Enhanced expression and activation of pro-inflammatory transcription factors distinguish aneurysmal from atherosclerotic aorta: IL-6- and IL-8-dominated inflammatory responses prevail in the human aneurysm

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ABSTRACT

Inflammation plays a key role in the pathogenesis of an AAA (abdominal aortic aneurysm); however, the nature of the inflammatory factors and cellular response(s) involved in AAA growth is controversial. In the present study, we set out to determine the aortic levels of inflammatory cytokines in relation to downstream inflammatory transcription factors and cellular responses. A comparison of AAA wall samples with atherosclerotic wall samples taken from the same aortic region allowed AAA-specific inflammatory parameters to be identified that distinguish AAAs from ASD (aortic atherosclerotic disease). RT-PCR (real-time PCR), ELISA, Western blotting and immunohistochemistry were combined to assess cytokines and transcription factors at the mRNA and protein level, and their activation status. Compared with ASD, inflammatory parameters associated with Th1-type [T-bet, IL (interleukin)-2, IFN-γ (interferon-γ), TNF-α (tumour necrosis factor-α), IL-1α and cytotoxic T-cells] and Th2-type [GATA3, IL-4, IL-10, IL-13 and B-cells] responses were all increased in AAA samples. Evaluation of major downstream inflammatory transcription factors revealed higher baseline levels of C/EBP (CCAAT/enhancer-binding protein) α, β and δ in the AAA samples. Baseline p65 NF-κB (nuclear factor κB) and c-Jun [AP-1 (activator protein-1)] levels were comparable, but their activated forms were strongly increased in the AAA samples. Downstream target genes of p65 NF-κB, c-Jun, IL-6 and IL-8 were hyperexpressed. Molecular and cellular processes associated with IL-6 and IL-8 hyperactivation were enhanced in the AAA samples, i.e. the expression of phospho-STAT-3 (signal transducer and activator of transcription-3) and perforin were elevated, and the content of plasma cells, neutrophils and vasa vasorum was increased. In conclusion, our findings demonstrate that an AAA is a general inflammatory condition which is characterized by enhanced expression and activation of pro-inflammatory transcription factors, accompanied by IL-6 and IL-8 hyperexpression and exaggerated downstream cellular responses, which together clearly distinguish an AAA from ASD.

Key words: abdominal aortic aneurysm, atherosclerosis, cytokine, inflammation, interleukin, transcription factor.

Abbreviations: AAA, abdominal aortic aneurysm; AP-1, activator protein-1; ASD, aortic atherosclerotic disease; C/EBP, CCAAT/enhancer-binding protein; CI, confidence interval; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN-γ, interferon-γ; IHC, immunohistochemical; IL, interleukin; IL-6R, IL-6 receptor; MCP-1, monocyte chemoattractant protein-1; MIF, migration inhibitory factor; MIP-1β, macrophage inflammatory protein-1β; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NF-κB, nuclear factor κB; RT-PCR, real-time PCR; sIL-6R, soluble IL-6R; STAT-3, signal transducer and activator of transcription-3; TNF-α, tumour necrosis factor-α.

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INTRODUCTION

An AAA (abdominal aortic aneurysm) is a focal balloon-like dilation of the terminal aortic segment. The atherosclerotic aneurysm, the common fusiform form of an AAA, is a frequent pathology and a major cause of death due to rupture [1]. The hallmark pathology of an atherosclerotic AAA is a chronic inflammatory condition of the arterial wall that is accompanied by a proteolytic imbalance [2]. Increased proteolytic activity results in excessive matrix degradation and progressive weakening of the vessel wall [3]. Despite considerable knowledge of the pathomorphology of AAAs, understanding the cellular and molecular inflammatory processes which underlie the increased protease expression and that drive aneurysmal growth is limited [3]. Available human studies analysing the cellular composition of AAAs indicate an extensive heterogeneous inflammatory response that involves macrophages, neutrophils and cytotoxic T-cells as well as Th1 and Th2 (T-helper 1 and 2) cell subsets [4–6].

It is well established that the Th1-/Th2-type balance is controlled tightly, and that exaggerated Th1 or Th2 responses are causatively associated with the development of inflammatory pathologies [7,8]. Th1- and Th2-type cellular responses involve distinct (and partly even opposite) immune and inflammatory processes, which are mediated by specific sets of cytokines and transcription factors. Although Th1-type responses are typically associated with enhanced expression of the cytokines IL (interleukin)-2 and IFN-γ (interferon-γ) and the transcription factor T-bet, Th2-type responses are characterized by a dominance of IL-4, IL-5 and/or IL-10 and elevated levels of the transcriptional regulator GATA3 [9].

In the context of a developing AAA, the Th1/Th2 balance is thought to play an important regulatory role in the control of matrix remodelling and inflammatory processes relevant for AAA growth and rupture [3]. However, the nature of the predominant cellular response (Th1 or Th2) and the inflammatory factors predominantly involved in human AAA growth is controversial. For example, Galle and co-workers [10] reported a predominance of Th1-type cellular and cytokine responses, whereas other studies have demonstrated the predominance of a Th2-type immune response [11], which is reflected by the enhanced expression of Th2-associated cytokines and minimal expression of the Th1-associated cytokine IFN-γ [3,11]. A recent protein array study assessing the cytokine and chemokine profile of aortic wall tissue from advanced (>5.5 cm) AAAs has reported the up-regulation of both Th1-associated [IL-1 and TNF-α (tumour necrosis factor-α)] and Th2-associated (IL-10) inflammatory mediators [12]. The downstream consequences of these and similar [13,14] observations remain uncertain as, at the transcription factor level, cytokines can exert opposite effects. For example, IL-1 can activate NF-κB (nuclear factor κB), whereas IL-10 can suppress its activation. With a comprehensive analysis of transcription factors lacking in the human studies performed so far, it remains unclear whether, and if so how, the observed changes in cytokine expression in the human AAA wall affect the basal expression of major inflammatory transcription factors and their degree of activation.

