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### Permalink

<https://escholarship.org/uc/item/4mj1f110>

### Journal

Journal of the American Heart Association, 8(8)

### Authors

Gu, Bon-Hee

Choi, Justin

Shen, Ying

et al.

### Publication Date

2019-04-16

### DOI

10.1161/JAHA.118.011671

Peer reviewed

# Elastin-Specific Autoimmunity in Smokers With Thoracic Aortic Aneurysm and Dissection is Independent of Chronic Obstructive Pulmonary Disease

Bon-Hee Gu, MS; Justin C. Choi, MD; Ying H. Shen, MD, PhD; Li-Zhen Song, MD; Michael E. Scheurer, PhD, MPH; Amber Luong, MD, PhD; Antony Rodriguez, PhD; Prescott Woodruff, MD; Laura Koth, MD; David B. Corry, MD; Farrah Kheradmand, MD; Scott A. LeMaire, MD

**Background**—Thoracic aortic aneurysm (TAA) and dissection (TAD) are characterized by progressive disorganization of the aortic wall matrix, including elastin, a highly immunogenic molecule. Whether acquired autoimmune responses can be detected in TAA/TAD patients who are smokers is unknown. The objectives of this study were to determine whether TAA/TAD smokers have increased T-cell responses to human elastin fragments, and to determine whether autoimmune responses in TAA/TAD smokers are dependent on chronic obstructive pulmonary disease.

**Methods and Results**—In a cross-sectional study (N=86), we examined peripheral blood CD4<sup>+</sup> T cell responses to elastin fragments in never-, former-, or current-smokers with or without TAA/TAD. CD4<sup>+</sup> T cells were co-cultured with irradiated autologous peripheral blood CD1a<sup>+</sup>/CD14<sup>+</sup> antigen presenting cells pulsed with or without elastin fragments to measure cytokine production. Baseline plasma concentration of anti-elastin antibodies and elastin-degrading enzymes (eg, matrix metalloproteinase-9, and -12, and neutrophil elastase) were measured in the same cohort. elastin fragment-specific CD4<sup>+</sup> T cell expression of interferon- $\gamma$ , and anti-elastin antibodies were dependent on history of smoking in TAA/TAD patients but were independent of chronic obstructive pulmonary disease. Matrix metalloproteinase-9, and -12, and neutrophil elastase plasma concentrations were also significantly elevated in ever-smokers with TAA/TAD.

**Conclusions**—Cigarette smoke is associated with loss of self-tolerance and induction of elastin-specific autoreactive T- and B-cell responses in patients with TAA/TAD. Development of peripheral blood biomarkers to track immunity to self-antigens could be used to identify and potentially prognosticate susceptibility to TAA/TAD in smokers. (*J Am Heart Assoc.* 2019;8:e011671. DOI: 10.1161/JAHA.118.011671.)

**Key Words:** aneurysm • immune system • immunology • inflammation

Thoracic aortic aneurysm (TAA) and dissection (TAD) are interrelated diseases characterized pathologically by fragmentation and architectural distortion of the medial layer of the aortic wall.<sup>1</sup> Although some patients develop TAA/TAD because of genetic mutations, at least 70% of cases are unrelated to heritable conditions.<sup>2</sup> In those unrelated to genetically-triggered causes, hypertension confers the highest

risk of disease development and progression.<sup>3</sup> Smoking has been strongly associated with increased risk for development of abdominal aortic aneurysms, although a smaller effect has been reported for TAA/TAD.<sup>4</sup> Globally, temporal trends in mortality from TAA/TAD patients show a positive linear relationship between hypertension, but not with smoking prevalence.<sup>5</sup> Importantly, both abdominal aortic aneurysms

From the Department of Medicine, Pulmonary and Critical Care (B.-H.G., L.-Z.S., A.R., D.B.C., F.K.), Division of Cardiothoracic Surgery, Michael E. DeBakey Department of Surgery (J.C.C., Y.H.S., S.A.L.), Cardiovascular Research Institute (Y.H.S., F.K.), Section of Hematology-Oncology, Department of Pediatrics (M.E.S.), Departments of Pathology and Immunology (A.R., D.B.C., F.K.), and Biology of Inflammation Center (A.R., D.B.C., F.K.), Baylor College of Medicine, Houston, TX; Department of Cardiovascular Surgery, Texas Heart Institute, Houston, TX (Y.H.S., S.A.L.); Department of Otorhinolaryngology – Head and Neck Surgery, McGovern Medical School, University of Texas Health Science Center, Houston TX (A.L.); University of California San Francisco, San Francisco, CA (P.W., L.K.); Center for Translational Research in Inflammatory Diseases, Michael E. DeBakey VA, Houston, TX (D.B.C., F.K.); CHI St Luke's Health—Baylor St Luke's Medical Center, Houston, TX (S.A.L.).

