ORIGINAL RESEARCH

Tumor Necrosis Factor-Like Weak Inducer of Apoptosis or Fn14 Deficiency Reduce Elastase Perfusion-Induced Aortic Abdominal Aneurysm in Mice

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***Background*—**Abdominal aortic aneurysm (AAA) involves leukocyte recruitment, inflammatory cytokine production, vascular cell apoptosis, neovascularization, and vascular remodeling, all of which contribute to aortic dilatation. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a cytokine implicated in proinflammatory responses, angiogenesis, and matrix degradation but its role in AAA formation is currently unknown.

***Methods and Results*—**Experimental AAA with aortic elastase perfusion in mice was induced in wild-type (WT), TWEAK deficient (TWEAK KO), or Fn14-deficient (Fn14 KO) mice. TWEAK or Fn14 KO deficiency reduced aortic expansion, lesion macrophages, $CD3^+$ T cells, neutrophils, $CD31^+$ microvessels, CCL2 and CCL5 chemokines expression, and MMP activity after 14 days postperfusion. TWEAK and Fn14 KO mice also showed a reduced loss of medial vascular smooth muscle cells (VSMC) that was related to a reduced number of apoptotic cells in these animals compared with WT mice. Aortas from WT animals present a higher disruption of the elastic layer and MMP activity than those from TWEAK or Fn14 KO mice, indicating a diminished vascular remodeling in KO animals. In vitro experiments unveiled that TWEAK induces CCL5 secretion and MMP-9 activation in both VSMC and bone marrow-derived macrophages, and decrease VSMC viability, effects dependent on Fn14.

***Conclusions*—**TWEAK/Fn14 axis participates in AAA formation by promoting lesion inflammatory cell accumulation, angiogenesis, matrix-degrading protease expression, and vascular remodeling. Blocking TWEAK/Fn14 interaction could be a new target for the treatment of AAA. **(*J Am Heart Assoc*. 2014;3:e000723 doi: 10.1161/JAHA.113.000723)**

Key Words: aneurysm • Fn14 • inflammation • MMP activity • TWEAK

Abdominal aortic aneurysm (AAA) is a disease that affects ≈5% of elderly men and is responsible for a high number of deaths in Western countries.[1] AAA is characterized in most cases by the formation of intraluminal thrombus, destructive remodeling of structural connective tissue, and chronic adventitial inflammation.[2] AAA rupture can be prevented by elective open surgical or endovascular aneurysm repair.[3] However, surgery is currently the only therapy for individuals with AAA. No drug treatments have been approved for use in this disease,[1,4] which greatly highlights the need for a better understanding of disease pathophysiology in order to implement novel management procedures and therapeutic strategies.

Little is understood about the mechanisms that initiate AAA. The formation and development of AAA is characterized by aortic wall inflammation characterized by infiltration of macrophages, T cells, neutrophils, and dendritic cells into the pathological aortic wall that plays a key role in driving the progressive and pathological remodeling of the aorta.[5–7] In addition, pathological features of aneurysm include smooth muscle cell apoptosis, increased oxidative stress, and significant matrix remodeling.[8]

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a proinflammatory cytokine belonging to a TNF superfamily that induces a high number of physiological and pathological processes depending on cell type and environment.[9] The sole functional receptor for TWEAK is fibroblast

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growth factor-inducible gene family that codes for a 14 kDa protein (Fn14). In the vasculature, TWEAK is expressed in normal arteries,[10,11] while Fn14 is expressed at low levels or is absent in healthy tissues.[10] In previous studies, we have demonstrated that both TWEAK and Fn14 are expressed in pathological wall including human atherosclerotic plaques[10] and AAA.[12] TWEAK is implicated in different processes associated with vascular remodeling such as inflammation,[13] proliferation and migration of vascular cells,[14] thrombosis,[15] and angiogenesis.[14,15] In fact, systemic administration of TWEAK aggravates atherosclerotic plaque development in the aortic root of apolipoprotein E (ApoE) knockout mice and conversely short-term anti-TWEAK mAb treatment reduced lesion severity and local inflammation.[16]

The role of TWEAK/Fn14 axis in AAA development has not been previously studied. We hypothesized that altering expression of the TWEAK/Fn14 axis would affect extracellular matrix remodeling and local inflammatory response, allowing us to modify AAA disease progression and thereby potentially offer a novel molecular target for therapy.

Methods

Cell Culture

Aortic vascular smooth muscle cells (VSMC) were isolated from aorta of wild-type and Fn14-knockout mice. Briefly, adhering fat and connective tissue were removed by blunt dissection from the thoracic aorta. Vessels were opened longitudinally and preincubated in DMEM (Whitaker) containing 1 mg/mL collagenase (type II, 290 U/mg), penicillin (100 U/mL), streptomycin (100 μg/mL), and glutamine (2 mmol/L) (Sigma) for 15 to 20 minutes at 37°C in 95% air/5% CO_2. Then aortas were minced into 1-mm pieces, incubated for an additional 1.5 to 2 hours, and rinsed twice with PBS to remove the cells, which were counted and seeded at a concentration of 10^4 cells/cm^2 in plastic culture flasks (Costar) in DMEM with 10% FBS. Cells were harvested for passaging at 2- to 3-day intervals and used between the second and seventh passages. For experimental analysis, cells were made quiescent by 24-hour incubation in medium without FBS.

Bone marrow-derived macrophages (BMDM) were isolated from femurs of WT, TWEAK, and Fn14 KO mice. Briefly, mice were sacrificed and hind legs were dissected and sterilized with 70% ethanol. Femurs were isolated removing all muscle tissue. The bones were cut at both ends and 1 mL of BMM medium (DMEM supplemented with 10% L929-Conditioned medium and 10% FBS) was flushed with a syringe and a 25-gauge needle. Cells were seeded and differentiated to macrophages in a humidified incubator with 5% CO_2 at 37°C. After 6 days, cells were depleted to 2% FBS for 18 hours and stimulated with recombinant murine TWEAK (R&D Systems) at different times.

Human AAA Samples

Ten AAA wall samples were collected during surgical repair and were obtained from patients undergoing surgery, enrolled in the RESAA protocol.[17] Ten control aortas were sampled from dead organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal and devoid of early atheromatous lesions.

Animals

Fn14-knockout mice (FN14 KO), TWEAK knockout mice (TWEAK KO), and wild-type (WT) counterparts (generously provided by Biogen Idec) have been reported previously and backcrossed onto the C57BL/6 strain.[18,19] Twelve-week-old male mice underwent aortic perfusion with 0.411 U/mL type I porcine pancreatic elastase (E1250; Sigma) (Fn14 KO [N=15], TWEAK KO [N=16] and littermates WT [N=16]) to produce experimental AAA or saline serum (Fn14 KO [N=6], TWEAK KO [N=5] or WT [N=5]) as a control, as previously described.[20]

Briefly, anesthetized mice underwent laparotomy, and the abdominal aorta was isolated from the level of the left renal vein to the bifurcation. Temporary silk ligatures were placed around the proximal and distal portions of the aorta, and an aortotomy was created at the bifurcation with a 30-gauge needle. Heat-tapered polyethylene tubing (PE-10; Baxter Healthcare Corp) was introduced through the aortotomy and secured with a silk tie. Using a syringe pump calibrated to 100 mm Hg (Sage Instruments), the aorta was filled with saline containing 0.414 U/mL Type I porcine pancreatic elastase (specific activity 5 U/mg protein; E1250; Sigma Chemical Co). For controls, saline was used. The perfusion catheter was then removed and the aortotomy was closed with a 10-0 suture to avoid constriction.

External preperfusion and 14-day postperfusion aortic diameters were measured to a resolution of 0.01 mm with a calibrated ocular grid while mice were under anesthesia and physiological blood pressure. Aortic diameter expansion ≥100% of that before perfusion defined AAA.[20] To harvest AAA tissues, we removed the aortic zone of maximal expansion and OCT or paraffin embedding for cross-section preparation.

All mice were maintained under barrier conditions. Water and normal laboratory diet was available ad libitum. The housing and care of animals and all the procedures carried out in this study were strictly in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committee of IIS-Fundación Jimenez Diaz.

Immunohistochemical Analysis

To analyze the presence of TWEAK, Fn14, and MMP-9, human AAA samples were stored in paraformaldehyde for 24 hours and later in ethanol until paraffin embedded. Tissues were cross-sectioned into 5-μm-thick pieces, dewaxed and rehydrated, and incubated overnight with anti-TWEAK (AF1090; R&D Systems), anti-Fn14 (4403; Cell Signaling Technology), and anti-MMP-9 (LS-B2486; LifeSpan BioSciencesm Inc). Negative controls using the corresponding IgG were included to check for non-specific staining. Biotinylated secondary antibodies were applied for 1 hour. Then avidin-biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories) was added for 30 minutes. Sections were stained with 3,3′-diaminobenzidine (Dako), counterstained with haematoxylin, and mounted in Pertex.