In the present study, we set out to address this issue and characterized AAA samples >5.5 cm with respect to their cytokine expression profile (at the mRNA and protein levels) and their cellular composition (using immunohistochemistry). We subsequently analysed the basal expression levels of major inflammatory transcription factors [p65 NF-κB, c-Jun, C/EBP (CCAAT/enhancer-binding protein) $\alpha/\beta/\delta$ and STAT-3 (signal transducer and activator of transcription-3)] and their activation status (active p65 NF-κB, phospho-c-Jun and phospho-STAT-3) in relation to relevant inflammatory processes controlled by these transcription factors, i.e. the expression of putative target genes (IL-6 and IL-8) and their associated cellular effects (B-cell, plasma cell and neutrophil content, and perforin expression).

Because a putatively exaggerated Th2 response in an AAA would clearly distinguish this pathology from ASD (aortic atherosclerotic disease), which is governed by Th1-type cellular and cytokine responses [3], a comparison of AAAs with ASD was considered relevant. The more so as AAAs and ASD share common pathological features and risk factors, but differences in their pathogenesis are only poorly defined to date [3].

The results of the present study demonstrate elevated levels of the three C/EBP isoforms (α, β and δ), and comparable basal but enhanced activated levels of the inflammatory transcription factors NF-κB and c-Jun in the AAA wall. These findings at the transcription factor level are in accordance with hyperexpression of downstream genes, in particular the cytokines IL-6 and IL-8, and subsequent functional effects at the molecular (increased phospho-STAT-3) and cellular (increased plasma cell and cytotoxic T-cell content) level. Taken together, these differences characterize AAAs as an inflammatory condition and clearly distinguish them from ASD.

MATERIALS AND METHODS

Patients

All human arterial wall samples were provided by the Vascular Tissue Bank (Department of Vascular Surgery, Leiden, The Netherlands). Sample collection and handling was performed in accordance with the guidelines of Medical Ethical Committee of the Leiden University Medical Center, Leiden, The Netherlands. All samples were obtained following consent of the patients. None of the patients in the present study had a history of diabetic or chronic inflammatory disease. The

primary cause of the fatal brain injury in the ASD group was a major head trauma or subarachnoidal bleeding.

Anterior lateral aneurysm wall samples were obtained from patients with an AAA > 5.5 cm undergoing elective open repair (AAA group: $n = 17$; age, 72.4 ± 6.2 years (value is the mean $\pm$ S.D.); 14 males/three females; and AAA diameter, 6.7 ± 1.1 cm). Due to reticent statin use only one patient with an AAA used a statin. For comparison of the AAA wall with the atherosclerotic aortic wall, we used non-aneurysmal aortic wall samples from brain-dead kidney donors. Only patches displaying advanced atherosclerosis with advanced atherosclerotic lesions (equalling the characteristics of grade IV–VI lesions according to the Stary classification [15]) were selected (ASD group; $n = 11$; age, 55.6 ± 10.2 years; seven males/four females; aortic diameter, < 2.0 cm). Of note, all ASD samples were obtained at the level of the renal artery and during a laparotomy, i.e. from a comparable region and during a similar procedure as the AAA samples.

Any adhering thrombus was carefully removed and the aortic walls (either AAA or ASD) were divided in two. One half was immediately snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}$C for mRNA [RT-PCR (real-time PCR)] and protein (Western blot, Multiplex and ELISA) analysis. The other half was fixed in formaldehyde for 24 h, decalcified using Kristensen's solution for 120 h and embedded in paraffin for histological analysis.

RNA extraction and mRNA analysis

Total RNA extraction was performed using RNAzol (Campro Scientific) and glass beads according to the manufacturer's instructions. Subsequently, cDNA was prepared using a Promega kit for RT-PCR. The mastermix (Eurogentec), an ABI-7700 system (Applied Biosystems) and established primer/probe sets (Applied Biosystems; Table 1) were used according to the manufacturer's instructions and as reported previously [16]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a reference and for normalization.

Tissue homogenization for protein analysis

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in 2 vols of lysis buffer [10 mmol/l Tris/HCl (pH 7.0), 0.1 mmol/l $CaCl_2$, 0.1 mol/l NaCl and 0.25 % Triton X-100]. This protocol releases both soluble as well as membrane-bound proteins. Samples were subsequently centrifuged at 10 000 *g* for 15 min at 4 °C, snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until use. Protein content in the homogenates was determined with a BCA (bicinchoninic acid) protein assay kit (Pierce).

Multiplex assay and ELISAs

IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte/macrophage colony-stimulating factor), IFN-γ, MCP-1 (monocyte chemoattractant protein-1), MIP-1β (macrophage inflammatory protein-1β) and TNF-α protein levels in tissue homogenates were determined using a Bio-Plex 17 panel for multiple cytokines (Bio-Rad Laboratories). Detection thresholds for the cytokine panel was < 0.5 pg/ml for IL-1, IL-2, IL-5, IL-6, IL-7, IL-8, IL-10 and TNF-α, < 1 pg/ml for IL-12 and IL-17A, and < 5 pg/ml for IL-4, IL-13, G-CSF, GM-CSF, IFN-γ and MCP-1. IL-6, IL-8 and MCP-1 levels exceeded the upper detection limit of the Bio-Plex 17 panel and were therefore determined accurately using separate specific ELISAs [PeliKane compact kit (Sanquin Reagents) for IL-6 and IL-8 and a Quantikine kit (R&D Systems) for MCP-1]. sIL-6R [soluble IL-6R (IL-6 receptor)] levels were determined using an ELISA specific for human sIL-6Rs (R&D Systems).

Table 1 ***Taq*Man® gene expression assays (Applied Biosystems) or probe/primer combinations (IL-6 and MIF) used in RT-PCR**

FAM, 6-carboxyfluorescein; TGF-β, transforming growth factor-β.