Accompanying Figures S1 through S6 are available at <https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.011671>

**Correspondence to:** Farrah Kheradmand, MD, and Scott A. LeMaire, MD, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mails: farrahk@bcm.edu; slemaire@bcm.edu

Received November 30, 2018; accepted February 26, 2019.

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## Clinical Perspective

### What Is New?

- We present a newly discovered association between activation of acquired immunity and smoking in patients with thoracic aortic aneurysm and dissection (TAA/TAD), which is independent of smoke-induced chronic obstructive pulmonary disease.
- We show that cigarette smoke is associated with a loss of self-tolerance and induction of elastin-specific autoreactive T cells in TAA/TAD patients.

### What Are the Clinical Implications?

- The clinical implications of this work include evidence for autoimmune T-cell activation associated with smoking in patients with TAA/TAD.
- These findings provide novel insights into factors that underlie the heterogeneous nature of TAA/TAD patients and could provide the rationale for developing peripheral blood biomarkers to track immune responses to self-antigens.
- These strategies could identify and prognosticate susceptibility to TAA/TAD in smokers.

and atherosclerosis have been tightly linked to the activation of innate and acquired immunity in smokers.<sup>6–9</sup> In contrast, it remains unknown whether the pathophysiological changes in TAA/TAD in smokers are linked to immune responses to matrix molecules.

Innate and acquired immune cells (eg, activated macrophages, B and T cells) form well-organized inflammatory foci within abdominal aortic aneurysms lesions.<sup>10,11</sup> Several proinflammatory chemokines, including interferon-inducible protein-10 and interferon-inducible T-cell alpha chemoattractant, are associated with the induction of several elastolytic enzymes including matrix metalloproteinases (MMP-9 and MMP-12) in macrophages and antigen presenting cells (APCs).<sup>10,12,13</sup> We and others have shown that in response to cigarette smoke, activated T cells that recognize elastin fragments (EFs) in the context of major histocompatibility class II molecules secrete 2 canonical cytokines, interferon- $\gamma$ , and interleukin (IL)-17A, that are associated with T-helper type 1 (Th1) and Th17 subset of T cells, respectively.<sup>12,14,15</sup> Consistently, interferon- $\gamma$  production<sup>16</sup> in aneurysmal aortic tissue significantly correlates with elastin degradation, indicating a close pathophysiological association.<sup>10</sup> Furthermore, the indispensable role of Th1 cells in aortic aneurysm development was demonstrated in animal models using CD4, interferon- $\gamma$ , or Cys-X-Cys chemokine receptor 3-deficient mice.<sup>13,16</sup> In humans with TAA/TAD, however, it is unclear whether a direct association exists between environmental factors (eg, active or former smoking) and acquired

immune responses (eg, circulating antigen-specific antibody or T cells).

In prior cross-sectional studies, we have shown significant associations between CD4<sup>+</sup> T cell reactivity to specific elastin peptides and the severity of emphysema in active or former smokers with chronic obstructive pulmonary disease (COPD).<sup>12,17,18</sup> Therefore, in this study, we used a similar approach to examine the role of autoreactive T cells in a well-characterized cohort of never-, former- or current-smokers with or without TAA/TAD. This approach allowed us to explore the concurrent effects of smoking on lung and vascular disease, and the role of autoimmune responses to the most abundant component of the elastic organs, elastin. Our findings suggest that cigarette smoking induces systemic inflammation that may reduce immune tolerance against self-antigens.

## Materials

The authors declare that all supporting data are available within the article.