Immunohistochemical analysis was done in all animals included in each group. Immunohistochemistry was carried out as previously described.[16] Primary antibodies were the monocyte/macrophage marker MOMA-2 (MCA519G; AbD Serotec), the smooth muscle cell marker smooth muscle actin (Clone 1A4; Sigma), the neutrophil marker Ly-6B.2 (MCA7716A; AbD Serotec), the endothelial cell marker CD31 (550274; BD Pharmingen), the lymphocyte marker CD3 (A0452; Dako), the chemokines CCL2 (sc-1785; Santa Cruz Biotechnology) and CCL5 (AB2109P; Chemicon), the proliferation marker Ki-67 (Clone SP-6; Abcam), and the apoptosis marker cleaved-PARP (ab32064; Abcam). Apoptosis in the AAA was also determined using the TUNEL method as recommended by the manufacturer (ApopTag Peroxidase In Situ Apoptosis Detection Kit, S7100; Millipore Ibérica).

Donkey anti-goat biotin, donkey anti-rabbit biotin, and goat anti-rat biotin (Amersham) were used as secondary antibodies. ABComplex/HRP was then added and sections were stained with 3,3′-diaminobenzidine (DAKO), counterstained with hematoxylin, and mounted in Pertex (Medite). Incubation without primary antibodies and/or irrelevant species- and isotype-matched immunoglobulins was used as a negative control for all immunostaining. Computer-assisted morphometric analysis was performed with the Image-Pro Plus software (version 1.0 for Windows). The threshold setting for area measurement was equal for all images. Samples from each animal were examined in a blinded manner. Results were expressed as % positive area versus total area of macrophages, neutrophils, MCP-1, and RANTES and as positive cells per square millimeter of whole lesion of CD3 and CD31, Ki-67, cleaved-PARP, and TUNEL.

Elastin Degradation and VSMC Paucity Grade

Verhoeff Van Gieson stain was performed using Elastic Stain Kit (Sigma-Aldrich, HT25A-1KT) according to manufacturer's protocol. Elastin fragmentation and VSMC content were graded as described previously.[21] Briefly, elastin preservation was graded as followed: grade 1, intact, well-organized elastin laminae; grade 2, elastic laminae with some interruptions and breaks; grade 3, elastic laminae with multiple interruptions and breaks; grade 4, severe elastin fragmentation or loss. VSMC content in the tunica media was graded using the following key: grade 1, intact VSMC; grade 2, minimal abnormalities; grade 3, loss of few VSMC; and grade 4, loss of VSMC in prolonged areas of the tunica media. Thus, the higher grade indicates poorer VSMC preservation.

Fluorescence Molecular Tomography

To detect areas of vascular remodeling, mice were injected intravenously with the imaging probe MMPSense-680 (2 nmol in 150 μL PBS; PerkinElmer) 13 days post elastase perfusion. MMPSense-680 is activated by key matrix metalloproteinases (MMPs) including MMP-2, -3, -9 and -13 and becomes highly fluorescent following activation. Twenty-four hours later, aortas were removed under deep anesthesia with Ketamine/Xylazine (100 and 10 mg/kg body weight, respectively). Fluorescent molecular tomography was performed with a chamber imaging system (FMT 1500; VisEn Medical, PerkinElmer). Tomographic images were acquired in ex vivo aortas, and the amount of MMP activity was evaluated with the aid of the FMT1500 software package.

Gelatin Gel In Situ Zymography

Mouse VSMC or BMDM were seeded in 24-well multiplates (Corning) at an initial concentration of 10^4 cells/mL in 1 mL of DMEM containing 10% FBS per well and cultured overnight. Cells were then serum starved at 2% FBS per well and cultured overnight. After that, medium was replaced with 0.5 mL of 2% FBS DMEM containing different concentrations of mouse recombinant TWEAK (R&D Systems). After 72 hours, VSMC supernatant was collected, centrifuged for removing debris, and concentrated with a 10 kDa microcon (Millipore) to a final volume of 25 μL. The MMP activity was assessed by in-gel gelatinase zymogram (Zymogran gels, Life Technologies), following the manufacturer's instructions. For tissue, AAA wall, or thoracic aorta from different animal groups were homogenized and 10 μg were also assessed by in-gel gelatinase zymogram.

RNA Extraction and Real-Time PCR

Human AAA wall tissues were snap frozen in N_2 liquid and homogenates (0.2 g) were divided and resuspended for mRNA analysis. Total RNA from VSMC or AAA tissues was obtained by TRIzol method (Life Technologies) and quantified by

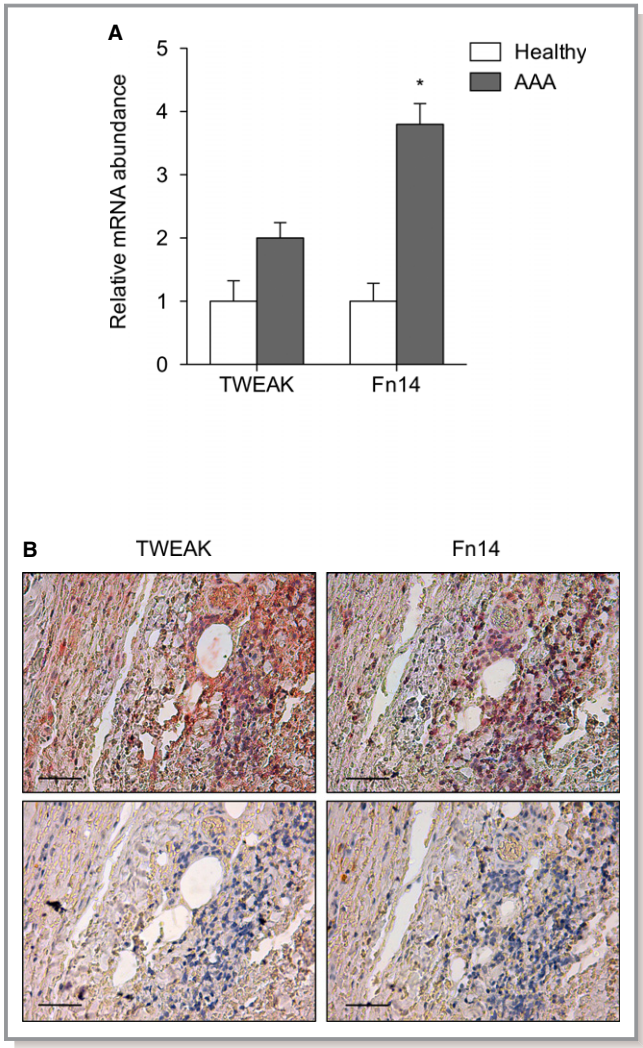

Figure 1. TWEAK and Fn14 are expressed in human AAA. A, Quantitative real-time polymerase chain reaction analysis of TWEAK and Fn14 mRNA expression from healthy aorta or AAA tissue. Values shown are mean±SE. N=6 per group. *P<0.005 vs healthy subjects. B, Representative staining of TWEAK and Fn14 expression in serial section of human AAA lesions. Negative controls were incubated with non-specific IgG. Scale bar, 100 μm. AAA indicates abdominal aortic aneurysm; TWEAK, tumor necrosis factor-like weak inducer of apoptosis.

absorbance at 260 nm in duplicate. Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated TaqDNA polymerase (Amplitaq Gold). After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. 18S rRNA served as housekeeping gene and was amplified in parallel with the genes of interest. 18S rRNA served as housekeeping gene and was amplified in parallel with the genes of interest. The expression of target genes was normalized to housekeeping transcripts. The following PCR primers and TaqMan probes were purchased from Applied Biosystems and optimized according to the manufacturer's protocol: 18S, human TWEAK (Hs00387540) and Fn14 (Hs00171993), and mouse MMP2 (Mm00439498), MMP3 (Mm00440295), MMP9 (Mm00442991), and MMP13 (Mm00439491). All measurements were performed in triplicate. The amount of target mRNA in samples was estimated by the 2ΔCT relative quantification method. Values of each sample were obtained as multiples of their baseline values.

