CD95-ligand contributes to abdominal aortic aneurysm progression by modulating inflammation

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Aims

Abdominal aortic aneurysm (AAA) is one of the number of diseases associated with a prominent inflammatory cell infiltration, matrix protein degradation, and smooth muscle cell apoptosis. CD95 is an inflammatory mediator and an apoptosis inducer. Previous studies have shown elevated expression of CD95 or CD95L in the aortic tissue of AAA patients. However, how the CD95L/CD95 contributes to aneurysm degeneration and whether blocking its signalling would be beneficial to disease progression remains largely unknown. In the present study, we sought to determine the role of CD95L and its downstream target, caspase 8, in AAA progression.

Methods and results

By using the CaCl₂ murine model of AAA, abdominal aortic aneurysms were induced in C57BL/6 mice. We found that both mRNA and protein levels of CD95L were increased in aneurysm tissue compared with NaCl-treated normal aortic tissue. To determine whether CD95L contributes directly to aneurysm formation, we used CD95L null (CD95L^{-/-}) mice to examine their response to CaCl₂ aneurysm induction. Six weeks after periaortic application of CaCl₂, aortic diameters of CD95L^{-/-} mice were significantly smaller compared to CaCl₂-treated wild-type controls. Connective tissue staining of aortic sections from CaCl₂-treated CD95L^{-/-} mice showed minimal damage of medial elastic lamellae which was indistinguishable from the NaCl-treated sham control. Furthermore, CD95L deficiency attenuates macrophage and T cell infiltration into the aortic tissue. To study the role of CD95L in the myelogenous cells in AAA formation, we created chimaeric mice by infusing CD95L^{-/-} bone marrow into sub-lethally irradiated wild-type mice (WT/CD95L^{-/-}BM). As controls, wild-type bone marrow were infused into sub-lethally irradiated CD95L^{-/-} mice (CD95L^{-/-}/WT^{BM}). WT/CD95L^{-/-}BM mice were resistant to aneurysm formation compared to their controls. Inflammatory cell infiltration was blocked by the deletion of CD95L on myeloid cells. Western blot analysis showed the levels of caspase 8 in the aortas of CaCl₂-treated wild-type mice were increased compared to NaCl-treated controls. CD95L deletion inhibited caspase 8 expression. Furthermore, a caspase 8-specific inhibitor was able to partially block aneurysm development in CaCl₂-treated aneurysm models.

Conclusion

These studies demonstrated that inflammatory cell infiltration during AAA formation is dependent on CD95L from myelogenous cells. Aneurysm inhibition by deletion of CD95L is mediated in part by down-regulation of caspase 8.

Keywords

Abdominal aortic aneurysm • CD95L • Caspase 8 • Inflammation

1. Introduction

Abdominal aortic aneurysm (AAA) is a common and potentially life-threatening condition. It predominantly affects the segment of aorta

below the renal arteries. At least 15 000 deaths per year in the United States are attributed to ruptured aortic aneurysms making this the 15th leading cause of mortality.¹ Currently there is no effective medical therapy to induce regression or slow AAA growth. The standard of care is

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mechanical intervention once the aneurysm reaches 5.0 cm in women or 5.5 cm in man.² At the histopathology level, AAA is characterized by inflammatory cell infiltration,^{3,4} extracellular matrix (ECM) degradation, matrix metalloproteinase (MMP) upregulation,⁵ and smooth muscle cell (SMC) apoptosis. Murine models of AAA recapitulate many features of human AAA.^{6–13}

SMCs are the only cell type within the aortic media; they are uniquely responsible for synthesis of matrix proteins in angiogenesis and vessel repair after injuries. Apoptotic death of SMCs has been observed both in human tissue of AAA^{14,15} and mouse models of AAA.^{9,10} Studies from human aortic samples have shown that loss of SMCs may contribute aetiologically to aneurysmal disease. Levels of CD95/CD95L proteins were higher in AAA patients than in normal aortic tissue.¹⁴ However, the cell type expressing the majority of CD95/CD95L in AAA is uncertain.

CD95 and CD95L are members of the tumour necrosis factor (TNF) receptor and TNF family, respectively. CD95 expression is observed in a variety of immune and non-immune cells. CD95L was found to be primarily expressed at the surface of activated T lymphocytes, macrophages,¹⁶ and natural killer (NK) cells.¹⁷ In addition to its critical role in regulating the balance between cell survival and cell death, CD95/CD95L fulfils diverse functions in different tissues *in vivo* beyond apoptosis.¹⁸ CD95/CD95L promotes inflammatory cell migration to the injury site, and inhibition of CD95-mediated inflammatory infiltration decreases cell death.¹⁹ CD95L itself can act as a proinflammatory mediator.^{20,21} It is unclear whether the predominate effect of CD95L in AAA progression is through induction of apoptosis or its role in enhancing the inflammatory process.