## Study Participants

A total of 86 subjects who were either lifetime non-smokers (n=33) or had a significant history of smoking (n=53) were enrolled prospectively from 3 independent cohorts: Cohort 1: TAA/TAD subjects who underwent pulmonary function testing were recruited from the Molecular Mechanisms of Thoracic Aortic Aneurysm and Dissection (n=60) cohort at Baylor College of Medicine; 11 subjects with known evidence for genetic and autoimmune diseases (Marfan syndrome n=7, giant cell arteritis n=1, rheumatoid arteritis n=1, polymyalgia rheumatica n=1, and idiopathic thrombocytopenic purpura n=1) were excluded. TAA/TAD subjects in this cohort did not have any known history of heart disease and were not actively seeking treatment for coronary artery disease. At the time of enrollment patients were diagnosed with TAA without dissection (TAA, n=35), or TAA and TAD (TAA+TAD, n=14) based on radiographic findings. Cohort 2: Healthy smokers with no evidence of airway obstruction or emphysema were recruited from the Sub Population and Intermediate Outcome Measures in COPD cohort at the University of California in San Francisco (n=19). Cohort 3: We recruited healthy non-smoker controls from the HSC-MS-13-0443 study at the McGovern Medical School of the University of Texas Health Science Center (n=18). Smokers were further characterized by their current or former (>1 year) smoking habits. Studies were approved by the Institutional Review Boards at Baylor College of Medicine, University of Texas Health Science Center, and University of California in San Francisco, respectively; informed written

consent was obtained from all patients. Full patient demographic data are provided in Table 1 and Figure S1.

## Peripheral Blood Mononuclear Cell Isolation and Immune Cell Purification

Human CD4<sup>+</sup> T cells were purified from peripheral blood mononuclear cells isolated from heparinized blood samples using Ficoll-Paque Plus (GE Healthcare) centrifugation technique.<sup>17</sup> We obtained 10 to 15 mL of heparinized blood samples after subjects enrolled in the study. Approximately 10 to 30×10<sup>6</sup> peripheral blood mononuclear cells were isolated from whole blood and were labeled with MicroBeads conjugated to anti-CD4 antibody (Miltenyi Biotec) followed by AutoMACS (Miltenyi Biotec) positive selection. Antigen presenting cells (APCs) were positively selected from CD4<sup>+</sup> T cell depleted fraction of the same peripheral blood mononuclear cells using anti-CD1a and anti-CD14 MicroBeads (Miltenyi Biotec).

## T Cell Stimulation With EFs and Cytokine Measurement

In some studies, we used a range of elastin peptide (EFs; QP45, Elastin Products Company) concentrations of 0.05, 0.1, and 0.5 μmol/L in T cell cultures. The optimum dose of EFs was determined to be 30 μg/mL (0.5 μmol/L) EFs. Therefore, in all subsequent assays, human CD4<sup>+</sup> T cells (5×10<sup>5</sup>) were co-cultured with γ-irradiated (30 Gy [SI unit of absorbed dose of ionizing radiation]) autologous APCs (5×10<sup>4</sup>) in 10:1

ratio (T cells to APCs) in the presence or absence of 30 μg/mL EFs in duplicate or triplicate conditions. After 3 to 4 days of co-culture, supernatants were collected and stored at −80°C for batch analysis of cytokines. LINCO-plex (Millipore) was used to measure concentrations of a selected group of cytokines: interferon-γ, IL-1β, IL-17A, IL-6, IL-10, and IL-13. T cell response, reported as fold-change, was calculated based on cytokine concentration detected in the presence of EFs divided by that detected in the absence of EFs (nil stimulation).

## Flow Cytometry-Based Assessment of Intracytoplasmic Cytokine and Proliferation

In a selected group of subjects, flow cytometry was used to detect the optimum range of elastin peptide using a range of 0.05, 0.1, and 0.5 μmol/L in T cell co-cultures using intracytoplasmic cytokine expression of interferon-γ and IL-17A, as described above.<sup>12</sup> CD4 T cells were cultured for 3 to 4 days in the presence or absence of APCs (CD1a<sup>+</sup>/CD14<sup>+</sup>) and were stimulated with phorbol 12-myristate 13-acetate (10 ng/mL; Sigma) and ionomycin (200 ng/mL) supplemented with monensin (10 ng/mL; Sigma) for 4 hours. Cells were stained for surface markers with FITC-CD3 and PE/Cy5-CD4 (BD Biosciences), fixed with 1% paraformaldehyde, permeabilized with 0.5% saponin, and stained with allophycocyanin (APC)–interferon-γ (BD Biosciences) and phycoerythrin (PE)–IL-17A (eBioscience) antibodies for analysis of intracytoplasmic cytokine production by flow cytometry. Data were analyzed by FlowJo software.

