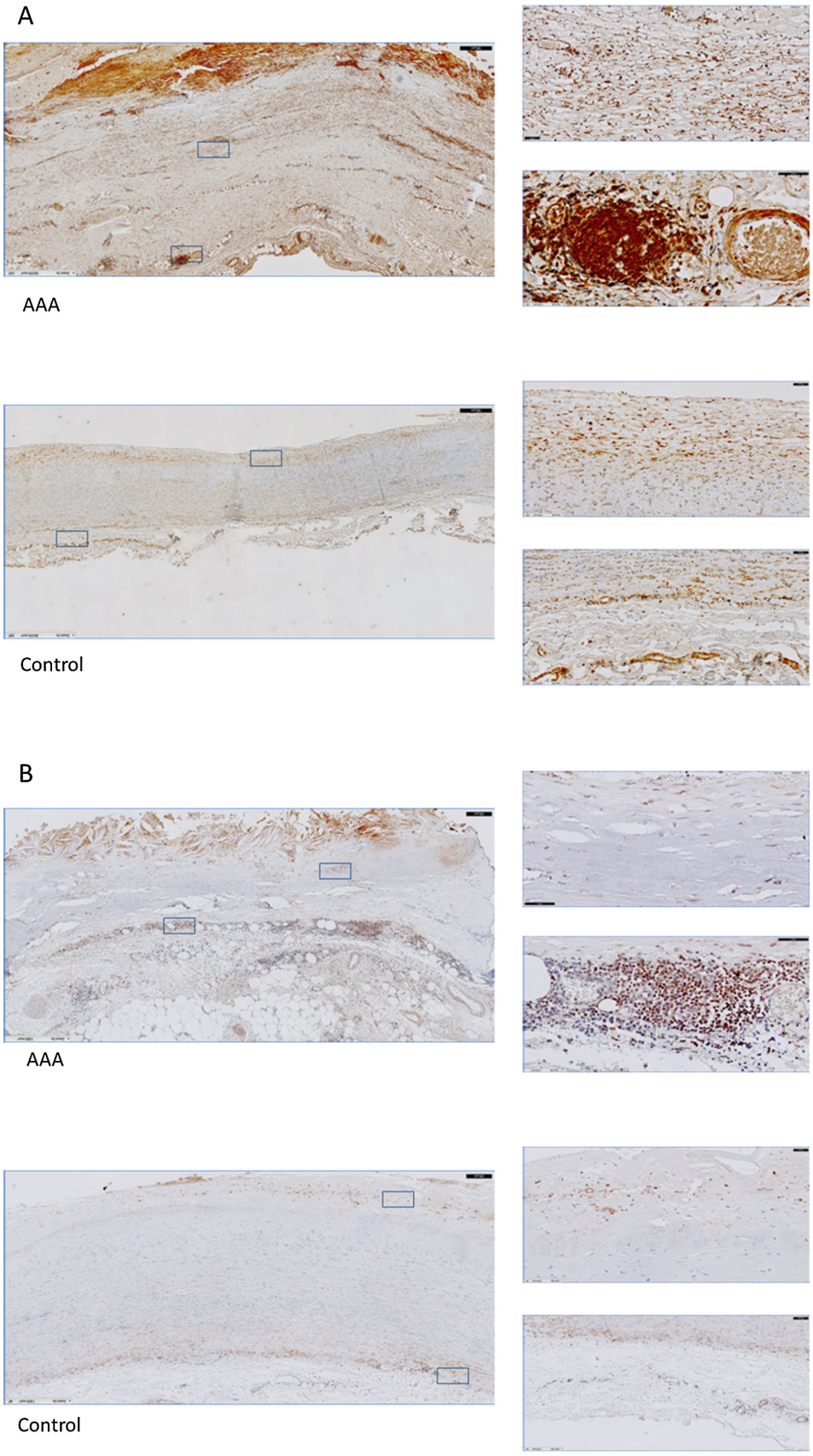

Fig. 2. CXCR1 and CXCR2 expression in human AAA and atherosclerotic ,control aorta wall. (A) Representative immunohistochemical staining showing aortic wall CXCR1 distribution in AAA and atherosclerotic control aorta. Overview 5X, detail 40×. (B) Representative immunohistochemical staining showing aortic wall CXCR2 distribution in AAA and atherosclerotic control aorta. Overview 5X (bar = 500 μm), details 40× (bar = 100 μm).