Methylene Blue Assay

Mouse VSMC were seeded in 24-well multiplates (Corning) at an initial concentration of 10^4 cells/mL in 1 mL of DMEM containing 10% FBS per well and cultured overnight. Cells were then serum starved during a 24-hour period. After that, medium was replaced with serum-free DMEM containing from 0 to 0.5 U/mL of elastase from porcine pancreas Type I (Sigma, E1250) and with or without mouse recombinant TWEAK (R&D Systems) at 100 ng/mL. After 18 hours, the number of VSMC was assessed by methylene blue incorporation assay. Cells were fixed in 10% formaldehyde saline for 30 minutes, and then incubated with 1% (w/v) methylene blue in 0.01 mol/L phosphate buffer for 30 minutes. The remaining dye was washed off by serially dipping the plate into each of 4 tanks of 0.01 mol/L borate buffer (pH 8.5). To elute the dye, 100 μL of 1:1 (v/v) ethanol and 0.1 mol/L HCl were added to each well. Plates were then gently shaken and absorbance at 650 nm (A650) was measured for each well using a microplate photometer. The percent changes in A650 were calculated and compared.

Enzyme-Linked Immunosorbent Assay

CCL-2 and CCL-5 concentrations in cell supernatants were determined in duplicate with commercially available enzyme-limked immunosorbent assay (ELISA) kits (MJE00 and MMR00; R&D Systems, respectively).

Statistical Analysis

Statistical analysis was performed using SPSS 11.0 statistical software. Due to abnormal data distribution, we selected the nonparametric Mann-Whitney U test for non-paired data sets. In vitro experiments were replicated at least 3 times. A probability value of 0.05 was considered to be statistically significant.

Results

TWEAK and Fn14 are Expressed in Human AAA

It has been demonstrated that although TWEAK is expressed in healthy aortic wall, Fn14 expression is almost

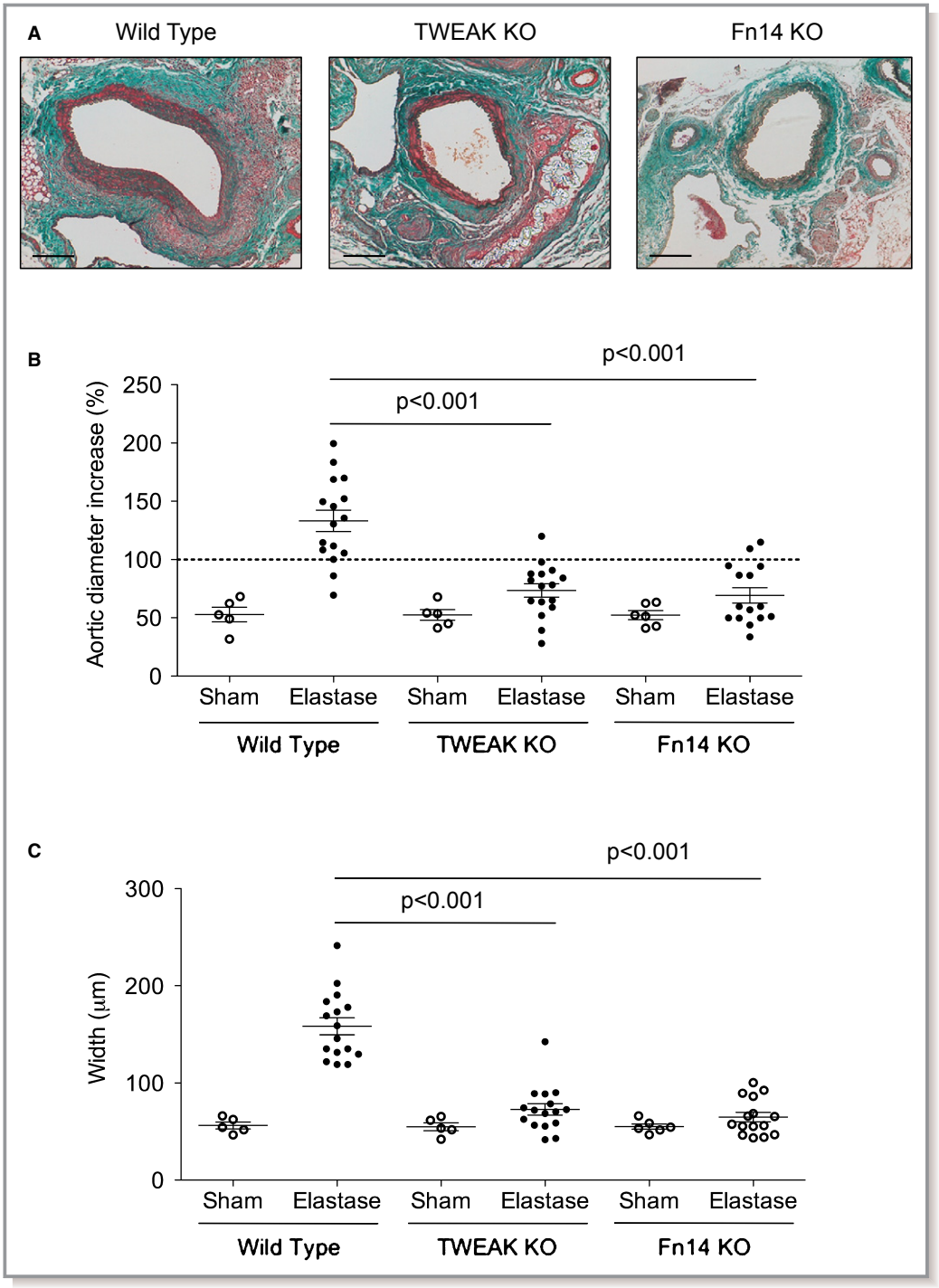

Figure 2. Reduced abdominal aortic aneurym formation in TWEAK KO and Fn14 KO mice. A, Representative Masson's trichrome staining in AAA lesions from WT, TWEAK KO and Fn14 KO mice at 14 days postperfusion Scale bar, 100 μm. B, Aortic diameters increase (percentage), and (C) maximal intimal-medial thickness (thickness of the artery wall) from mice at day 14 postperfusion with elastase (WT [N=16], TWEAK KO [N=16] and Fn14 KO [N=15]) or saline (WT [N=5], TWEAK KO [N=5] and Fn14 KO [N=6]). AAA indicates abdominal aortic aneurysm; KO, knockout; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; WT, wild type.

absent.[10] In order to know whether TWEAK and/or Fn14 is upregulated in human AAA wall, mRNA was extracted from AAA wall tissue and healthy aorta (N=6). Real-time PCR showed that TWEAK is expressed and Fn14 is upregulated in human AAA ($P<0.05$ versus healthy aorta; Figure 1A). The presence of TWEAK and Fn14 was confirmed by