T-bet	Hs00203436_m1
GATA-3	Hs00231122_m1
IL-1α	Hs00174092_m1
IL-1β	Hs00174097_m1
IL-2	Hs00174114_m1
IL-4	Hs00174122_m1
IL-8	Hs00174103_m1
IL-10	Hs00174086_m1
IL-13	Hs00174379_m1
IFN-γ	Hs00174143_m1
TNF-α	Hs00174128_m1
MCP-1	Hs00234140_m1
MIP-1β	Hs99999148_m1
TGF-β	Hs99999918_m1
IL-6	Forward, 5′-TGTAGCCGCCCCACACA-3′
	Reverse, 5′-AGATGCCGTCGAGGATGTACC-3′
	Probe, 5′-FAM-AGCCACTCACCTCTTCAGAACGAATTGACA A-3′
MIF	Forward, 5′-TGGCCGAGCGCCTG-3′
	Reverse, 5′-GCCGCGTTCATGTCGTAATA-3′
	Probe, 5′-FAM-CATCAGCCCGGACAGGGTCTACATCA-3′

Western blot analysis

Western blot analysis was performed essentially as described previously [17] using antibodies specific for human p65 NF-κB (both non-active and active; Santa Cruz Laboratories and Chemicon respectively), c-Jun (Santa Cruz Laboratories), phospho-c-Jun (Ser^{73}) (Santa Cruz Laboratories), perforin (Santa Cruz Laboratories), STAT-3 (Santa Cruz Laboratories), phospho-STAT-3 (Epitomics), C/EBPα (Santa Cruz Laboratories), C/EBPβ (Santa Cruz Laboratories), C/EBPδ (Santa Cruz Laboratories) and β-actin (Santa Cruz Laboratories) as described previously [18].

The corresponding secondary antibodies were obtained from Santa Cruz Biotechnology [donkey anti-(goat IgG)] and Pierce [goat anti-(rabbit IgG) and goat anti-(mouse IgG)]. Immunoblots were visualized and quantified using the Super Signal West Dura Extended Duration Substrate (Perbio Science), LabWorks 4.6 software and a luminescent image workstation (UVP). Protein expression levels in aortic wall samples were normalized for β-actin, and separate anti-(β-actin) immunoblots were performed for each sample.

Immunohistochemistry

Immunohistochemistry was performed using deparaffinized ethanol-dehydrated tissue sections (4 μm thick) essentially as described previously [16,19]. Sections were incubated overnight with polyclonal antibodies specifically staining human MPO (myeloperoxidase; 1:4000 dilution; DAKO), CD20 (1:1000 dilution; DAKO), CD138 (1:1000 dilution; Serotec), IL-6 (1:2000 dilution; Biogenesis) or IL-8 (1:200 dilution; Biogenesis). Conjugated biotinylated anti-(goat IgG) or anti-(rabbit IgG) were used as secondary antibodies. Sections were developed with Nova Red® (Vector Laboratories) and counterstained with Mayer's haematoxylin to allow morphological analysis. The specificity of the antibody staining was confirmed by omitting the primary antibody. The presence of eosinophils was evaluated morphologically by haematoxylin/eosin staining.

Statistical analysis

All values are expressed as means $\pm$ S.D. for normally distributed data or medians (range) for non-normally distributed data. Normally distributed continuous variables were analysed using a Student t test, whereas non-normally distributed continuous data were analysed with a Wilcoxon–Mann–Whitney test using (SPSS 11.5 for Windows). CIA (Confidence Interval Analysis; version 2.0.0. 41; https://www.som.soton.ac.uk/cia/) was used to calculate the non-parametric 95% CI (confidence interval) for differences in mRNA expression. Possible relationships between aneurysm diameter and inflammatory markers were evaluated by Pearson's correlation test. The level of statistical significance was set at $P < 0.05$. The present study incorporates multiple statistical comparisons. For the sake of clarity, non-corrected data are provided; however, a Bonferroni correction should be considered when interpreting non-correlated data.

RESULTS

Characterization of aneurysmal and atherosclerotic aortic walls based on cytokine expression profiles

Aortic aneuysmal (AAA) and atherosclerotic (ASD) wall samples used in the present study were characterized for the expression of markers of inflammation. RT-PCR and the Bio-Plex assay were used to determine the expression level of specific Th1-associated, Th2-associated and general inflammatory factors on the mRNA and/or protein level.

Compared with ASD, the AAA group had significantly higher aortic mRNA expression levels of the Th1-associated transcription factor T-bet (24-fold increase) and the Th2-associated transcription factor GATA3 (12-fold increase; Table 2). The T-bet/GATA3 ratio was similar in both experimental groups ($P = 0.52$).

A subsequent analysis of cytokines in aortic mRNA extracts revealed substantially increased mRNA levels of the Th1-associated cytokines IL-2, IFN-γ, TNF-α, IL-1α and IL-1β in AAA samples. In line with this observation, the protein levels of IL-2, IFN-γ, TNF-α and IL-1α were significantly higher in AAA homogenates (Table 3).

With respect to Th2-associated cytokines, higher mRNA levels of IL-4, IL-10 and IL-13 ($P < 0.01$) were found in the AAA samples (Table 2). An increase in IL-4 and IL-13 protein levels was also observed, whereas IL-5 and IL-10 levels were below the detection limit of the Bio-Plex assay (Table 3).

In addition to these typical Th1- and Th2-associated factors, cytokines/chemokines reflecting a general pro-inflammatory status, such as MCP-1, MIP-1β, MIF (migration inhibitory factor), G-CSF and GM-CSF, were also significantly expressed in the AAA samples compared with the ASD samples (at the mRNA and/or protein level; Tables 2 and 3).

Taken together, the above molecular characterization of aortic wall samples by cytokine profiling does not indicate a clear Th1/Th2 polarization in AAAs, but points to a generic enhancement of inflammation compared with ASD.

Characterization on the basis of cellular markers of Th1/Th2 polarization

To characterize aneurysmal (AAA) and atherosclerotic (ASD) aortas further, their cellular composition was evaluated with particular emphasis on Th1- and Th2-associated cellular responses.

Absolute and relative cytotoxic T-cell infiltration (Th1-associated) was assessed by analysing aortic CD4 and CD8 mRNA expression levels [20]. AAA samples had higher CD8 (26-fold; $P < 0.001$) and CD4 (12-fold; $P = 0.24$) mRNA expression levels and a higher CD8/CD4 ratio ($P < 0.001$), suggesting an enhanced influx of cytotoxic T-cells in AAA tissue. Specific IHC (immunohistochemical) staining of cytotoxic T-cells in sections prepared from AAA samples confirmed an increased abundance of cytotoxic T-cells compared with ASD samples (results not shown).