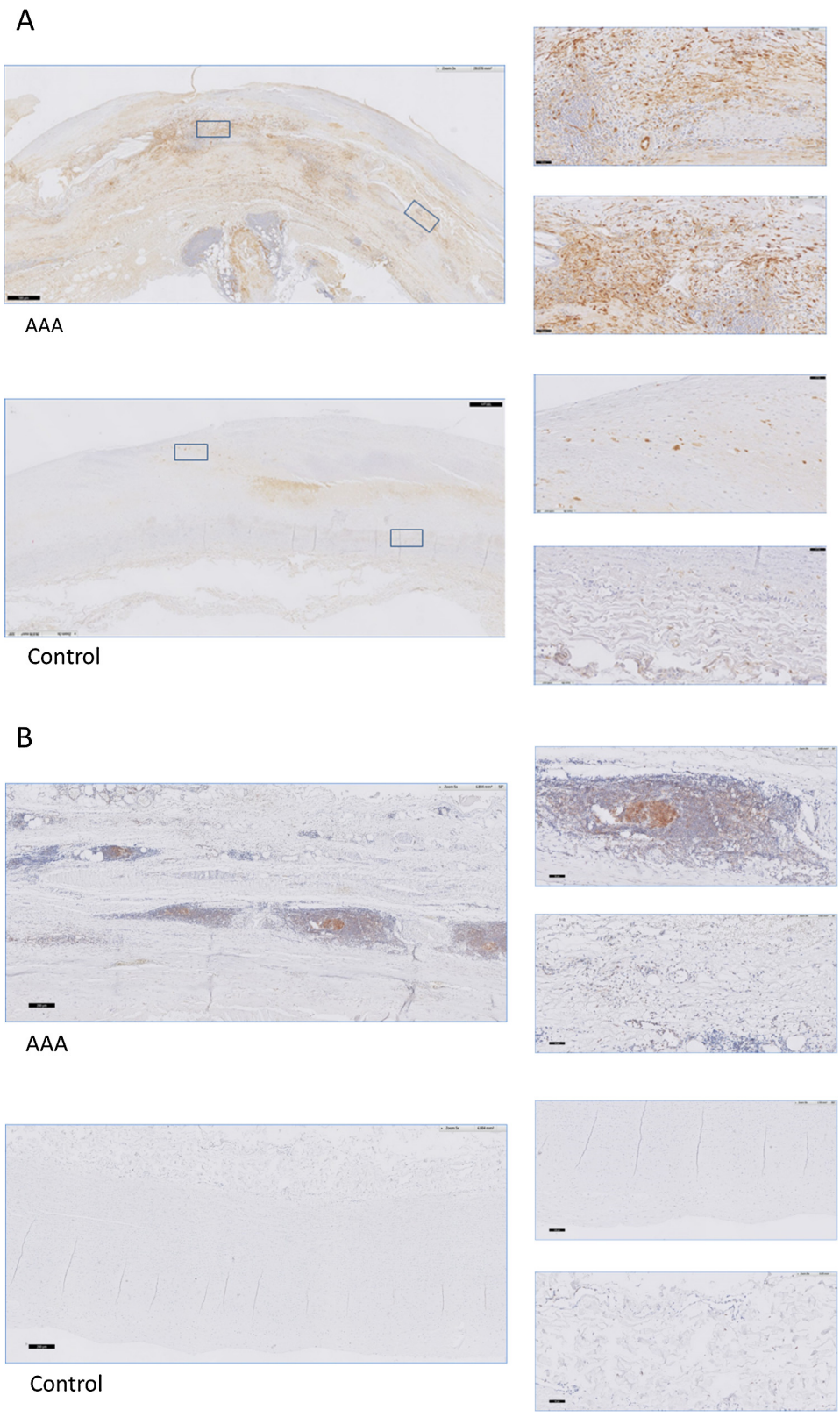

Fig. 3. CXCL-8 signaling in human AAA and atherosclerotic ,control aorta wall. (A and B) Representative immunohistochemical staining showing aortic wall phosphoERK1/2 (A) and pPCKΔ/ϕ (B) distribution in AAA and control aorta. Overview 5X (bar = 500 μm), details 40× (bar = 100 μm). (C) CXCL-8 signaling pathway is upregulated in AAA vs atherosclerotic control aorta (Z-score for AAA vs. atherosclerotic control: 2.97 ($p < 0.0001$), Ingenuity Pathway Analysis). Map shows the significantly up (red) and downregulated (green) genes and their putative localization in signaling cascade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

both $p < 0.0001$).

CXCL8 has particularly strong effects on neutrophil chemotaxis, stabilization and activation, and is described to be the dominant promoter of CXC chemokine-mediated angiogenesis. Histologic evaluation shows abundant and dispersed neutrophils (MPO staining) in human aneurysms, while neutrophils are absent in control