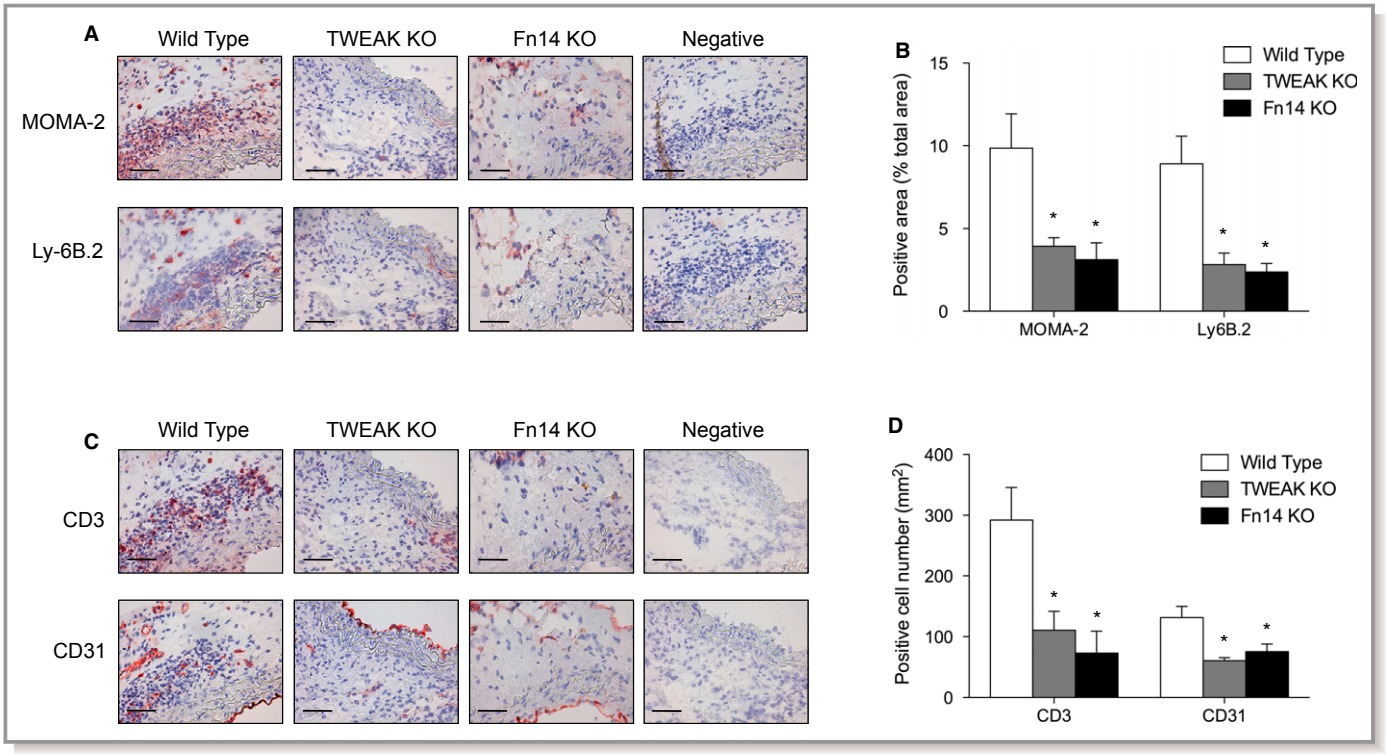

Figure 3. TWEAK or Fn14 deletion reduced inflammatory cell content in abdominal aortic aneuryms. A, Representative staining and (B) quantification of positive staining of macrophages by MOMA-2 and neutrophils by Ly-6B.2 in AAA lesions from TWEAK KO (N=9), Fn14 KO (N=9) and WT (N=10) mice at 14 days postperfusion. Data are mean±SE. **P*<0.05 vs WT. Negative controls were incubated with non-specific IgG. Scale bar, 50 μm. C, Representative staining and (D) quantification of $CD3^+$ and $CD31^+$ cells in AAA lesions from TWEAK KO (N=9), Fn14 KO (N=9) and WT (N=10) mice at 14 days postperfusion. Data are mean±SE. **P*<0.05 vs WT. Negative controls were incubated with non-specific IgG. Scale bar, 50 μm. AAA indicates abdominal aortic aneurysm; KO, knockout; MOMA, monocyte macrophage antibody; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; WT, wild type.

immunohistochemistry in serial sections from human AAA tissues (Figure 1B).

TWEAK or Fn14 Deficiency Protected Mice From Elastase Perfusion-Induced AAA

To test the role of TWEAK/Fn14 axis in AAA development, we performed elastase aortic perfusion to induce AAA in TWEAK and Fn14-deficient mice and their littermates WT mice. Development of AAA was defined by a 100% increase in aortic diameter.[20] Analysis at 14 days postperfusion showed significant attenuation of aortic diameter in TWEAK KO and Fn14 KO mice compared with control animals (Figures 2A and 2B; $P<0.001$ for both). Whereas 14/16 WT mice developed AAA 14 days after elastase perfusion (87% incidence) only 1/16 animal developed AAA in TWEAK KO and 2/15 in Fn14 KO group (6% and 13% incidence, respectively) ($P<0.001$ for both). Cross-sectioning of the infrarenal region of the aorta also revealed a reduction in maximal intimal-medial thickness in TWEAK and Fn14 KO animals compared with WT mice (Figure 2C). As a control, sham-operated animals of each genetic modified group and WT mice were analyzed and they did not show a significant aortic dilation (Figures 2B and 2C).

TWEAK or Fn14 Deficiency Reduces AAA Lesion Inflammatory Cell Content

The presence of inflammatory cell infiltration in AAA lesions is a hallmark of disease progression.[8] The absence of TWEAK or its functional receptor Fn14 reduced monocyte/macrophages ($P<0.05$ and $P<0.01$, respectively) and neutrophils ($P<0.005$ for both) accumulation in AAA lesions compared with WT mice (Figures 3A and 3B). In addition, the number of $CD3^+$ T-cell was also diminished in TWEAK or Fn14 KO mice compared with control animals ($P<0.01$ and $P<0.001$, respectively; Figures 3C and 3D). Angiogenesis within the wall of an aortic aneurysm may play key roles in aneurysm progression as well as rupture.[22] Reduced AAA growth and inflammatory cell content in TWEAK or Fn14-deficient mice might be caused by impaired angiogenesis. To test this hypothesis, aortic sections of WT, TWEAK KO, and Fn14 KO mice were immunostained with anti-mouse CD31, a marker of endothelial cells. The presence of microvessels was reduced in both TWEAK and

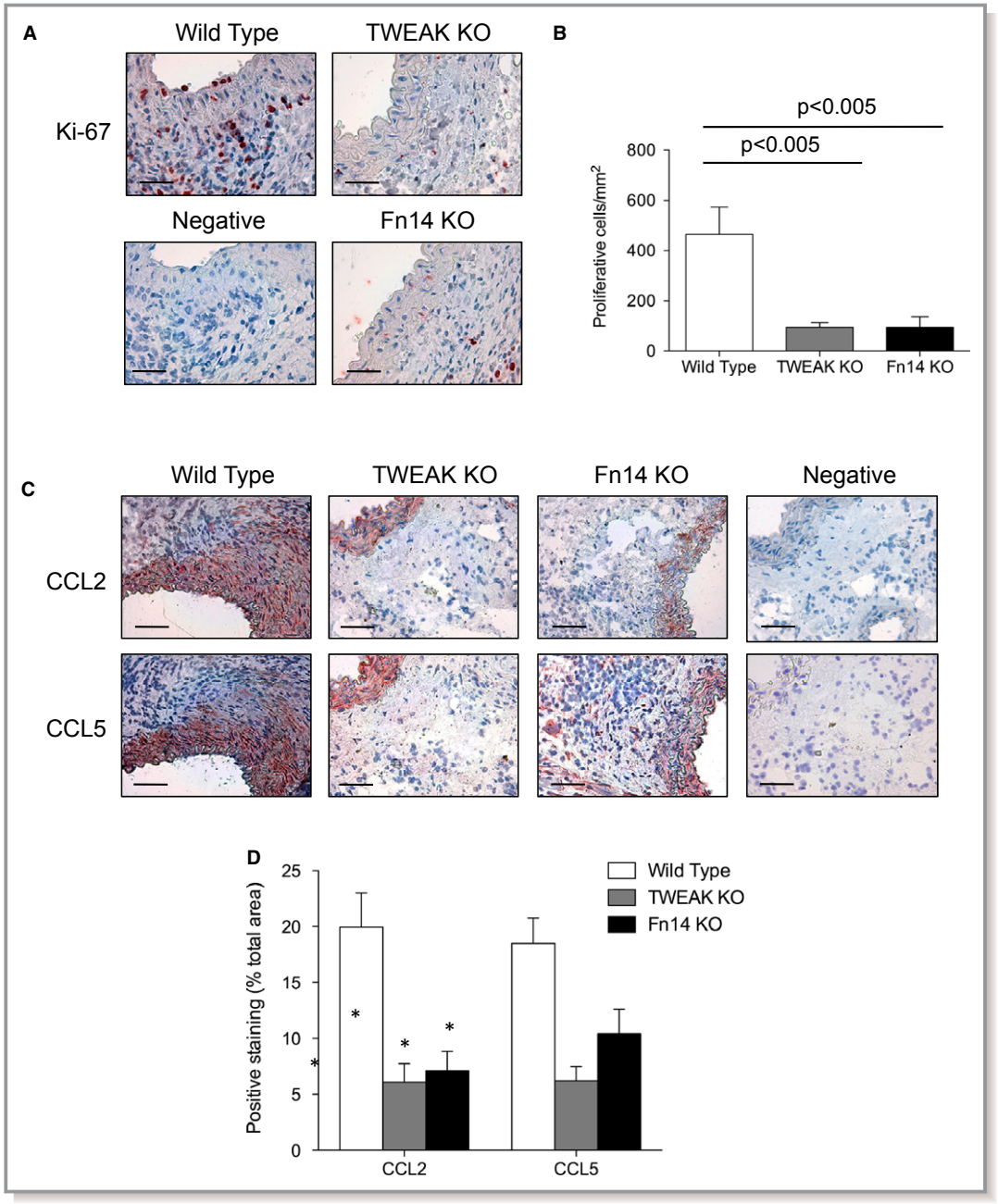

Figure 4. TWEAK or Fn14 deletion diminished pro-inflammatory chemokine expression in abdominal aortic aneurysm. A, Representative staining and (B) quantification of positive staining of Ki-67 in AAA lesions from TWEAK KO (N=7), Fn14 KO (N=7) and WT (N=7) mice at 14 days postperfusion. Values shown are mean±SE. Negative controls were incubated with non-specific IgG. Scale bar, 50 μm. C, Representative staining and (D) quantification of positive staining of CCL2 and CCL5 in AAA lesions from TWEAK KO (N=9), Fn14 KO (N=9) and WT (N=10) mice at 14 days postperfusion. Values shown are mean±SE. **P*<0.005 vs WT. Negative controls were incubated with non-specific IgG. Scale bar, 50 μm. E, Effect of TWEAK on CCL2 and CCL5 secretion in cultured VSMC. WT or Fn14 KO VSMC were incubated with 0.01 to 0.1 μg/mL TWEAK during 24 hours and supernatants were tested by ELISA for CCL2 or CCL5. Values shown mean±SE. **P*<0.005 vs basal. F, Effect of TWEAK on CCL2 and CCL5 secretion in cultured BMDM. WT or Fn14 KO VSMC were incubated with 0.01 to 0.1 μg/mL TWEAK during 24 hours and supernatants were tested by ELISA for CCL2 or CCL5. Values shown mean±SE. **P*<0.01 vs basal. AAA indicates abdominal aortic aneurysm; BMDM, bone marrow-derived macrophages; CCL2, Chemokine Ligand 2; CCL5, Chemokine Ligand 5; ELISA, enzyme-limked immunosorbent assay; KO, knockout; MCP-1, Monocyte Chemoattractant Protein 1; RANTES, Regulated on Activation, Normal T-cell Expressed and Secreted; TW, TWEAK; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; VSMC, vascular smooth muscle cells; WT, wild type.