Potential Th2-related cellular effects were evaluated by IHC staining of B-cells (CD20) and an assessment of eosinophil infiltration. AAA samples had a dispersed CD20

Table 2 Fold differences in gene expression of markers of Th1-associated, Th2-associated and general inflammatory responses

mRNA expression was analysed in aneurysmal wall (AAA; $n = 17$) and atherosclerotic wall (ASD; $n = 11$) samples. Gene expression level of the measured genes in ASD was set at 1, and relative gene expression levels in AAA tissues are expressed as the median fold increase, together with the corresponding 95% CI of the median fold increase and the *P* value. The level of mRNA expression for each gene of interest was calculated using Ct values. Ct values are defined as the number of PCR cycles at which the fluorescent signal generated during the PCR reaches a fixed threshold. For each sample, the Ct for the target gene and for the housekeeping gene was determined to calculate ΔCt ($C_{t,\ \text{target gene}} - C_{t,\ \text{housekeeping gene}}$). The relative expression of a gene can be calculated from the formula $2^{-\Delta Ct}$. The corresponding ΔCt values for AAA and ASD are means $\pm$ S.D. (for normally distributed data) or medians (range) (for non-normally distributed data). High (low) ΔCt values reflect low (high) mRNA expression levels. A ΔCt value of 20 represents the detection limit of the assay. TGF-β, transforming growth factor-β.

			ΔCt	
Response	Fold increase in AAA	*P* value	AAA	ASD
Th1-associated				
T-bet	19.6 (2.6–380)	$\leqslant 0.05$	8.7 (4.3–20)	13.3 (5.2–20)
IL-2	104 (7.6–849)	< 0.001	9.7 (6.0–13.3)	16.2 (10.7–20)
IFN-γ	265 (8.8–1746)	< 0.001	9.0 (5.8–13.5)	18.3 (8.6–20)
TNF-α	3.0 (0.8–15.9)	0.09	8.1 (3.9–11.1)	9.3 (6.0–20)
IL-1α	1105 (11.2–8540)	< 0.01	8.3 (4.0–20.0)	16.6 (5.7–20.0)
IL-1β	12.4 (3.7–44.6)	< 0.001	2.7 ± 1.5	6.2 ± 2.8
Th2-associated				
GATA-3	20.1 (2.6–380)	< 0.01	6.8 (3.1–11.4)	10.4 (5.4–20)
IL-4	2.1 (0.7–10.3)	0.12	1.9 (7.1–15.2)	12.7 (9.9–20)
IL-10	1.4 (0.4–5.3)	0.55	5.8 (3.2–9.4)	6.3 (3.3–20)
IL-13	300 (2.2–948.8)	< 0.01	11.0 (4.8–20)	20.0 (11.8–20)
General inflammatory				
MCP-1	2.8 (1.03–10.2)	< 0.05	-1.7 ± 1.8	0.0 ± 1.7
MIP-1β	4.9 (1.6–14.9)	< 0.05	1.2 ± 1.3	3.1 ± 3.0
MIF	2.0 (1.3–3.6)	< 0.01	-2.0 ± 1.2	-0.9 ± 0.9
TGF-β	1.3 (0.81–2.38)	0.19	-0.9 ± 0.9	-0.5 ± 1.1

immunoreactivity, which was infrequently observed in ASD samples (limited to infiltrates in the medial-adventitial transition zone in some ASD controls), clearly demonstrating enhanced B-cell infiltration in AAAs (Figures 1A and 1B). Eosinophils were not found in AAA or ASD samples (results not shown).

The results of our cellular characterization are consistent with the cytokine expression findings and demonstrate the over-representation of cells important for Th1- and Th2-type responses. Subsequently, the downstream consequences were examined by assessing the basal expression level and/or the activation status of relevant inflammatory transcription factors, i.e. transcription factors mainly involved in the signal transduction of the cytokines expressed in AAAs {C/EBPα, C/EBPβ and C/EBPδ, NF-κB and c-Jun [AP-1 (activator protein-1)]}.

Enhanced expression of (activated) pro-inflammatory transcription factors in AAAs

Protein homogenates of AAA and ASD samples were prepared and subjected to Western blot analysis. Aortic transcription factor concentrations were quantified relative to β-actin. When compared with the ASD samples, the aortic concentrations of the C/EBPα, C/EBPβ, C/EBPδ isoforms were 6.3-, 3.1- and 4.2-fold higher ($P < 0.05$) respectively, in the AAA samples (Figure 2A). Baseline p65 NF-κB and c-Jun expression levels were comparable in the AAA and ASD samples (Figure 2B), but the aortic wall concentrations of their activated forms, p65 NF-κB_{active} and phospho-c-Jun, were 3.7- and 3.3-fold (both $P < 0.001$) higher respectively, in the AAA samples as assessed by antibodies specifically detecting activated/phosphorylated epitopes (Figure 2B). Taken together, these results demonstrate an enhanced pro-inflammatory status at the transcription factor level in AAAs, despite enhancement of expression of cytokines with anti-inflammatory properties (e.g. IL-10).

Enhanced IL-6 and IL-8 expression in AAAs

Next, we examined whether inflammatory factors that are positively regulated by C/EBPs, p65 NF-κB and c-Jun are also elevated in AAAs when compared with ASD. IL-8 transcription and IL-6 expression rely on the concomitant activation of signalling pathways activating NF-κB and AP-1 (i.e. c-Jun) or NF-κB and C/EBPs [21,22]. Indeed, IL-6 and IL-8 mRNA expression was strongly and significantly elevated in AAA samples compared

Table 3 Differences in protein expression of markers of Th1-associated, Th2-associated and general inflammatory responses

Aortic wall protein expression levels of markers of Th1-associated, Th2-associated and general inflammatory responses were determined in aneurysmal wall (AAA; $n = 14$) and atherosclerotic wall (ASD; $n = 11$) samples using a Bio-Plex assay or ELISA for MCP-1. Values are medians (range). ND, not detectable (i.e. below the detection limit of the Bio-Plex assay). NA, not applicable.