AAA is a chronic inflammatory disease in which inflammatory cell infiltration and increased levels of proinflammatory cytokines in the aorta play a crucial role in both human and animal models. Although it is known that CD95/CD95L levels are increased in human AAA tissue, the actual source of CD95L and the mechanism by which the CD95/CD95L system contributes to the aneurysm formation has not been completely addressed.¹⁴ Here, we report that the critical source of CD95L in the aneurysm development is mainly from infiltrating myeloid cells. Exclusive deletion of CD95L in myeloid cells inhibited inflammatory cell infiltration in the CaCl₂-induced AAA model. We provide the evidence that the pathway through which CD95L/CD95 impacts aneurysm progression is by affecting the trafficking of inflammatory cells.

2. Methods

2.1 Human aneurysmal and normal aortic tissue

Human AAA segments were obtained from patients undergoing elective repair ($n = 6$). Normal aortic specimens were obtained from organ donors at autopsy from six subjects who had no evidence of aneurysm. The consent was obtained from patients for the use of the tissue. The study of normally discarded human tissue was approved by the Institutional Review Board at the University of Nebraska Medical Center. The study conforms to the declaration of Helsinki.

2.2 Mice

CD95L-null (CD95L^{-/-}) mice on a C57Bl/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) as were the C57Bl/6-Green Fluorescent Protein (GFP) mice and wild-type, C57Bl/6 mice. All experiments were carried out in accordance with the guidelines

of the University of Nebraska Medical Center Animal Care Committee for the use and care of laboratory animals. All mouse experiments conform to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. All mice were maintained in the pathogen-free animal facility.

2.3 Generation of chimaeric mice lacking CD95L expression in myelogenous cells

CD95L^{-/-} mice or wild-type mice were euthanized by cervical dislocation. The femur and tibia were isolated from the surrounding tissue, sterilized in 70% ethanol for 15 s, and then washed in PBS. The bone marrow was flushed out with Phosphate Buffered Saline (PBS). Bone marrow cells were then passed through a cell strainer, washed with PBS.

In order to understand the contribution of CD95L from invading inflammatory cells, we generated chimaeric mice lacking CD95L expression in myelogenous cells. Five-week-old wild-type mice were irradiated (1200 rads) and transplanted with 5×10^6 bone marrow cells from CD95L^{-/-} mice (designated as WT/CD95L^{-/-BM} mice) via the lateral tail veins.²² As controls, CD95L-null mice were irradiated and transplanted with 5×10^6 bone marrow cells from wild-type mice (designated as CD95L^{-/-}/WT^{BM} mice). A second control group included irradiated wild-type mice that were transplanted with wild-type bone marrow (designated as WT/WT^{BM} mice). Mice that were irradiated but did not receive bone marrow transplantation died within a week. Expression of CD95L and mutated CD95L in mouse bone marrow and spleen was measured by real-time PCR. To further assess bone marrow transplantation efficacy, a group of irradiated CD95L deficient mice were transplanted with bone marrow from C57Bl/6-GFP mice. One week (circulating cells only) and 4 weeks after transplantation, GFP-positive cells in the circulation, spleens, and bone marrow of recipient mice were measured with FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed using the FlowJo V10 software (FlowJo LLC, Ashland, OR, USA).

2.4 Aneurysm induction

Mice at 8 weeks of age underwent surgery as described previously.^{7,8} Briefly, mice were anaesthetized with isoflurane in 100% oxygen with a flow of 1.5 L/mL administered by means of a facemask connected to co-axial circuit (Patterson Scientific, Waukesha, WI, USA). The abdominal aorta between the renal arteries and bifurcation of the iliac arteries was isolated from surrounding retroperitoneal structures. The diameter of the aorta was measured in triplicate in the mid-infrarenal aorta using a Leica Application System (Leica Microsystems Inc., Buffalo Grove, IL, USA). After baseline measurements were obtained, 0.25 M CaCl₂ was applied to the external surface of the aorta using a cotton applicator cut to size for 15 min. The aorta was then rinsed with 0.9% NaCl (sterile saline) and the incision was closed. After the operation buprenorphine (0.1 mg/kg body weight) was given subcutaneously daily for 2 days. NaCl (0.9%) was substituted for CaCl₂ in sham control mice. Six weeks later, the mice underwent repeat laparotomy. The aortic diameter was measured as described above. Then, mice were sacrificed under general anaesthesia by exsanguination. Mouse aortas were collected for analysis. For the treatment of caspase 8 inhibitor, Z-IETD-FMK (Sigma, St Louis, MO, USA), WT, C57Bl/6, mice were treated with CaCl₂. The aortic diameters were assessed before aneurysm induction. Mice were injected i.p. with Z-IETD-FMK (25 µg/kg body weight) twice weekly beginning at the day of aneurysm induction for 6 weeks until sacrifice. For controls,

mice were injected with 4 μ L/g body weight of Dimethyl Sulfoxide (DMSO) (solvent for Z-IETD-FMK).