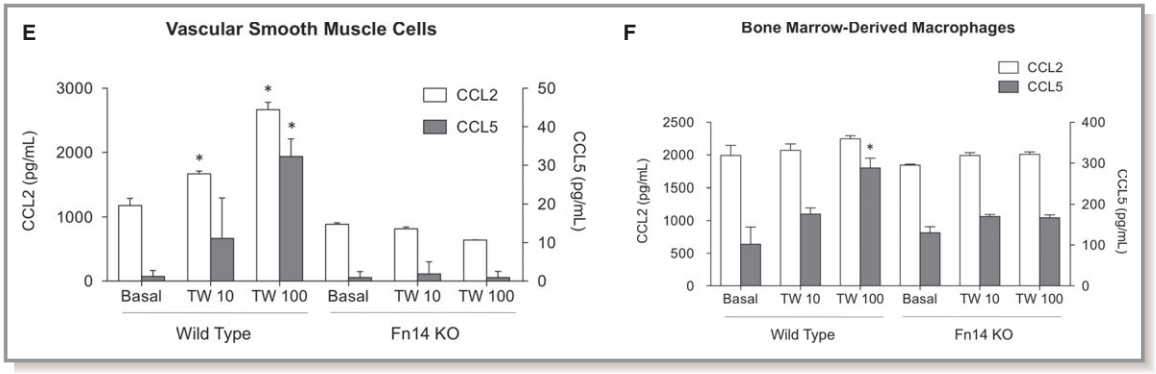

Figure 4. (Continued).

Fn14 KO mice compared with WT mice ($P<0.001$ and $P<0.05$, respectively; Figures 3C and 3D).

Reduction in inflammatory cells content observed by TWEAK or Fn14 deletion in mouse AAA lesions suggests a role of TWEAK/Fn14 axis in leukocyte recruitment or proliferation. In AAA lesions, Ki67$^+$ proliferating cells were significantly reduced in either TWEAK or Fn14 KO compared with wild-type mice ($P<0.005$ for both; Figures 4A and 4B). Most of Ki67$^+$ cells were located outside of the media layer. In addition, CCL2 and CCL5 participate in the recruitment of inflammatory cells such as monocyte/macrophages and neutrophils in human AAA.[23,24] In AAA lesions, we detected lower CCL2 and CCL5 expression in TWEAK KO or Fn14 mice that in WT animals ($P<0.005$ for all; Figures 4C and 4D). To confirm a direct effect of TWEAK on CCL2 and CCL5 protein expression, VSMC or BMDM were cultured in the absence or presence of recombinant mouse TWEAK (0.01 to 0.1 μg/mL) during 24 hours, and CCL2 and CCL5 secretion was analyzed by ELISA. TWEAK increased both CCL2 and CCL5 secretion in VSMC but only CCL5 secretion in BMDM in a dose-dependent manner ($P<0.01$ versus basal for all; Figures 4E and 4F). This effect was related with the presence of Fn14 since TWEAK did modify neither CCL2 nor CCL5 secretion in VSMC or BMDM from Fn14 KO mice (Figures 4E and 4F).

These observations suggest that reduced amount of macrophages, neutrophils, and CD3$^+$ T cells in TWEAK or Fn14 KO mouse AAA lesions were caused by impaired inflammatory cell recruitment, neoangiogenesis, and/or proliferation.

TWEAK or Fn14 Deficiency Reduces Vascular Cells Apoptosis

Loss of medial VSMC density is a characteristic of human AAA tissues and is related to elastica disruption in tunica media. Immunostaining for α-actin showed a pronounced disorganization of medial VSMC in WT mice compared with either TWEAK or Fn14 KO animals (Figure 5A). Accordingly, lesion medial smooth-muscle cell loss was significantly diminished in TWEAK or Fn14 KO animals compared with WT animals ($P<0.01$ for both; Figure 5B).

Apoptosis may also contribute to loss of medial VSMC.[25] To analyze the contribution of TWEAK/Fn14 axis to apoptosis, aortic sections of WT, TWEAK KO, and Fn14 KO mice were analyzed by TUNEL staining. TUNEL$^+$ cells were reduced in the wall (media and adventitia) and in the media (mainly VSMC) from both TWEAK and Fn14 KO mice compared with WT animals (Figures 5C and 5D). To confirm the presence of apoptosis in AAA lesions, aortic sections of WT, TWEAK KO, and Fn14 KO mice were also immunostained with anti-cleaved-PARP. The presence of cleaved-PARP$^+$ cells were also reduced in the wall and in the media from both TWEAK and Fn14 KO mice compared with WT animals (Figures 5C and 5E).

To analyze the contribution of TWEAK on VSMC viability, we induce VSMC death with recombinant elastase.[26] As expected, elastase decreased VSMC viability in a dose-dependent manner (not shown). In addition, no effect on VSMC viability was observed after incubation with TWEAK alone. However, TWEAK decreased VSMC viability in cells exposed to a non-lethal dose of elastase (≤0.1 U/mL). This effect was dependent on the expression of Fn14 since TWEAK failed to diminish cell viability in Fn14 KO VSMC exposed to any dose of elastase (Figure 5F). These results indicate that TWEAK plays an important role in VSMC death when proteases are activated.

TWEAK is Implicated in Medial Elastin Degradation

Medial elastin fragmentation is a major molecular mechanism of AAA development.[27] Verhoeff-van Gieson staining showed a reduction in elastin fragmentation in AAA lesions from TWEAK and Fn14 KO mice compared with those from WT mice (Figures 6A and 6B). We also monitored MMP activity in ex vivo aorta with a protease activatable fluorescent imaging agent (MMPSense 680) using Fluorescence Molecular