	Protein expression (ng/mg of total aortic protein)		
Response	AAA	ASD	*P* value
Th1-associated			
IL-2	0.3 (0.04–0.6)	ND	< 0.001
IFN-γ	8.2 (2.3–14.9)	0.0 (0–4.4)	< 0.001
TNF-α	0.2 (0.0–0.8)	ND	< 0.001
IL-1β	4.3 (1.1–19.1)	0.42 (0.00–2.00)	< 0.001
Th2-associated			
IL-4	0.3 (0–1.4)	ND	0.12
IL-5	ND	ND	NA
IL-10	ND	ND	NA
IL-13	1.4 (0.3–5.4)	ND	< 0.01
General inflammatory			
MCP-1	213 (85–1781)	116 (48–923)	0.17
MIP-1β	7.5 (5.1–27.4)	7.0 (0.5–14.2)	0.23
G-CSF	6.0 (1.3–31.4)	0.0 (0–0.75)	< 0.001
GM-CSF	0.3 (0–1.5)	ND	< 0.01
IL-7	0.2 (0–0.6)	ND	< 0.01
IL-12	< 0.05 (0–0.3)	ND	0.12
IL-17	< 0.05 (0–11.7)	ND	0.53

with ASD samples (Table 4). Analysis of IL-6 and IL-8 expression levels in AAA and ASD tissue homogenates by ELISA confirmed that the AAA samples contained very large amounts of IL-6 and IL-8 (Figure 3A). IL-6 and IL-8 levels exceeded the levels of all other cytokines determined in the AAA samples and were increased more than 100-fold ($P < 0.001$) compared with the ASD samples. No relationship was found between the IL-6 or IL-8 level and the aneurysm diameter.

Hyperexpression of IL-6 and IL-8 clearly demonstrates an inflammatory divergence between AAAs and ASD. IHC analysis of IL-6 and IL-8 expression (Figure 3B) confirmed the abundant expression of both cytokines in AAA samples. Of note, IL-6 and IL-8 immunoreactivity in AAA samples were dispersed throughout all layers of the aortic wall and not essentially confined to the intimal layer and intimal border zone of the media as in ASD samples. Refined analysis of the cell types expressing IL-6 and IL-8 in AAA samples revealed that IL-6 immunoreactivity was predominantly associated with plasma cells and macrophages, whereas IL-8 immunoreactivity was mainly associated with lymphocytes and neutrophils. IL-6 and IL-8 immunoreactivity in ASD samples on the other hand was predominantly limited to foam cells/macrophages, vascular smooth muscle cells and lymphocytes. Taken together, these results demonstrate a transmural hyperexpression of IL-6 and IL-8 in AAAs.

Examination of the molecular and cellular effects downstream of IL-6

IL-6-mediated responses depend on the availability of cell-bound IL-6R or sIL-6R. sIL-6R can induce IL-6 signalling in cells not constitutively expressing IL-6R (IL-6 trans-signalling) but expressing its receptor dimer gp130 [23]. Figure 4(A) shows that sIL-6R was present in the aortic wall, and that levels were comparable in AAA and ASD samples. Quantification of phospho-STAT-3, a downstream transcriptional effector of IL-6, showed that the AAA wall contained higher levels of phospho-STAT-3 (20-fold increase; $P < 0.01$) compared with the ASD wall (Figure 4B). The protein levels of the inactive form (non-phosphorylated STAT-3) were lower in the AAA wall (Figure 4B). Taken together, these results indicate that hyperexpression of IL-6 in AAAs is associated with elevated levels of phospho-STAT3, i.e. enhanced IL-6 signalling.

Well-established functional downstream effects (read-outs) of IL-6 include the differentiation/maturation of B-cells into plasma cells and the activation of cytotoxic T-cells. Evaluation of the plasma cell content in AAA and ASD samples using IHC demonstrated an abundance of plasma cells in the AAA wall, whereas plasma-cell-specific immunoreactivity (CD138) was not, or hardly, present in the ASD wall (Figures 1C and 1D). Expression of perforin, a factor which indicates cytotoxic T-cell activation, was significantly elevated in AAA samples, both at the transcriptional (13-fold, $P = 0.006$; results not shown) and protein (Figure 4C) expression levels.

Examination of the molecular and cellular effects downstream of IL-8

IL-8 strongly promotes neutrophil infiltration and activation, and can exert strong pro-angiogenic activities. Quantification of infiltrated neutrophils by IHC staining of MPO demonstrated a pronounced recruitment of these cells in AAA samples, but only a scattered presence of neutrophils in the adventitial layer of ASD samples (Figures 1E and 1F).

To examine whether increased pro-angiogenic responses were associated with AAAs, the number of vasa vasorum in AAA and ASD samples were quantified. The doubling of the number of vasa vasorum (9/mm^2 in AAA samples compared with 4/mm^2 in ASD samples) points to an enhanced angiogenic response in AAAs (results not shown).

DISCUSSION

In the present study, we set out to determine the aortic levels of inflammatory cytokines in relation to