2.5 Histology and immunohistochemistry

For Verhoeff-Van Gieson (VVG) connective tissue staining, mouse abdominal aortic tissues were embedded in paraffin and stained as described.^{12,23} For immunohistochemistry, aortic tissue sections were incubated with monoclonal rat anti-mouse Mac3 antibody (BD Bioscience, San Jose, CA, USA), polyclonal rabbit anti-CD3 antibody (Abcam, Cambridge, MA, USA),^{7,8} anti-CD178 polyclonal rabbit antibody (Thermo Fisher Scientific, Waltham, MA, USA), and anti-MMP-2 and anti-MMP-9 polyclonal rabbit antibodies (Abcam). Positive macrophages and T cells were graded by observers blinded to the genotypes and treatment of the mice in a high-power field (40 $\times$ objective). Five or more samples in each group were stained and evaluated; the mean grade was reported.

2.6 Reverse transcription polymerase chain reaction

Total RNA from human aorta and mouse aortic tissue, bone marrow, and spleen was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Thermoscript RT-PCR system (Invitrogen) with glyceraldehyde 3-phosphate dehydrogenase, β -actin, or 18S rRNA as internal references. The real-time PCR of human and mouse was performed on the ABI StepOne Real-time PCR System (Applied Biosystem). Genotyping was done by using specific primers and Taqman probes for CD95L and TaqMan Universal PCR Master Mix (Applied Biosystem) as described from the Jackson Laboratory.

2.7 Gelatin zymography

Aortic proteins were extracted as previously described.⁸ The protein concentration was standardized with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Gelatin zymography was performed as described previously by Longo *et al.*,⁸ with 0.8% gelatin in a 10% SDS-polyacrylamide gel. The molecular sizes of gelatinolytic activities were determined using protein standards (Bio-Rad Laboratories). The intensity of each band was quantified using NIH ImageJ software (National Institute of Health).

2.8 Western blot analysis

Extracts from mouse and human aorta were loaded into 10% SDS-PAGE. Following electrophoresis, the gel was transferred onto a 0.45 μ m Polyvinylidene Fluoride membrane (Bio-Rad Laboratories). The membrane was incubated overnight at 4°C with antibodies directed against CD95L, caspase 8, β -tubulin, or β -actin (Cell Signaling, Beverly, MA, USA). Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated, species specific, secondary antibodies (Cell Signaling) using the Clarity Western ECL system (Bio-Rad Laboratories).

2.9 TUNEL assay

Mouse aortic sections were deparaffinized and rehydrated. The sections were stained for cell death using the DAB *In Situ* Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA) as per manufacturers' instruction. Briefly, aortic tissue sections were incubated with 20 μ g/mL of proteinase K for 30 min at room temperature, incubated with 0.3% hydrogen peroxide in PBS to quench endogenous peroxidase activity, and

incubated with 1XTdT enzyme in reaction buffer for an hour at 37°C. After washing with PBS, sections were incubated with 50 μ L anti-BrdU antibody solution for 30 min at 37°C, then with 50 μ L of Strep-HRP solution for 10 min at room temperature. The staining was developed by incubation of DAB solution. Counterstaining was done by incubating with methyl green for 5 min at room temperature. TUNEL-positive cells were graded.

2.10 Statistical analysis

Measurements of aortic diameter are expressed as mean value $\pm$ the standard error (SE) of the mean. For continuous variables, if the data were normally distributed, the Student's *t*-test (comparison between two groups) or ANOVA with the appropriate *post hoc* test (comparison among groups of three or more) were used. A Fisher's exact test was used for the analyses evaluating non-continuous variables. Statistical significance was accepted at a $P < 0.05$. The aorta was considered to be aneurysmal if the diameter increased by 50% or more from the baseline measurement as defined by the joint councils of the Society for Vascular Surgery and the North American Chapter of the International Society for Cardiovascular Surgery.²⁴