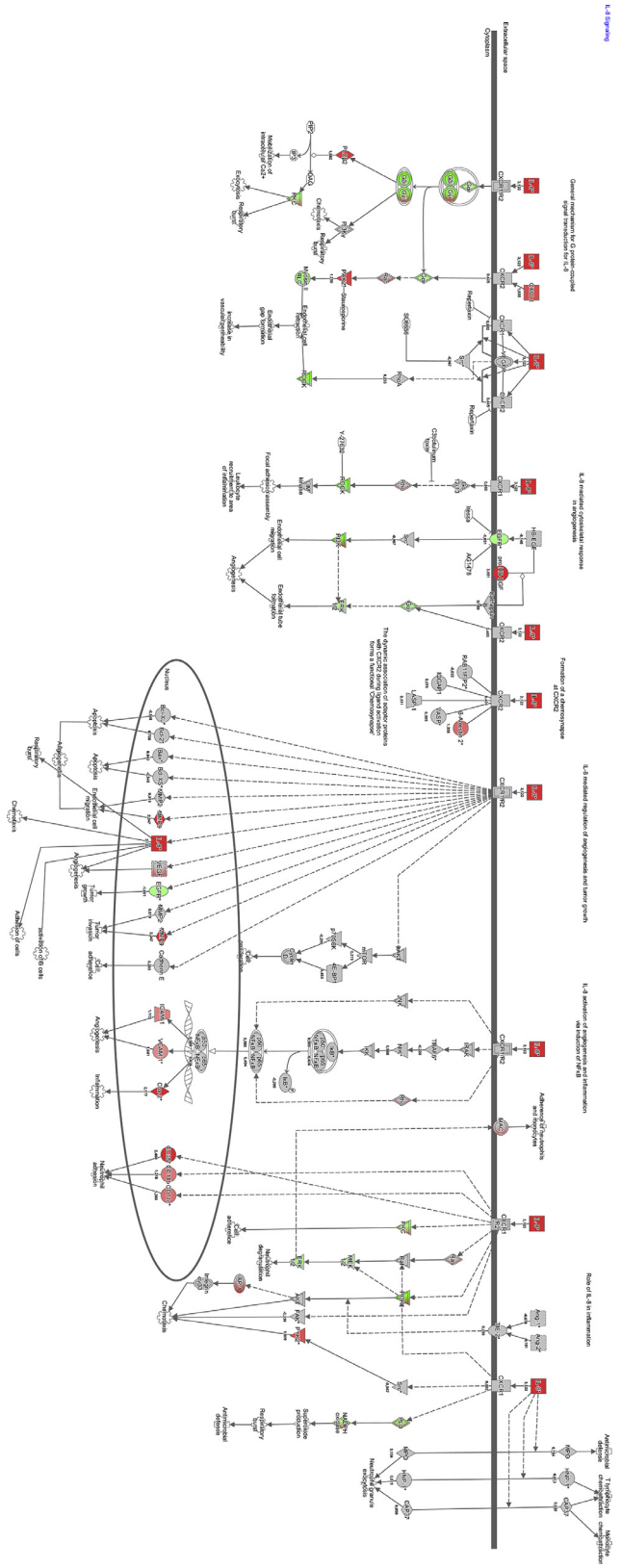

Fig. 3. (*continued*)

atherosclerotic samples (Fig. 4). Occasional neutrophils in the vaso vasora confirmed the validity of the staining. This characterizes neutrophils abundance as a clearly distinctive feature of human AAA.

3.3. CXCR1/2 inhibition abrogates aneurysm formation.

To evaluate possible involvement of the CXCL8 in aneurysm formation, we tested whether the oral CXCR1/CXCR2 inhibitor (DF2156A) influences aneurysm formation in the established murine elastase model of the disease. Mice (n = 10) received DF2156A during two weeks via daily oral gavage starting from the day before elastase perfusion. Control animals (n = 10) received daily oral gavage with saline. At the day before elastase perfusion, 7 days and 14 days after perfusion the aortic diameter was measured via ultrasound.

Aortic dilatation at day 7 was similar in both groups (12.7% SD ± 8.5% (DF2156A) and 21.5% SD ± 14.9% (vehicle), $p = 0.16$). A daily gavage completely abolished aneurysm formation, measured at day 14, in all animals (17.7% dilatation SD ± 9.6%, (DF2156A) and 71.9% SD ± 26.7% (vehicle) $p < 0.001$) (Fig. 5).

DF2156A treatment almost completely quenched leucocyte infiltration and preserved the integrity of the vessel wall as shown by an increased collagen content and less elastin breaks (Fig. 6). Furthermore, treatment with the CXCR1/CXCR2 inhibitor resulted in significantly less leucocytes ($p < 0.05$) and reduced MMP9 content ($p < 0.05$) compared to the controls (Fig. 6a and b). While all mice revealed similar macrophage counts ($p = 0.98$) (Fig. 6b), indicating that the effect on vascular inflammation is highly selective.

4. Discussion

This study confirms CXCL8 hyper-expression and enhanced activation of the CXCL8 axis as a distinctive feature of AAA disease. Interference with the oral CXCL8 antagonist fully abrogated AAA formation, characterizing this axis as a potential pharmaceutical target for AAA.