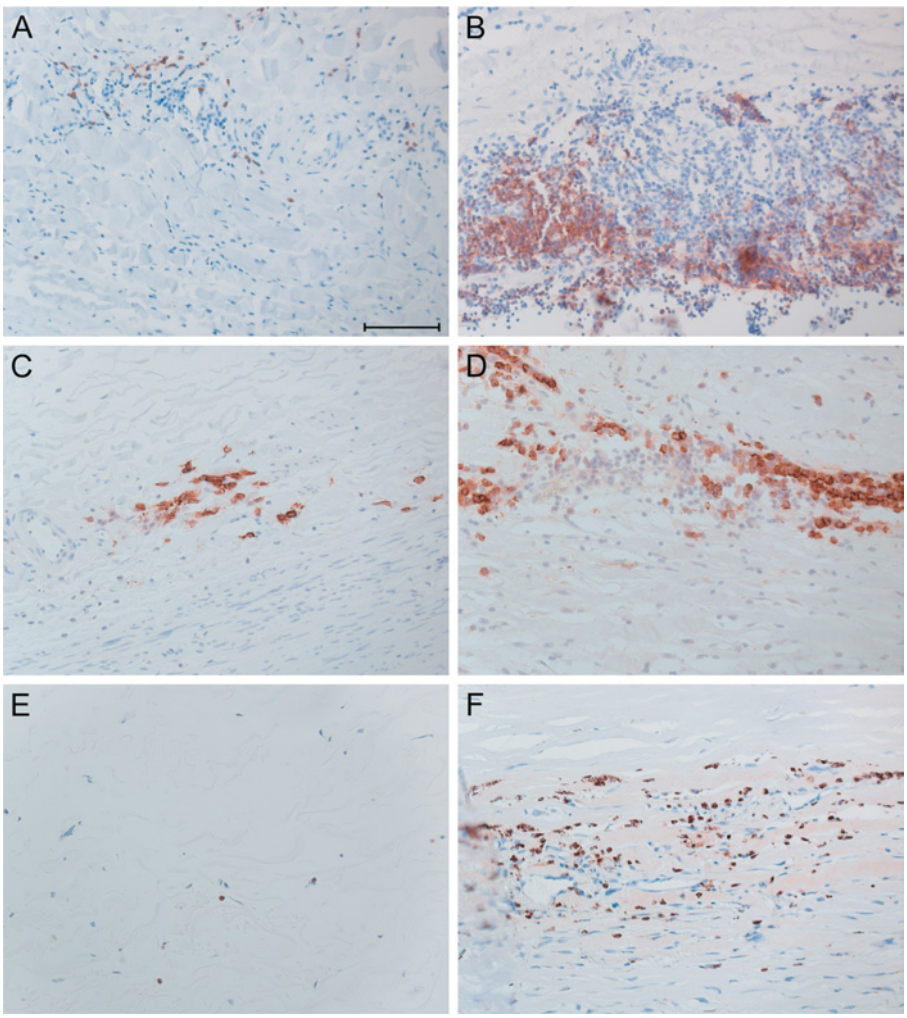

Figure 1 IHC analysis of the cellular composition of AAA and ASD tissue

Representative photomicrographs (similar magnification) of ASD (left) and AAA (right) tissue stained with antibodies specifically detecting (A and B) B-lymphocytes (B-cells; anti-CD20), (C and D) plasma cells (anti-CD138), and (E and F) neutrophils (MPO). Scale bar, 100 μm.

downstream inflammatory transcription factors and cellular responses in human AAAs. Comparison of growing AAAs with ASD using biopsies taken from the same aortic region allowed us to define inflammatory characteristics of AAAs that distinguish the disease from ASD.

The results of the present study demonstrate an enhanced expression of factors associated with both Th1- and Th2-type responses in AAAs. Our findings do not, therefore, support a clear Th1 or Th2 polarization in AAAs. At the transcription factor level, AAAs clearly differ from ASD by having higher concentrations of the pro-inflammatory transcription factors C/EBPα, C/EBPβ and C/EBPδ, and the activated forms of p65 NF-κB and c-Jun, a component of AP-1. Consistent with this, AAAs are characterized by hyperexpression of IL-6 and IL-8, both of which are positively regulated by NF-κB and AP-1. We demonstrate that molecular and cellular processes that are associated with hyperactivation of IL-6 and IL-8 are exaggerated in AAAs: the aortic phospho-STAT-3 and perforin concentrations are elevated in the AAA wall, and B-cells, plasma cells and neutrophils are abundantly present in AAA samples, clearly distinguishing this pathology from ASD.

The large majority of patients with an AAA also suffer from advanced atherosclerotic disease, and AAAs have long been considered to be one of the classical manifestations of atherosclerotic disease. However, traditional atherosclerotic risk factors, such as cholesterol and diabetes, are not associated with an AAA [24], suggesting that an AAA may be a separate entity. Although AAAs and ASD share an inflammatory component relevant for disease evolution, the exact molecular processes causatively involved in the progression of AAAs or ASD are not fully understood. In addition, clear-cut mechanistic differences allowing the discrimination of AAAs from ASD have not been identified to date.

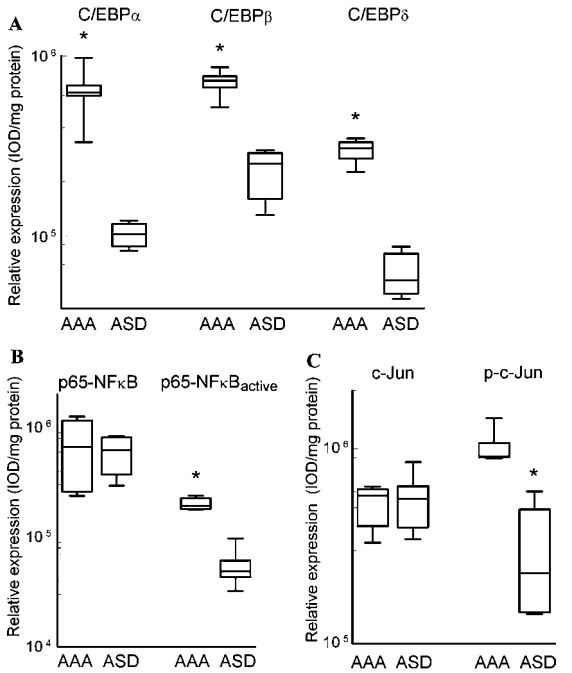

Figure 2 Elevated aortic concentrations of inflammatory transcription factors are characteristic of the AAA wall

Basal levels of (A) C/EBPα, C/EBPβ and C/EBPδ, and (B) p65 NF-κB, c-Jun protein and their activated forms [NF-κB_{active} and phospho-c-Jun (p-c-Jun)] were determined by Western blotting in homogenates of AAA and ASD samples. Transcription factor expression was determined relative to β-actin for each sample. Results are presented in boxplots (median value is indicated by the solid horizontal line, the lower and upper quartiles are indicated by the box, and the range is indicated by the error bars). $^*P < 0.001$ compared with ASD. IOD, integrated optical density.

Table 4 Increase in IL-6 and IL-8 gene expression in the AAA wall

mRNA expression of IL-6 and IL-8 was analysed in aneurysmal wall (AAA; $n = 17$) and atherosclerotic wall (ASD; $n = 12$) samples. The gene expression level of the measured genes in ASD was set at 1, and relative gene expression levels in AAA tissues are expressed as median fold increase, together with the corresponding 95% CI of the median fold increase and the P value. The mean ($\pm$S.D.) ΔCt values are provided in the last two columns. High (low) ΔCt values reflect low (high) mRNA expression levels.