3. Results

3.1 CD95L deficiency in mice prevented AAA formation

Increased CD95/CD95L in human aneurysm tissue could impact aneurysm formation by inflammatory cell recruitment and/or aortic SMC apoptosis. To further investigate the potential causal roles that CD95L plays in AAA progression, we used a well-established CaCl_2 murine model of AAA.^{8,25} Both mRNA and protein levels of CD95L are increased in aneurysmal, CaCl_2 -treated aorta compared to non-aneurysmal sham controls (Figure 1A,B). This is confirmed with immunostaining of CD95L in aortic tissue (Figure 1C,D). Importantly, these results were consistent with studies in human aortic samples. The aortic levels of CD95L mRNA and protein were significantly higher in AAA patients relative to age-matched controls (Figure 1E,F). The increase of CD95L in human or murine aneurysm tissue is not proof that it has a causal role since the increased levels could simply reflect the presence of the local inflammatory response. To determine whether CD95L contributes directly to aneurysm formation, we used CD95L null (CD95L^{-/-}) mice to test their response to CaCl_2 aneurysm induction. Six weeks after periaortic application of CaCl_2 , the aortic diameter in CD95L^{-/-} mice showed a small increase with only 1 (6%) of 16 of the aortas becoming aneurysmal (>50% increase) (Table 1). The histologic appearance of NaCl-treated CD95L^{-/-} mice was indistinguishable from the NaCl-treated sham control (Figure 2B,D). The WT background-matched control mice showed a 60% increase ($P < 0.0001$) in aortic diameter after CaCl_2 treatment with 8 (73%) of 11 developing aneurysms (Table 1). The difference in aneurysm incidence is highly significant ($P < 0.001$). Connective tissue staining of aneurysmal aortic sections from WT mice showed disruption and fragmentation of medial elastic fibres (Figure 2C), while CaCl_2 -treated CD95L^{-/-} mice showed minimal damage of medial elastic lamellae (Figure 2D). These observations demonstrated that CD95L has a critical role in aneurysm progression.

Studies from human aortic samples showed that levels of CD95L protein were higher and the number of SMCs were lower in the aortic aneurysm tissue than in normal aorta, indicating that CD95L may play a role in SMC apoptosis during the aneurysm development.¹⁴ To assess the role of CD95L in aortic SMC apoptosis in CaCl_2 -treated mice, we

Figure 1 CD95L levels were increased in aortic aneurysm tissue. (A) Aortic CD95L mRNA expression in NaCl or CaCl₂-treated WT mice ($n = 6$ /group); (B) western blot analysis of aortic CD95L protein from NaCl- ($n = 5$) or CaCl₂-treated ($n = 7$) WT mice; the dot graphs show relative CD95L levels of mRNA and protein in the aorta. Representative images are shown at the upper right corner of the graphs. * $P < 0.05$, # $P < 0.01$ compared to controls, Student's t -test, GAPDH, and β -actin were used as the internal standard; (C,D) immunohistochemical staining of CD178 (for CD95L) in the aortas of NaCl or CaCl₂-treated WT mice ($n = 5$ /group). Arrows and circles indicate CD178-positive cells. CD178-positive cells in the aorta were evaluated in a high-power field (40 $\times$). The value reflect the mean $\pm$ SE. # $P < 0.01$, compared to NaCl treatment, Student's t -test; (E) aortic CD95L mRNA expression in human tissue ($n = 6$ /group); and (F) western blot analysis of CD95L protein in human aortas ($n = 6$ /group). The dot graphs show relative CD95L levels of mRNA and protein in the aorta. Representative image for western blot is shown at the upper right corner of the graph. * $P < 0.05$, # $P < 0.01$ compared to controls, Student's t -test, 18s rRNA, and β -actin were used as the internal standard. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

examined DNA fragmentation using the TUNEL assay (Figure 2F). Aortic tissue from CaCl₂-treated WT mice contained few TUNEL-positive cells (Figure 2H) which was not different from CaCl₂-treated CD95L^{-/-} mice (Figure 2J). These results suggest that CD95L may have an additional role other than induction of apoptosis.

3.2 Inflammatory cell infiltration was affected by CD95L

There is increasing evidence that CD95L is involved in processes other than apoptosis.²⁶ CD95/CD95L signalling plays a critical role in cell activation and migration.^{27,28} One of the remarkable histological changes in

both human AAA and CaCl₂-treated aortas is the inflammatory infiltration in the adventitia and media.⁸ In consideration of the possible effects of CD95L on inflammatory cell recruitment into the aorta, we examined the aortic tissue for the presence of CD3-positive cells (T cell-specific-antigen) and Mac3-positive cells (macrophage-specific-antigen). As can be seen from Figure 3A–D, T cell and macrophage infiltration in CaCl₂-treated CD95L^{-/-} mice was markedly decreased compared with CaCl₂-induced WT mice. These data demonstrate that CD95L plays an important role in T lymphocyte and macrophage recruitment and suggest that the aneurysm inhibitory effects seen in the CD95L^{-/-} mice are due primarily to decreased inflammatory cell recruitment.