Our previous work identified CXCL8 hyper-expression as a distinctive and prominent feature of human AAA disease.[13] We first performed an independent confirmatory validation of the CXCL-8 hyper-expression on aneurysm wall samples from a distinct, and large patient cohort (Aneurysm Express) using a different analysis platform (Luminex). Immunohistochemistry for CXCL8 distribution in AAA disease indicated comprehensive expression in both leucocytes as well as mesenchymal cells. This pattern was clearly distinct from advanced atherosclerotic disease in which expression was predominantly confined to the intima, in particular to foam cells.

In humans CXCL8 signals through the CXCR1 and -2 receptors. These receptors have different affinities, suggesting distinct responses at varying CXCL8 levels. Although the activities are thought to largely overlap, there are indications that they may mediate distinct aspects of CXCL-8 mediated inflammation [22]. Reportedly, both receptors share a broad expression pattern, which includes a wide variety of leucocytes , mesenchymal cells (smooth muscle cells, fibroblasts) and endothelial cells. Immunohistochemical double staining (not shown) for CXCR1 and 2 showed abundant receptor expression both leucocytes as well as smooth muscle cells/myofibroblasts of the aneurysm wall. Observations above not only identify CXCL8 hyper-expression as a clear distinctive feature between AAA and atherosclerotic disease, but also show that the transcriptional machinery required for CXCL8 signaling is present in AAA. Exaggerated CXCL8 signaling in AAA compared to atherosclerotic wall specimens was shown by Ingenuity-based pathway which ranked CXCL-8 signaling and granulocyte adhesion and diapedesis among the top upregulated pathways in AAA, and by enhanced ERK1/2 and PKCΔ/ϕ phosphorylation in AAA wall.