			ΔCt	
Cytokine mRNA	Fold increase in AAA	P value	AAA	ASD
IL-6	29.9 (10.9–86.8)	0.001	2.8 ± 1.7	7.7 ± 2.0
IL-8	21.1 (3.48–65.3)	0.003	1.4 ± 2.1	5.8 ± 4.5

Recent studies assessing the aortic expression of Th1- and Th2-specific inflammatory mediators in human AAAs report controversial observations and claim a predominance of either Th1-type or Th2-type responses [3,10,11]. Our present findings do not support a clear Th1/Th2 polarization in AAAs and indicate an up-regulation of both immune cell responses (as compared with ASD), together with a profound general inflammatory response, which is characterized by the high expression of cytokines, chemokines and growth factors [e.g. MCP-1, MIF, TGF-β (transforming growth factor-β), MIP-1β and G-CSF]. Our present observations are consistent with a recent protein array study by Middleton and co-workers [12], who characterized the AAA as a classical inflammatory condition which is dominated by a generic inflammatory response involving similar pro-inflammatory (e.g. IL-1β and TNF-α) and anti-inflammatory cytokines (e.g. IL-10) [25].

The net effect at the downstream transcription factor level has not been investigated to date. Our present findings demonstrate for the first time that the aortic concentrations of major inflammatory transcription factors at baseline (C/EBPα, C/EBPβ and C/EBP δ) or their activated forms (p65 NF-κB_{active} and phospho-c-Jun) are significantly increased in AAAs relative to ASD. Elevated aortic levels of phospho-c-Jun are in accordance with an increased activation of JNK (c-Jun N-terminal kinase) in an AAA as reported previously [26]. As IL-8 and IL-6 gene expression depend on simultaneous activation of NF-κB and AP-1 [21,22], the aortic concentrations of these cytokines in AAAs and ASD were examined at the gene transcription level. Indeed, IL-6 and IL-8 mRNA were markedly elevated in AAA samples and this was paralleled with a significant increase in IL-6 and IL-8 protein levels. High aortic expression levels of IL-6 and IL-8 in human AAAs have also been reported by others [13,14,27], but the extreme disparity compared with ASD has not been recognized to date. The hyperexpression of IL-6 and IL-8 in the AAA samples observed in the present study thus constitutes an important difference between the pathologies of AAAs and ASD. Predominance of IL-6 and IL-8 in the growing AAA and responses mediated by these cytokines point to a central role of these factors in the development of AAAs.

IL-6 is a well-recognized inducer of the hepatic acute-phase response and a possible cardiovascular risk factor [28]. Because the IL-6R is expressed predominantly by hepatocytes and leucocytes, an extrahepatic role of IL-6 was uncertain. With the discovery of sIL-6R allowing 'IL-6 trans-signalling' in cells normally not expressing IL-6R [23], IL-6 has gained importance in vascular disease, and in atherosclerosis IL-6 has been associated with lipid homoeostasis, vascular remodelling and plaque remodelling [29]. Our present results demonstrate that sIL-6R is expressed in AAA and ASD walls to a comparable extent. We have also demonstrated that a key mediator and downstream effector of IL-6, STAT-3, is present in the aorta, together with its activated (phosphorylated) form phospho-STAT3. We have shown that the large differences in IL-6 protein expression in AAA and ASD samples are paralleled at the downstream transcription factor level: AAA walls contain higher phospho-STAT-3

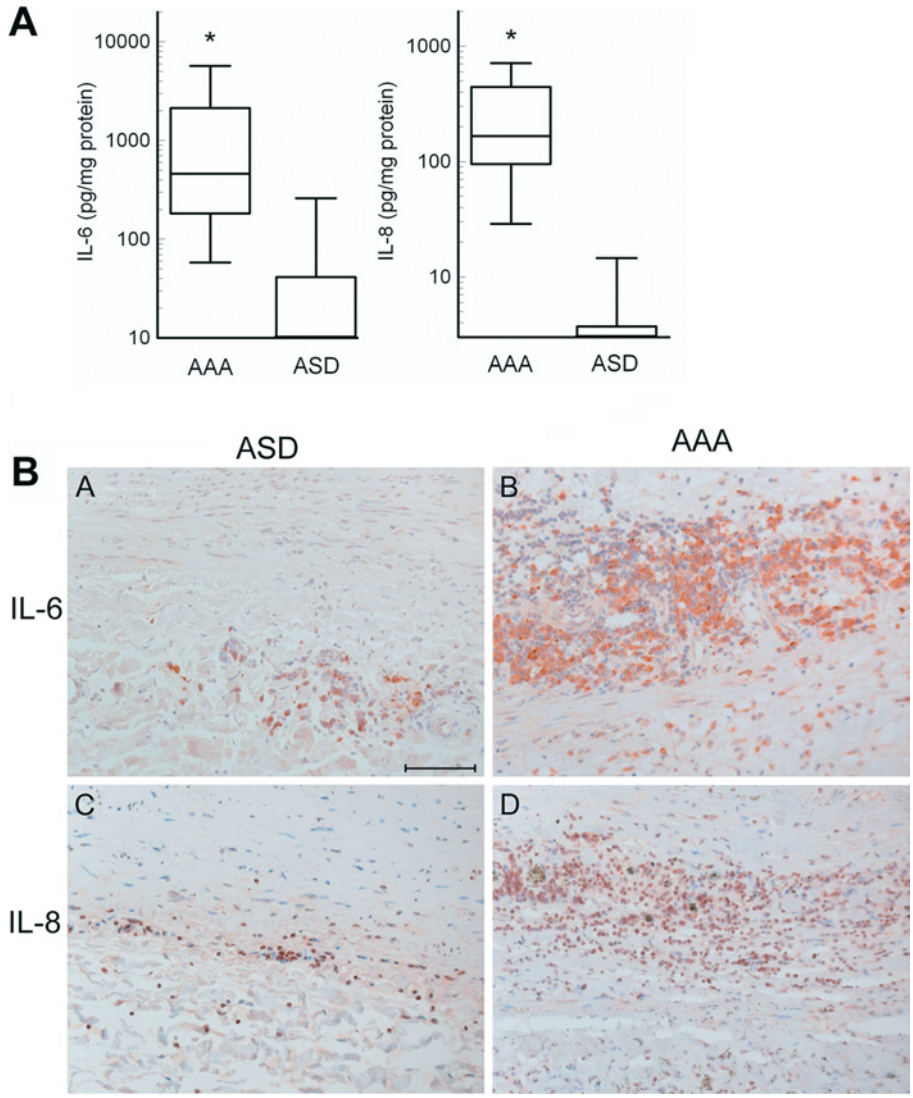

Figure 3 Elevated aortic concentrations of IL-6 and IL-8 protein are characteristic of the AAA wall