Table 1 Changes in aortic diameter in WT and CD95L^{-/-} mice after treatment of NaCl and CaCl₂

Treatment	WT		CD95L ^{-/-}	
	NaCl	CaCl ₂	NaCl	CaCl ₂
Number	11	11	9	16
Pre-treatment (μm)	503 ± 3.9	515 ± 9.9	500 ± 5.0	491 ± 6.6
Post-treatment (μm)	565 ± 15.0	827 ± 22.5 ^a	560 ± 15.6	638 ± 12.7 ^{b,c}
AAA development (%)	0	72.7	0	6.3 ^d
Percent of increase (%)	16.7	60.6 ^e	17	30.2 ^{d,f}
Range of increase (%)	8–31	43–77	9–25	9–51

Aortic diameters were measured before NaCl (or CaCl₂) incubation (Pre) and at sacrifice (Post). Measurements of aortic diameter were expressed as mean ± SE. The percent of increase was represented as a percent compared with pre-treatment. The development of aneurysm was defined as at least 50% increase relative to original diameter of aorta.

^a*P* < 0.0001, Student's *t*-test, compared to pre-treatment.

^b*P* < 0.0001, compared to CaCl₂-treated WT.

^c*P* < 0.05, compared to pre-treatment.

^d*P* < 0.001 compared to CaCl₂-treated WT mice.

^e*P* < 0.0001 compared to NaCl-treated WT mice.

^f*P* < 0.05 compared to NaCl-treated CD95L^{-/-} mice.

Our previous studies have demonstrated that MMP-2 and MMP-9 are essential for the connective tissue degradation in the aortic wall leading to AAA. It has been shown that aortic SMCs are the primary source of MMP-2 while MMP-9 is mainly derived from infiltrating macrophages^{4,8,29} (Supplementary material online, Figure S1). Using zymography, we examined MMP-2 and MMP-9 expression in the mouse aorta. As expected, aortic levels of MMP-2 and MMP-9 were up-regulated in CaCl₂-treated WT mice. However, CD95L deficiency prevented an increase of MMP-2 and MMP-9 after CaCl₂ aneurysm induction (Figure 3E).

3.3 Deletion of CD95L in the myeloid cells attenuated aneurysm formation and reduced inflammatory cell infiltration

To address the contribution of CD95L expressed by myeloid cells compared to mesenchymal cells, we generated chimaeric mice by transplanting wild-type or CD95L^{-/-} bone marrow into sub-lethally, gamma-irradiated wild-type recipients (i.e. WT/WT^{BM} or WT/CD95L^{-/-BM}, respectively) and transplanting WT bone marrow into sub-lethally, gamma-irradiated CD95L^{-/-} recipients (i.e. CD95L^{-/-}/WT^{BM}). After recovery from bone marrow transplantation, mice

Figure 2 CD95L deletion prevented aneurysm formation. (A) Effect of CD95L deficiency on aneurysm development in the CaCl₂-induced aneurysm model. Aortic diameter increased in WT and CD95L^{-/-} mice after NaCl and CaCl₂ treatment are shown in the dot graph. The 10% increase in NaCl-treated mice is attributable to normal growth and development that occurs between 8- and 14-weeks. ^a*P* < 0.0001 compared to NaCl-treated WT mice; ^b*P* < 0.05 compared to NaCl-treated CD95L^{-/-} mice; ^c*P* < 0.0001 compared to CaCl₂-treated WT, ANOVA with *post hoc* test; (B–E) histological changes in mouse aortas (VVG staining). NaCl-treated (B) (*n* = 5) and CaCl₂-treated (C) (*n* = 6) aortas from WT mice; NaCl-treated (D) (*n* = 5) and CaCl₂-treated (E) (*n* = 5) aorta from CD95L^{-/-} mice. Each section shown is representative of 5–6 samples with similar results. (G–J) TUNEL staining of aortic tissue from NaCl- or CaCl₂-treated WT and CD95L^{-/-} mice (*n* = 5/group). TUNEL-positive cells (indicated with arrows) in the aorta were evaluated in a high-power field (40×). The dot graph shows the mean ± SE. No statistical difference (NS) was detected in TUNEL-positive cells between WT and CD95L^{-/-} mice using ANOVA with *post hoc* test (F).