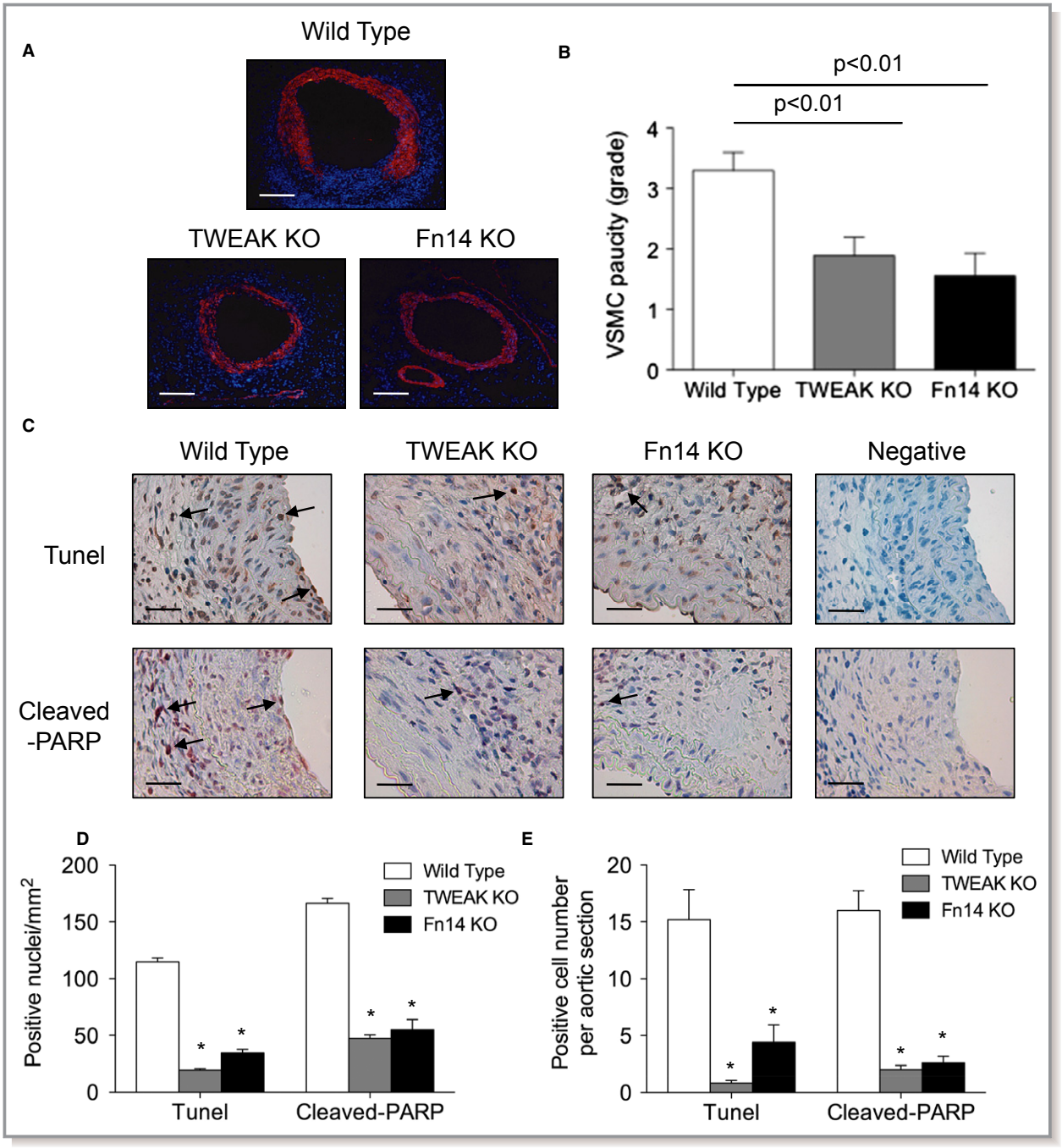

Figure 5. TWEAK or Fn14 deletion reduced VSMC loss in abdominal aortic aneurysm. A, Representative staining of α-actin (red) in AAA lesions from WT, TWEAK KO and Fn14 KO mice at 14 days postperfusion. Nuclei were counterstained with DAPI (blue). Scale bar, 100 μm. B, Evaluation of VSMC preservation by grading (higher scores indicate more irregularity and loss of VSMC) in TWEAK KO (N=9), Fn14 KO (N=9) and WT (N=10) mice at 14 days postperfusion. Values shown are mean±SE. C, Representative staining of TUNEL$^+$ and cleaved-PARP$^+$ cells in AAA lesions from WT, TWEAK KO and Fn14 KO mice at 14 days postperfusion. Arrow indicates TUNEL$^+$ or cleaved-PARP$^+$ cell. Scale bar, 50 μm. Negative controls were incubated with non-specific IgG for cleaved-PARP or without TdT for TUNEL. D, Quantification of TUNEL$^+$ or cleaved-PARP$^+$ cells per square milimeters of AAA lesion or (E) medial TUNEL$^+$ or cleaved-PARP$^+$ cell number per aortic section in WT (N=7), TWEAK KO (N=7) and Fn14 (N=7) KO mice at 14 days postperfusion. Values shown are mean±SE. *P<0.05 vs control. F, Effect of TWEAK in VSMC viability. WT or Fn14 KO VSMC were incubated with 100 ng/mL in the absence or presence of elastase (0.01 to 0.5 U/mL) during 18 hours. Number of VSMC was assessed by methylene blue incorporation assay. Values represent percentage of living cells relative to control cells. Values shown mean±SE. *P<0.05 vs control. AAA indicates abdominal aortic aneurysm; DAPI, 4'-6diamidino-2-phenylindole; KO, knockout; PARP, Poly ADP ribose polymerase; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; VSMC, vascular smooth muscle cells; WT, wild type.

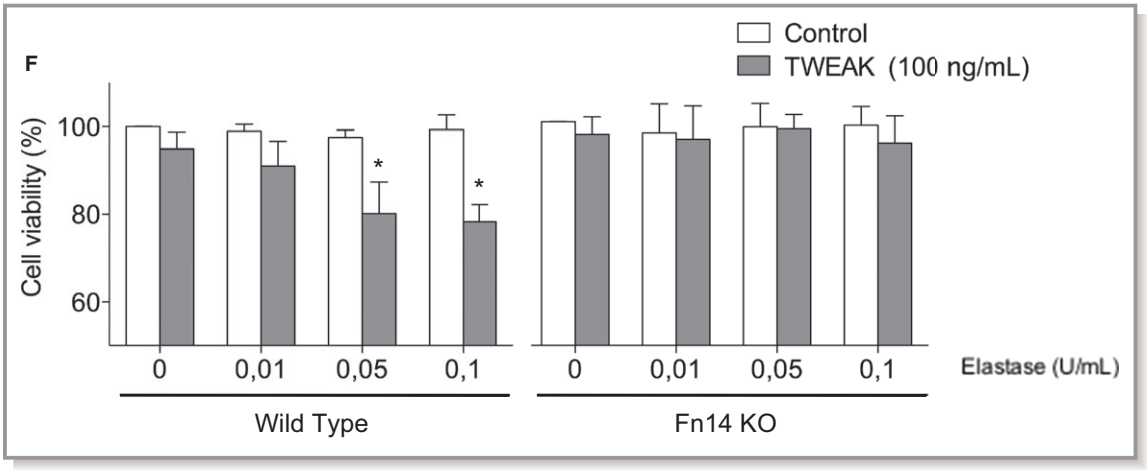

Figure 5. (Continued).

Tomography (FMT) imaging system. MMPSense 680 is activated by metalloproteinsases-2, -3, -9, and -13. Figure 6C represents a 2D data set of fluorescence derived from MMPSense 680 in WT, TWEAK, and Fn14 KO mice after induction of AAA. Clear fluorescence signal as a result of MMP activity was detected in the aorta from WT mice at the position where AAA was developed (Figure 6D). Aorta from TWEAK or Fn14 KO showed reduced MMP activity compared with WT mice ($P<0.05$ and $P<0.01$, respectively; Figure 6B). Furthermore, we observed that MMP-3 and MMP-9 activities were reduced in AAA wall from TWEAK or Fn14 KO mice compared with WT mice, as demonstrated by a gelatin gel zymogram assay (Figure 6E). No MMP activity was observed in thoracic aorta from any studied group.

To confirm a direct effect of TWEAK on MMP activity, VSMC or BMDM were cultured in the absence or presence of TWEAK (100 ng/mL) and MMP-2, -3, -9, and -13 mRNA expression was analyzed. TWEAK increased MMP-9 mRNA expression but not MMP-2, -3, or -13 in VSMC from WT mice ($P<0.01$ versus control; Figure 7A). In addition, TWEAK also increased MMP-3 and MMP-9 activity in BMDM from WT mice ($P<0.05$ versus control for both; Figure 7D) This effect was dependent on the presence of Fn14 since TWEAK did not modify MMP-3 or MMP-9 mRNA expression in VSMC or BMDM from Fn14 KO mice (Figures 7A and 7D). Gelatin gel zymogram also showed that TWEAK increased MMP-9 activity, but not MMP-2 or MMP-3, in cultured VSMC or BMDM from WT mice but not from Fn14 KO (Figures 7B, 7C, 7E, and 7F).

Discussion

Development, progression, and ultimate rupture of AAA involve complex pathological mechanisms. Identification of underlying causes and successful medical treatment remains a major challenge in modern vascular medicine. This study established that TWEAK/Fn14 axis is essential to AAA formation in an elastase perfusion experimental model. Previously, we have demonstrated that both TWEAK and Fn14 are highly expressed in VSMC-, macrophages-, and neutrophils-rich areas within human AAA.[12] Now, we observed an increase in Fn14 mRNA expression in aortic tissue from male AAA patients and confirmed that both TWEAK and Fn14 are present in human AAA. We dissected the importance of the TWEAK/Fn14 axis using in vitro and in vivo studies with 2 different genetic approaches namely TWEAK and Fn14 KO mice. We showed that TWEAK/Fn14 is essential to AAA progression by promoting lesion inflammatory cell accumulation, neoangiogenesis, CCL2 and CCL5 chemokine expression, loss of VSMC, and elastin degradation by augmenting MMP expression and activity. In vitro studies confirmed that TWEAK regulates proinflammatory chemokines secretion, VSMC viability, and MMP-9 activation.