CXCL8 classically associates with neutrophil influx and neutrophil-mediated inflammation. CXCL8 not only acts as a strong chemo-attracted for neutrophils, but it also increases neutrophil tissue content by increasing neutrophil half-life by preventing apoptosis [23]. Infiltrating neutrophils may critically contribute to the proteolytic imbalance of AAA disease through release of multiple matrix degrading proteases such as the serine protease neutrophil elastase, and metalloproteinases

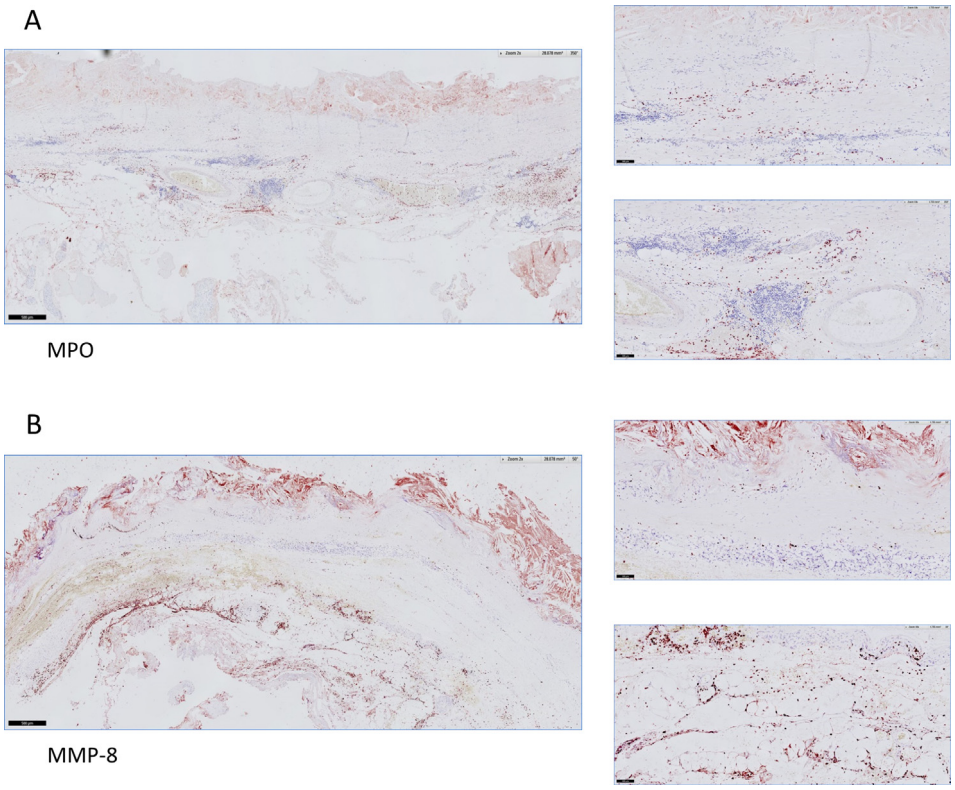

Fig. 4. Neutrophil abundance in human AAA. 4 Representative immunohistochemical staining showing aortic wall neutrophil distribution (Myeloperoxidase (MPO) and MMP-8 (Neutrophil collagenase)) in AAA and control aorta. Overview 5X (bar = 500 μm), details 40× (bar = 100 μm).