Figure 3 CD95L deletion inhibited inflammatory cell infiltration and MMP expression. (A–D) Immunohistochemical staining of macrophage and T cell in the aortas of WT and CD95L^{-/-} mice after CaCl₂ treatment. Aortas were collected from WT ($n = 5$) and CD95L^{-/-} ($n = 5$) mice at 6 weeks after CaCl₂ treatment. Paraffin sections were immunostained with anti-CD3 (A,B) and anti-Mac3 (C,D) antibodies. Arrows and circles indicate CD3 or Mac3 positive cells. CD3⁺ and Mac3⁺ cells in aortas were counted (cells/high-power field, 40 $\times$). The values reflect the mean $\pm$ SE. * $P < 0.01$, compared to WT, Student's t -test. (E) Gelatin zymographic analysis of MMP-2 and MMP-9. Six weeks after NaCl or CaCl₂ treatment, aortic proteins from WT and CD95L^{-/-} mice were extracted and separated by electrophoresis on a 10% SDS-PAGE containing 0.8% gelatin. Gelatin zymography is representative of aortic protein extract from 5 samples in each group. MMP levels were quantified and shown in the dot graph. * $P < 0.01$ compared to NaCl-treated; # $P < 0.05$ compared to CaCl₂-treated WT controls.

underwent CaCl₂ aneurysm induction. RT-PCR confirmed CD95L expression in CD95L^{-/-}/WT^{BM} mice and minimal expression of CD95L in WT/CD95L^{-/-}BM chimaeras from myeloid cells and spleen (Supplementary material online, Figure S2). Transplantation efficacy was also confirmed with flow cytometry (Supplementary material online, Figure S2). Considering the known high levels of expression of CD95L by T cells, we postulated that its deletion in the white

blood cell compartment would affect the same changes as whole body deletion. As observed previously,^{8,22} control, WT/WT^{BM}, mice developed aneurysms (5 of 7) at a rate similar to that reported for WT mice. CD95L^{-/-}/WT^{BM} mice developed aneurysms (5 of 7) at a rate that is similar to WT/WT^{BM} mice. Aortic diameter in the WT/WT^{BM} and CD95L^{-/-}/WT^{BM} chimaeric mice increase by 59% and 53%, respectively (Figure 4A). In marked contrast, however,

Figure 4 Deletion of CD95L in the myeloid cells inhibited aneurysm growth and T cell and macrophage infiltration. (A) Aortic diameter increase in CaCl_2 -treated WT/WT^{BM} ($n = 7$), CD95L^{-/-}/WT^{BM} ($n = 7$), or WT/CD95L^{-/-}BM ($n = 16$) chimaeric mice. (C–K) Histological changes in the aortas of CaCl_2 -treated WT/WT^{BM}, CD95L^{-/-}/WT^{BM}, or WT/CD95L^{-/-}BM chimaeric mice ($n = 5/\text{group}$). VVG staining of aortic sections (C–E); immunostaining of CD3 positive cells (F–H), and Mac3 positive cells (I–K) ($n = 5/\text{group}$). Arrows indicate positively stained cells. Quantitation of macrophage and T cell infiltration in the aorta is shown in the dot graph (B). * $P < 0.05$ compared to controls, WT/WT^{BM} and CD95L^{-/-}/WT^{BM}, ANOVA with *post hoc* test.

only 3 of 16 WT/CD95L^{-/-}BM mice developed aneurysms with a 37% increase in aortic diameter (Figure 4A). Furthermore, the CaCl_2 -treated WT/WT^{BM} and CD95L^{-/-}/WT^{BM} mice exhibit significant disruption of the elastic lamellae of the aortic wall (Figure 4C and D, respectively). The CaCl_2 -treated WT/CD95L^{-/-}BM chimaeras revealed only minor distortion of lamellar architecture (Figure 4E). Since AAA lesions are dominated by T cells as well as macrophages and whole body deletion of CD95 had a significant effect on inflammatory cell infiltration, T cell, and macrophage infiltration in the aortic wall was assessed by immunohistochemistry (Figure 4B,F–K). WT/

CD95L^{-/-}BM mice exhibited decreased T cell and macrophage infiltration in the CaCl_2 -treated aorta (Figure 4H,K) as compared with WT/WT^{BM} and CD95L^{-/-}/WT^{BM} mice (Figure 4F,I and G,J, respectively). SMC apoptosis in the aortic tissue was assessed by TUNEL assay. Few TUNEL-positive cells were detected in the aortic sections which was not different among three groups of mice (Supplementary material online, Figure S3). Taken together, these studies confirm a central role of myeloid cell-derived CD95L in supporting aneurysm progression and elastin degradation through recruitment of inflammatory cells.

Figure 5 Caspase 8 activity was increased in AAA but inhibited by CD95L deletion. Protein from aortic tissue of human and mice were extracted and caspase 8 levels were assessed by western blot analysis. (A) The caspase 8 levels in the NaCl- or CaCl₂-treated aorta of WT and CD95L^{-/-} mice ($n = 5$ /group) were shown in the dot graph. * $P < 0.05$ compared to CaCl₂-treated CD95L^{-/-} mice, ANOVA with *post hoc* test. (B) The caspase 8 levels in the CaCl₂-treated aorta of WT/WT^{BM}, CD95L^{-/-}/WT^{BM}, and WT/CD95L^{-/-}BM mice ($n = 5$ /group) were shown in the dot graph. * $P < 0.05$ compared to control mice, ANOVA with *post hoc* test. (C) The caspase 8 protein levels in the aortas of human control and aneurysm patients ($n = 6$ /group), * $P < 0.05$ compared to control, Student's *t*-test. Representative western blot is shown in right panels. β -Actin and GAPDH were used as internal loading controls. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3.4 CD95L deletion prevented aneurysm formation via inhibition of caspase 8 activity