Recent reports demonstrate the role of TWEAK/Fn14 in different models of tissue remodeling after injury.[28] Here, we observed that TWEAK or Fn14 KO mice present a reduced aortic diameter after elastase exposition. The reduction of aortic diameter was accompanied by a diminution in the inflammatory cell content. Specifically, we observed a reduction of 60% to 70% in macrophages, T lymphocytes, and neutrophils in AAA from TWEAK or Fn14 KO mice compared with WT animals. These results are consistent with previous data in which mice lacking TWEAK, its receptor, or treated with blocking TWEAK antibodies failed to recruit T cells, macrophages, or neutrophils to the injured tissue in models of cerebral artery occlusion or atherosclerosis.[16,29]

The mechanism/s implicated in the reduction of inflammatory cells content observed in TWEAK or Fn14-deficient mice is most likely due to the reduction in chemokine expression, diminution of neovessels formation, and decrease

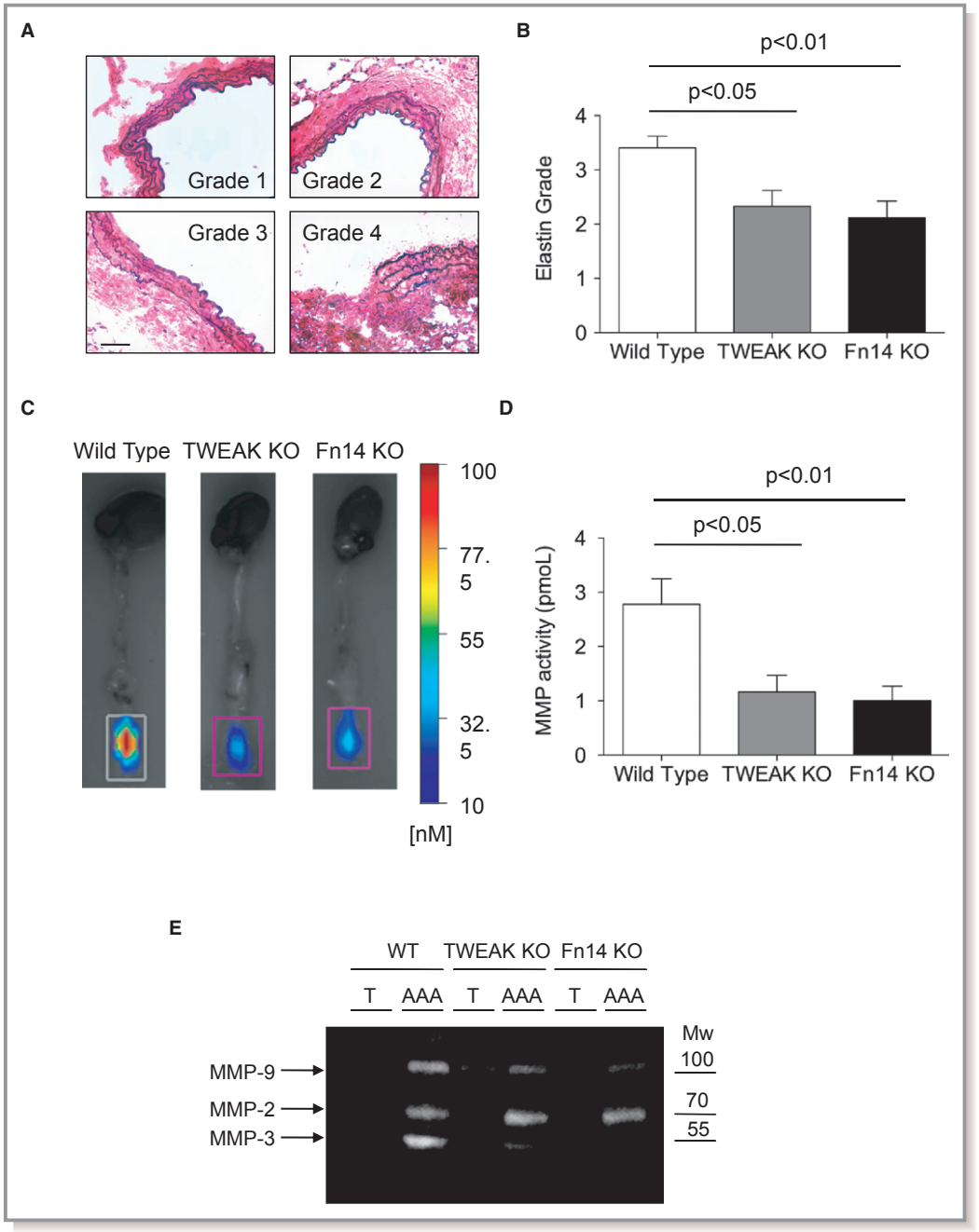

Figure 6. TWEAK or Fn14 deletion diminished elastin degradation and MMP-activity in abdominal aortic aneurysm. A, Representative Verhoeff-van Gieson staining showing elastin degradation–grading keys (4 grades). Scale bar, 50 μm. B, Quantification of medial elastin degradation in WT (N=10), TWEAK KO (N=9) and Fn14 KO (N=9) mice. Values shown are mean±SE. C, Representative FMT ex-vivo images and (D) quantification of FMT aorta fluorescence from WT (N=6), TWEAK KO (N=6) and Fn14 KO (N=6) mice imaging datasets. Imaging was performed 24 hours following MMPSense-680 injection. Values shown are mean±SE. E, Gelatin gel zymography to detect MMP activities in tissue extract from AAA or thoracic aorta (T) from WT, TWEAK or Fn14 KO mice. AAA indicates abdominal aortic aneurysm; FMT, fluorescence molecular tomography; KO, knockout; MMP, matrix metalloproteinase; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; WT, wild type.

in proliferative cells. TWEAK has proinflammatory properties and acts on different cell types involved in vascular remodeling to induce the expression of various cytokines and chemokines. Thus, TWEAK induces CCL2 secretion by human endothelial cells,[13] VSMC,[16] macrophages.[30] Herein, in vitro experiments showed that CCL2 and CCL5 expression are