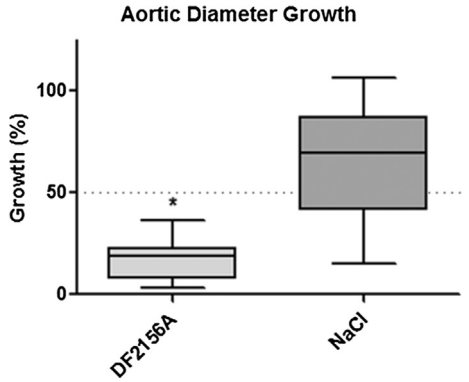

Fig. 5. The CXCR1/2 antagonist DF2156A fully abrogates AAA formation. Relative change (% change from baseline diameter) in aorta diameter 14 days after elastase treatment. The oral CXCR1/2 antagonist DF2156 prevents aneurysm formation in the elastase model of AAA disease ($p < 0.001$).

MMP8 (neutrophil collagenase) and MMP9 (neutrophil gelatinase). Neutrophil-derived proteases further impair the proteolytic imbalance by their ability to inactivated endogenous protease-inhibitors such as TIMPs (through the action of neutrophil elastase) and cystatin C (through MMP9 and neutrophil elastase) [5]. This latter response appears responsible for the secondary TIMP-1 and Cystatin C deficiencies in AAA [5]. Experimentally, a crucial role for neutrophils in AAA disease is emphasized by several animal studies in which interference with either neutrophil activation or infiltration alleviates AAA formation and/or progression [8,24,25].

Apart from its classical role on neutrophils, CXCL8 also influences other leucocytes (in particular M1 macrophages [26]) thereby further contributing to the perpetuation of a pro-inflammatory environment [27], and exerts potent pro-angiogenic effects. Angiogenesis is a characteristic feature that has been linked to vascular inflammation and AAA rupture, and as such has been brought forward as a therapeutic target for pharmaceutical AAA stabilization [28].

The CXCL8-signaling pathway has long been identified as potential pharmacological target for several acute and chronic inflammatory conditions [21,29-34]. Combined CXCR1/2 [30,30,32] and selective CXCR2 inhibitors are currently under clinical evaluation [31,32,34].

We tested the ability of the combined CXCR1/2 antagonist DF2156A to inhibit AAA formation in an established murine model of the disease. A single daily dose strongly reduced AAA formation; in fact the minimal dilatation observed presumably reflects the effect of pressure-perfusion and/or the loss of elastic recoil by the elastase treatment, and not the influx of any inflammatory cells. The effects exerted by DF2156A in the elastase model by far exceeded the effects reported for other established anti-inflammatory agents, such as doxycycline [35], indomethacin [36] or cyclosporin [37]. Apart from interference with CXCL8 signaling, CXCR1/2 inhibitors may also interfere with MIF an alternative, but more promiscuous CXCR1/2 ligand [38]. This ligand has been implicated in AAA disease [39], but is only moderately upregulated in human AAA disease compared the aortic atherosclerotic disease [13], and is not identified as a differentially upregulated pathway in the Ingenuity pathway analysis.

Considering the apparent failure of medical stabilization of small AAA so far, the potency of CXCR1/2 inhibition in vivo is remarkable and merits clinical evaluation. As mice only express CXCR2, we could not test the contribution of each receptor to the process of clinical AAA formation. Consequently it is unclear whether clinical trial with selective CXCR2 inhibitors would be equally effective as a combined CXCR1/2 antagonist.

In conclusion, to our knowledge, this study is the first to demonstrate full abrogation of aneurysm formation in the murine elastase model, emphasizing the critical role of the CXCR2-axis in aneurysm formation in the model. This and along with the clinical data, identifies activation of the CXCL8-pathway as a distinctive feature of AAA and characterizes this pathway as a promising (possibly the most promising)