(A) Aortic wall protein expression levels of IL-6 and IL-8 were determined by specific ELISAs for aneurysmal wall (AAA; $n = 14$) and atherosclerotic wall (ASD; $n = 12$) samples. Results are presented in box plots as the median, lower and upper quartiles and range. $^{*}P < 0.0001$ compared with ASD. (B) Representative photomicrographs (similar magnification) of ASD (left) and AAA (right) tissue stained with antibodies specifically detecting IL-6 or IL-8. IL-6 immunoreactivity was associated with plasma cells and macrophages; IL-8 immunoreactivity was associated with lymphocytes and neutrophils based on morphological characteristics. Scale bar, 100 μm.

concentrations than ASD, pointing to a pronounced activation of the IL-6 (trans)signalling route in AAAs.

Increased IL-6 (trans)signalling may, at least partly, explain the differences in cellular composition seen in AAA and ASD wall samples. IL-6 reportedly controls late B-cell differentiation and plasma cell formation, and is an established promoter of T-cell migration, retention and activation [30,31]. Enhanced activation of the IL-6 route may very well be responsible for the abundant infiltration of B-cells observed in the present study and by others [14,32]. Furthermore, the histological results in the present study indicate a high abundance of plasma cells (plasmacytosis) in the AAA samples, suggesting a higher B-cell differentiation rate [33]. Analysis of CD4 and CD8 expression indicates mainly increased infiltration of cytotoxic T-cells in AAA samples. Moreover, increased perforin expression, an established marker of cytotoxic T-cell activation [34], demonstrated an increased cytotoxic T-cell activation status in AAA compared with ASD samples, which is consistent with the observation that apoptosis is enhanced in AAAs [35].

Increased IL-8 expression has been reported in several inflammatory conditions, including Kawasaki's disease [36] and Behçet disease [37], two conditions that are associated with aneurysm formation. Consistent with this, expression of IL-8 in aortic aneurysmal tissue has been described [12,27]. To our knowledge, the hyperexpression of IL-8 associated with neutrophil cell infiltration

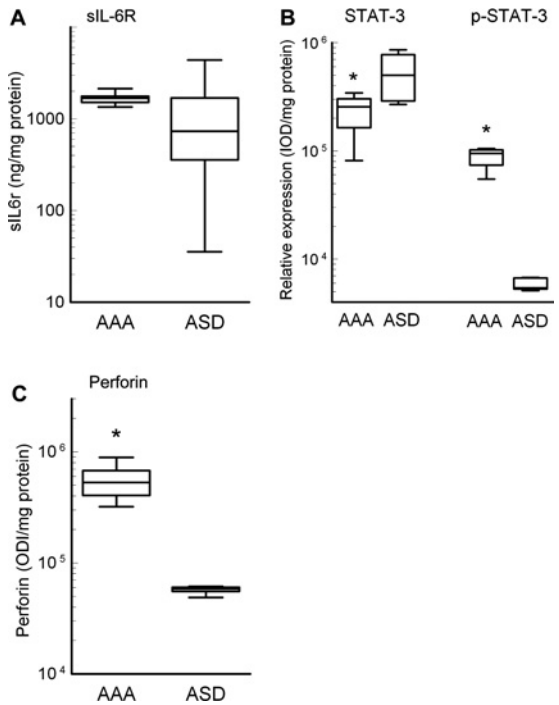

Figure 4 Assessment of the functional components of the IL-6 signalling pathway in AAA and ASD walls

(A) Aortic wall protein expression levels of sIL-6R was determined by ELISA from aneurysmal wall (AAA; $n = 14$) and atherosclerotic wall (ASD; $n = 12$) samples. (B) Basal levels of STAT-3 protein and levels of the activated form [phospho-STAT-3 (p-STAT-3)] were determined by Western blotting in AAA and ASD homogenates. Expression was determined relative to β-actin. $^*P < 0.0001$ compared with ASD. (C) Aortic wall protein expression levels of perforin was determined by ELISA from aneurysmal wall (AAA; $n = 14$) and atherosclerotic wall (ASD; $n = 12$) samples. Elevated perforin protein levels in the aneurysmal wall indicate increased cytotoxic T-cell activation in AAAs. Results are presented in box plots as medians, lower and upper quartiles and ranges. $^*P < 0.001$ compared with ASD. IOD (ODI), integrated optical density.

in the AAA samples observed in the present study has not been reported to date and suggests that neutrophil infiltration is a hallmark of an AAA, allowing the discrimination of AAAs and ASD. Furthermore, G-CSF, the simultaneous expression of which facilitates IL-8-dependent neutrophil cell recruitment [38], was highly expressed in the AAA, but not in ASD, samples. It is tempting to speculate that the concomitant expression of these factors is responsible for neutrophil infiltration which appears critical for the process of aneurysm formation [39,40].

In summary, our present results demonstrate that inflammation in the AAA wall is clearly distinct from inflammation in ASD, and that IL-6 and IL-8 hyperexpression and the dominance of IL-6- and IL-8-mediated responses prevail in an AAA. This comprehensive inflammatory response in an AAA may well be responsible for the broad, albeit not universal, up-regulation of selective members of the MMP (matrix metalloproteinase) class of proteases [2,16] {MMP-8 (neutrophil-derived) and MMP-9 (AP-1-regulated [26])}, and the cysteine proteases cathepsin K, L and S [16] (NF-κB- [41], C/EBP- [42] and IFN-γ- [43] regulated respectively) in the disease. Anti-inflammatory strategies attenuating IL-6 and IL-8 expression or activity [26], and/or IL-6 and IL-8 signalling, may prove effective for the pharmaceutical stabilization of AAAs.

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