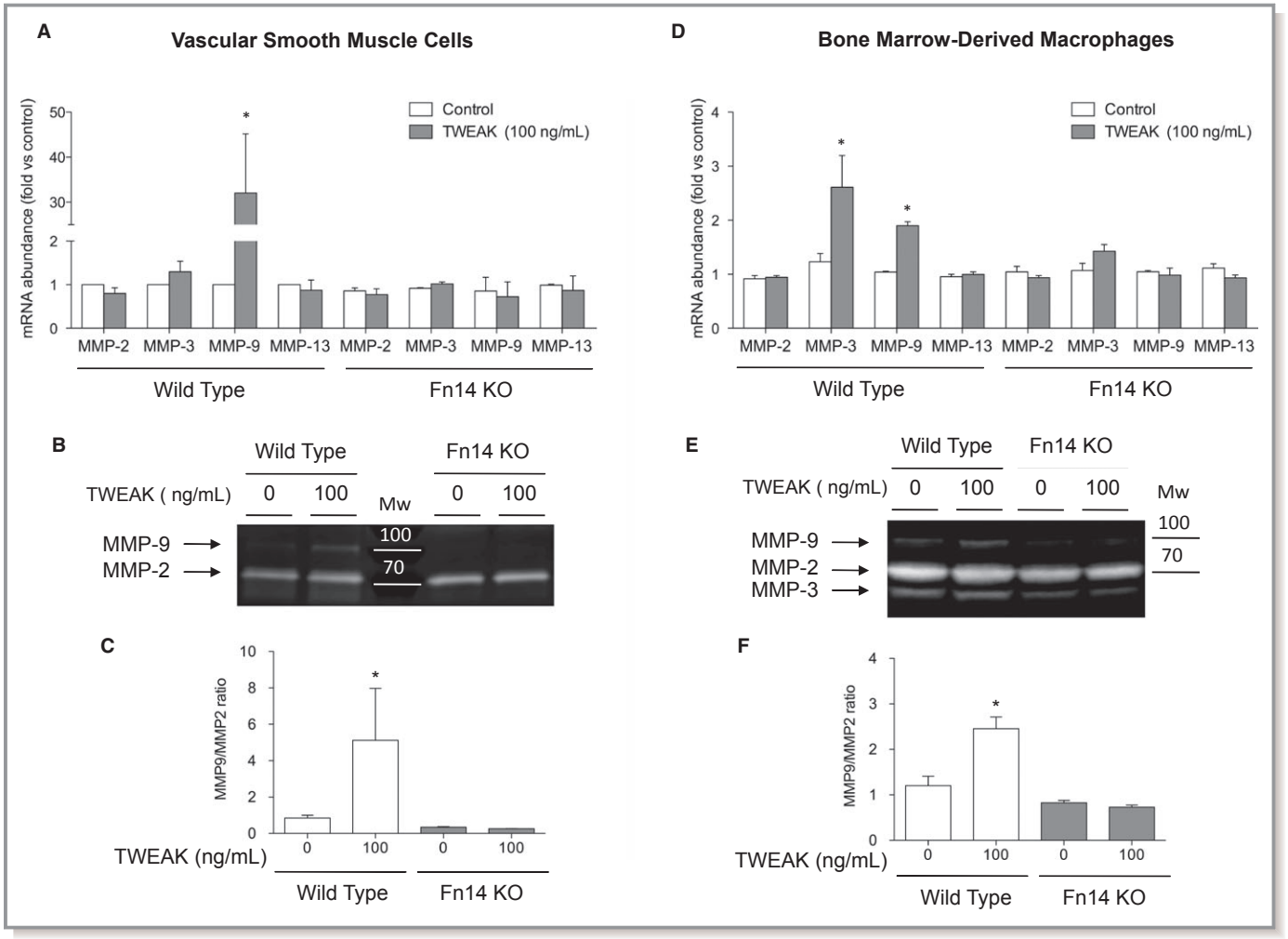

Figure 7. TWEAK increases MMP-9 expression and activity in cultured VSMC and BMDM. A, Quantitative real-time polymerase chain reaction analysis on MMP-2, -3, -9 and -13 in cultured VSMC from WT and Fn14 KO mice. Values shown are mean±SE of 3 independent experiments. B, Gelatin gel zymography to detect MMP activities in supernatants from cultured VSMC of WT and Fn14 KO mice in the absence or presence of TWEAK (100 ng/mL). C, Densitometric analysis of MMP activity in VSMC. Values shown are mean±SE of 3 independent experiments. *P<0.05 vs no TWEAK. D, Quantitative real-time polymerase chain reaction analysis on MMP-2, -3, -9 and -13 in cultured BMDM from WT and Fn14 KO mice. Values shown are mean±SE of 4 independent experiments. E, Gelatin gel zymography to detect MMP activities in supernatants from cultured BMDM of WT and Fn14 KO mice in the absence or presence of TWEAK (100 ng/mL). F, Densitometric analysis of MMP activity in BMDM. Values shown are mean±SE of 4 independent experiments. *P<0.05 vs no TWEAK. BMDM indicates bone marrow-derived macrophages; KO, knockout; MMP, matrix metalloproteinase; Mw, molecular weight; TWEAK, tumor necrosis factor-like weak inducer of apoptosis; VSMC, vascular smooth muscle cells; WT, wild type.

increased by TWEAK/Fn14 interaction in murine VSMC. In addition, CCL5 secretion was also increased by TWEAK in cultured BMDM. The increase expression of these chemokines VSMC or BMDM exposed to recombinant TWEAK is dependent on Fn14 availability, since Fn14 KO VSMC or BMDM failed to secret CCL2 or CCL5 in response to TWEAK. Both cytokines mediate experimental aneurysm formation[31,32] and are upregulated in human AAA,[23,24] suggesting that the interaction between TWEAK and Fn14 is an important mechanism in leukocyte recruitment in AAA.

Mural neoangiogenesis is a feature of both experimental and human AAA diseases.[22,33] The vessels of the vasa vasorum are the routes used by the inflammatory cells to access the aortic intima and media. Inflammatory leukocytes such as monocytes/macrophages produce the proangiogenic cytokine vascular endothelial growth factor A that participates in neovessels formation through endothelial cell proliferation. In this context, TWEAK increases proliferation of endothelial cells although this effect is independent of VEGF-A.[14] Furthermore, TWEAK induces angiogenesis in rat corneas[14] and anti-TWEAK mAb treatment diminishes angiogenesis in a model of arthritis.[34] Now, we observed that TWEAK or Fn14 deletion also substantially decreased aortic adventitial neovessel formation in our animal model. This diminution could be related to the reduction of Ki-67 positive endothelial cells observed in TWEAK or Fn14 KO mice compared with WT

animals since TWEAK induces endothelial cells proliferation.[14] However, we cannot discard that Ki-67 positive cells involves other cell types present in AAA lesion such as macrophages that proliferate during atherosclerotic tissue remodeling.[35] Overall, our data indicates that TWEAK participates in neoangiogenesis during AAA progression.

Induction of VSMC apoptosis makes an important contribution to the evolution of aneurysm degeneration.[25] Loss of VSMC observed in AAA is related with the presence of active proteases. Elastase is one of the most potent proteases reported to be present in the pathological arterial wall and is able to induce apoptosis subsequent to extracellular matrix degradation.[26] We have observed that WT mice lost more VSMC than either TWEAK or Fn14-null mice. In vitro experiments showed that TWEAK alone did not decrease VSMC viability. However, in the presence of non-lethal concentrations of elastase, TWEAK decreased VSMC viability. This effect required its interaction with Fn14 since TWEAK plus elastase did not affect Fn14 KO VSMC viability. These results indicate that TWEAK could participate in VSMC death in the presence of activated proteases.

Finally, elastin is a major structural component of the aorta and one of the most durable proteins of the extracellular matrix.[8] The dissolution of elastic fibers requires the presence of specific proteinases, and several elastolytic matrix MMPs are thought to contribute to aneurysm development, including MMP-2, MMP-9, and macrophage elastase MMP-12.[8] We have observed that WT animals present higher elastin degradation in their aorta compared with either TWEAK or Fn14 KO mice. Ex vivo FMT analysis showed that MMP activity was diminished ≈60% in the infrarenal aorta of either TWEAK or Fn14 KO mice. Additionally, we observed a reduced MMP-9 and MMP-3 activity in AAA wall from TWEAK or Fn14 KO mice compared with WT mice by gelatin gel zymogram assay. This diminution can be related with 2 potential mechanisms, reduction of leukocyte infiltration (a major source of MMP-9 in AAA) observed in KO animals or a potential diminution of its expression by resident cells (VSMC). In vitro experiments in VSMC or BMDM demonstrated that TWEAK, through interaction with Fn14, increased MMP-9 expression and activity. These data are consistent with previous reported studies in which it was demonstrated that TWEAK increased MMP-9 activity in macrophages[30] or myotubes and in myofibers of TWEAK transgenic mice.[36] Interestingly, MMP-9 seems to be essential for the development of AAA since MMP-9 KO animals are resistant to elastase-induced AAA.[20] These data indicate that TWEAK participates in matrix degradation during AAA progression through MMP-9 activation. However, we cannot discard the contribution of other MMPs such as MMP-3. In fact, MMP-3 mRNA expression was increased by TWEAK in cultured BMDM. However, we have not observed an increase of MMP-3 activity in BMDM after TWEAK treatment. In this sense, it is important to keep in mind that macrophages are a robust source of TWEAK.[37] In fact, BMDM from WT mice have increased MMP-3 activity compared with BMDM from Fn14 KO mice. This data could suggest that endogenous TWEAK may already be sufficient to increase MMP-3 activity and addition of recombinant TWEAK to the culture medium do not further enhance MMP-3 activity.

In conclusion, this is the first study to analyze the importance of TWEAK/Fn14 axis in experimental AAA formation. TWEAK contributes to the progression of AAA by promoting lesion inflammatory cell accumulation, angiogenesis, loss of VSMC, and protease expression. The multiple facets of TWEAK-mediated regulation on inflammation, aortic smooth muscle cells viability, and vascular remodeling makes the TWEAK/Fn14 pathway a potential target for treatment of aortic aneurysm.

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Disclosures

Burkly is an employee and stockholder in Biogen Idec, Inc.

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Tumor Necrosis Factor–Like Weak Inducer of Apoptosis or Fn14 Deficiency Reduce Elastase Perfusion–Induced Aortic Abdominal Aneurysm in Mice

In the article by Tarín et al., "Tumor Necrosis Factor–Like Weak Inducer of Apoptosis or Fn14 Deficiency Reduce Elastase Perfusion–Induced Aortic Abdominal Aneurysm in Mice," which published online August 4, 2014, and appears in the August 2014 issue of the journal (*J Am Heart Assoc.* 2014;3:e000723 doi: 10.1161/JAHA.113.000723), one of the author's affiliations appeared incorrectly. Carlos Zaragoza's affiliation originally appeared as "Spanish National Cardiovascular Research Center, Madrid, Spain" but has now been corrected to read "Cardiovascular Joint Research Unit, University Hospital Ramón y Cajal Hospital and University Francisco de Vitoria School of Medicine, Madrid, Spain."

The publisher regrets this error.

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Carlos Tarín, Valvanera Fernández-Laso, Cristina Sastre, Julio Madrigal-Matute, Mónica Gómez, Carlos Zaragoza, Jesús Egido, Linda C. Burkly, Jose L. Martín-Ventura and Luis M. Blanco-Colio

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