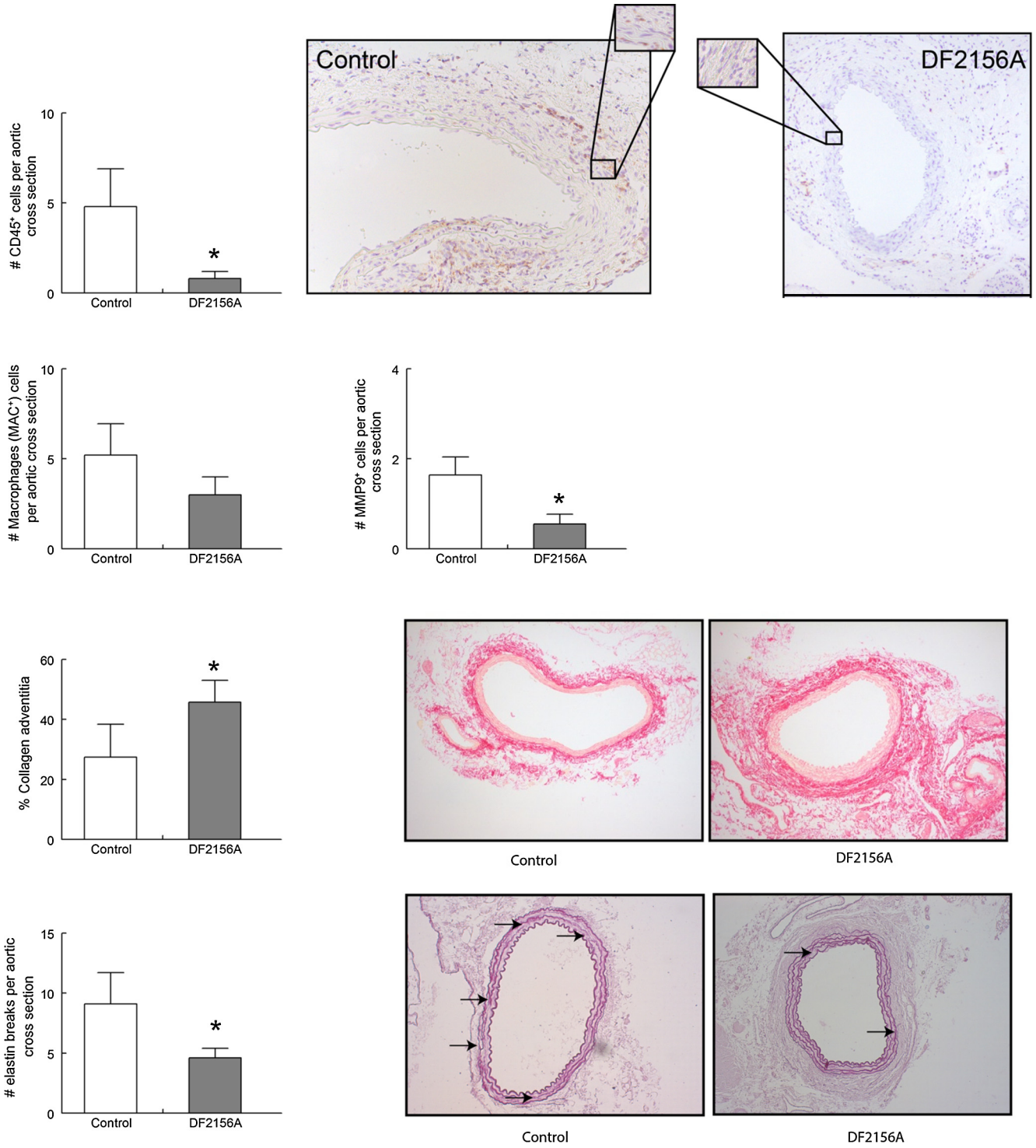

Fig. 6. The CXCR1/2 antagonist DF2156A quenches vascular inflammation and preserves the aortic matrix following elastase infusion. The oral CXCR1/2 antagonist DF2156 quenches vascular inflammation (reduced leucocyte (CD45 staining), macrophage content (MAC3 staining) and MMP9 content) and preserves the aortic matrix (adventitial collagen content and medial elastin breaks) following elastase infusion. All data shown is for day 14 following elastase exposure.

target for the medical stabilization of growing AAA.

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Conflict of interest

The authors declare that no conflict of interest exists.

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