Through a non-canonical pathway caspase 8 has proinflammatory properties.^{30–32} It can trigger the NLRP3 inflammasome and cleave pro-IL-1 β to its active form. We further investigated whether these differences

between WT and CD95L null mice were mediated by caspase 8 activation. We analysed aortic caspase 8 levels using western blot. Caspase 8 and cleaved caspase 8 proteins in the aorta of CaCl₂-treated WT mice were increased compared to NaCl-treated controls (Figure 5A). CD95L deletion in bone marrow of wild-type mice (WT/CD95L^{-/-}BM) inhibited caspase 8 production and activation (Figure 5B). Similarly, aortic caspase 8 activity was higher in human AAA tissue than controls and its

Figure 6 Caspase 8 inhibition blocked aneurysm progression and inflammatory cell infiltration. (A) Aortic diameter changes in WT mice after aneurysm induction and treatment with the caspase 8-specific inhibitor, Z-IETD-FMK, or DMSO are shown in the dot graph ($n = 10/\text{group}$). (B,C) VVG staining of aortic tissue from DMSO- or Z-IETD-FMK-treated WT mice after aneurysm induction. (D–G) Immunostaining of CD3-positive cells (D,E), Mac3-positive cells (F,G) ($n = 5/\text{group}$). Arrows indicate positively stained cells. Quantitation of macrophage and T cell infiltration in the aorta is shown in the dot graph on the right. * $P < 0.05$ compared DMSO-treated controls, Student's *t*-test.

downstream target protein, PARP1, was up-regulated in human AAA tissue (Figure 5C and Supplementary material online, Figure S4). These data suggest that findings in the murine model are highly relevant to AAA disease in patients and the effect of CD95L on aneurysm formation is associated with down-regulation of caspase 8. To determine whether caspase 8 inhibition could block aneurysm growth, WT mice underwent CaCl₂ aneurysm induction and then were treated with a caspase 8-specific inhibitor, Z-IETD-FMK (25 µg/kg body weight) twice weekly. Control mice were treated with DMSO, carrier for Z-IETD-FMK, (4 mL/kg body weight). The Z-IETD-FMK inhibited aortic caspase 8 activation (Supplementary material online, Figure S5). Six weeks after, control mice developed aneurysms (9 of 10) at a rate similar to that of WT mice using the standard clinical definition of a 50% increase in diameter. In marked contrast, however, only 3 of 10 Z-IETD-FMK-treated mice developed aneurysms. Aneurysm incidence was significantly decreased in the Z-IETD-FMK-treated group by the Fisher's exact test ($P = 0.0198$). The mean aortic diameter was also significantly decreased (Figure 6A). Furthermore, control mice exhibit significant disruption of the elastic lamellae of the aortic wall (Figure 6B) while the Z-IETD-FMK-treated mice revealed only minor distortion of lamellar architecture (Figure 6C). Importantly, the Z-IETD-FMK treatment inhibited T cell and

macrophage infiltration compared to control (Figure 6D–G). Taken together, these data demonstrate that aneurysm attenuation by deletion of CD95L is mediated by down-regulation of caspase 8 which, in turn, blocks inflammatory cell recruitment.

4. Discussion

AAAs are characterized by chronic inflammatory infiltration, SMC apoptosis, and elastin fragmentation of the aortic wall. Previous studies using human aneurysm tissue indicated that aortic SMC apoptosis was associated with increased CD95/CD95L expression.¹⁴ However, the precise role of CD95/CD95L in AAA is unknown. In this study, we showed that CD95L expression was increased in both human and murine aneurysm tissue. Furthermore, deletion of CD95L reduced the infiltration of macrophages and T cells, the predominant inflammatory cells seen in AAA tissue. This corresponded to reduced aortic MMP-2 and MMP-9 expression. Our results identified a mechanism by which the CD95/CD95L system mediated inflammatory cell recruitment to the injury site and aortic SMC phenotypic modulation via caspase 8 signalling (Figure 7). Furthermore, specific deletion of CD95L in myeloid cells prevented

CaCl₂-induced aneurysm formation and reduced inflammatory infiltration. This demonstrates that CD95L from myeloid cells plays a crucial role in the immunomodulation in AAA progression.

It was generally believed that CD95L modulates inflammation by promoting activation-induced T cell death.³³ Along this line, local production of CD95L by cells was thought to play a role in the immune-privilege status of the eye by killing virus-infected and damaged cells.³⁴ However, accumulating evidence indicates that CD95L-mediated signalling enhances proinflammatory responses through caspase-dependent or independent signalling pathways.^{35–37} CD95L-mediated signalling promotes proliferation of human T lymphocytes, growth factor-deprived fibroblasts, and allows for maturation of dendritic cells in culture.²⁷ In addition, it induces proinflammatory cytokine responses by human monocytes and monocyte-derived macrophages, such as TNF- α .³⁵ Mutations in CD95 ameliorate disease in mice with experimental autoimmune encephalomyelitis and collagen-induced arthritis providing indirect evidence for a role of CD95L in autoimmune disease.^{38,39} These observations demonstrate the existence of complex regulatory mechanism of CD95L-mediated signalling as well as the differential effect in various cell types.

Although previous studies indicated that aortic SMC apoptosis in AAA was associated with increased CD95/CD95L expression,¹⁴ whether CD95/CD95L signalling has a causal role in aneurysm formation or simply occurs because of inflammatory cell infiltration is unclear. The CaCl₂-AAA model recapitulates many features of human AAA, including increased CD95L expression as shown in this study. By using CD95L deficient mice we demonstrated that CD95L deletion attenuated aneurysm

formation, indicating CD95/CD95L signalling plays a causal role in aneurysm progression. This occurred in association with diminished inflammatory infiltrates and decreased MMP expression. Classically, CD95L/CD95 is considered to be part of the apoptosis pathway. Inhibition of MMP-2 production from SMCs of CaCl₂-treated CD95^{-/-} mouse aortas indicates the critical role of CD95/CD95L signalling in SMC phenotypic modulation. However, we saw few apoptotic cells in the CaCl₂-treated WT aorta, and there was no difference between WT and CD95 deficient mice. SMC apoptosis in human aneurysm tissue is seen at the end stage of the disease. Our study suggests that SMC apoptosis may not be a primary factor in the early process of aneurysm development and progression. The CaCl₂-induced aneurysm model has certain shortcomings that should be noted. This model is rather acute with no atherosclerosis development before aneurysm formation. It cannot mimic all the stages of aneurysm development in human, for instance, the late stage of the disease. However, this model has been extensively studied and recapitulates many hallmark pathologic features in human aneurysms, including infiltration by lymphocytes and macrophages, matrix degradation, and increased cytokine production.^{8,25,40–42}

Although CD95L has been widely reported to be expressed predominantly in antigen-stimulated T lymphocytes and NK cells,^{28,33,43} some reports indicate that certain non-hematopoietic tissue such as the testis, thyroid, and eye can express CD95L.⁴⁴ Activation of CD95/CD95L signalling transduction pathways can induce proinflammatory cytokines and chemokines in different cell types, such as endothelial cells, bronchial epithelial cells, human vascular SMCs, and neurons.^{45–47} In order to better understand the role of CD95L on myeloid cells in AAA, we generated chimaeric mice with deletion of CD95L in the myeloid compartment by bone marrow cell transplantation. Here we show that deletion of CD95L in myeloid cells displayed an inhibitory effect on aneurysm progression that was similar to the effects seen with total body deletion of CD95L. This demonstrates that CD95L in inflammatory cells has a key role in murine AAA formation.

There is mounting evidence that activation of caspase 8 triggers a non-apoptotic cascade that modifies or facilitates the responses of other signalling pathways within cells. Caspase 8 was found to be essential for cytokine-induced monocyte differentiation in culture.⁴⁸ It plays a critical role in attenuation of autophagic signalling.⁴⁹ This signal primes cells for undirected migration. The chemotactic gradient arising from the injured tissue directs those cells to the site of injury. Caspase 8 appears to work through its ability to activate NLRP3 inflammasomes and process pro-IL-1 β .^{31,32} Accordingly, we believe that caspase 8 has critical roles in CD95/CD95L-induced non-apoptotic function in AAA. We found that caspase 8 was increased in both human and murine AAA tissue. More importantly, specific inhibition of caspase 8 had similar effects to CD95L deletion, which inhibited aneurysm progression and inflammatory cell infiltration.

Collectively, our findings highlight the importance of the peripheral immune response in the progression in AAA. We have identified the CD95/CD95L signalling machinery as a crucial trigger of the inflammatory process in AAA. Furthermore, CD95L expression on inflammatory cells mediates aneurysm progression primarily through its effect on immune function. This appears to be mediated by activation of caspase 8. Our findings indicate that the primary role of CD95L in AAA progression involves recruitment of inflammatory cells rather than apoptosis. Given the multiple putative roles of CD95/CD95L in immune responses and diseases, therapeutic targeting of the CD95/CD95L pathway might be a potential therapy for AAA.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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