**Table 1.** Clinical and Demographic Information of the Study Participants

Characteristics	HC (n=37)		TAA/TAD (n=49)			
	NS (n=18)	S (n=19)	TAA NS (n=8)	TAA S (n=27)	TAA+TAD NS (n=7)	TAA+TAD S (n=7)
Smokers: current		6 (32%)		11 (41%)		3 (43%)
Smokers: former		13 (68%)		16 (59%)		4 (57%)
Age, mean±SD, y	54±15*	66±9	58±8	67±10	60±12	69±7
BMI, mean±SD, kg/m <sup>2</sup>	32±8	29±5	32±7	28±8	31±6	28±6
Sex, male (%)	6 (33)	11 (58)	6 (75)	17 (63)	5 (71)	6 (86)
Blood pressure±SD, mm Hg						
Systolic	ND	132±17	138±13	136±16	136±17	150±23
Diastolic	ND	79±11	66±26	80±11	75±15	88±13
Lung function						
FEV <sub>1</sub> /FVC±SD	ND	77±5	74±14	73±9	77±8	80±15
FEV <sub>1</sub> (L) ±SD	ND	2.9±0.5	2.7±0.7	2.2±1.1	2.4±0.9	2.8±0.7

BMI indicates body mass index; FEV<sub>1</sub>, forced expiratory volume in 1-second; FVC, forced vital capacity; HC, healthy control; L, liters; ND, not determined; NS, non-smokers; S, ever smokers; TAA, thoracic aortic aneurysm (TAA) without dissection; TAA/TAD, thoracic aortic aneurysm and dissection; TAA+TAD, TAA with dissection.

\*Significant difference between NS in HC and S in TAA by the Kruskal–Wallis test with Dunn post-test for multiple comparisons (*P*<0.05).

To measure cell proliferation, CD4<sup>+</sup> T cells isolated from peripheral blood mononuclear cells were labeled with carboxyfluorescein succinimidyl ester according to manufacturer's protocol (eBioscience) and co-cultured with APCs for 3 days as described. Briefly, T cells were labeled with 10 μmol/L carboxyfluorescein succinimidyl ester and relative proliferation was measured by flow cytometry. T cells treated with 2% phytohemagglutinin were used as positive control for proliferation.

### ELISA-Based Quantification of Elastin-Specific Antibody Titer

ELISA was used to detect elastin-specific antibody titer as previously described.<sup>17</sup> Briefly, anti-human elastin antibody titer was detected using polystyrene microtiter plates (flat bottom Fisher) that were coated with 40 μL of 25 μg/mL EFs dissolved in PBS and incubated at 4°C overnight. Plates were washed 3 times with PBS containing 0.05% Tween 20 (BioRad) and blocked with 200 μL of 0.2% I-block (BioRad) for 2 hours at 37°C. Plates were washed, and 2-fold serial dilution of plasma samples was incubated for 2 hours at 37°C before 3 serial washes. Horseradish peroxidase-conjugated goat anti-human IgG antibody (0.16 μg/mL) (Thermo Fisher) was added for 1 hour at 37°C, and the signal was developed using 50 μL of 3,3',5,5'-tetramethylbenzidine (TMB)-ELISA substrate solution (ThermoFisher). The colorimetric reaction was terminated using 0.5 N sodium hydroxide, and the optical density for each well was assessed at 405 nm using a microplate reader. One sample with the highest optical density ratio was selected to use as reference standard.<sup>17</sup>

### Quantification of MMPs and Neutrophil Elastase in Plasma

The concentration of MMP-12, MMP-9 and neutrophil elastase (NE) in plasma were measured using Luminex assay (Millipore) according to the manufacturer's protocol; MMP-12 (cat# HMMP1MAG), MMP9 (cat#HMMP2MAG), and NE (cat# HSP3MAG). As recommended in the manufacturer's protocol, plasma was diluted with assay buffer in 1:20 ratio for MMP-9 and 1:100 ratio for NE.

### Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5 for Mac OS X (GraphPad Software). Comparisons between continuous variables with >2 populations were performed using the Kruskal–Wallis test with Dunn post-test for multiple comparisons. Comparisons of unpaired continuous data from 2 populations were performed using the Mann–Whitney *U* test. For multiple comparisons,

family-wide error rates were maintained at 0.05 using the Bonferroni correction.

## Results

### Elastin-Specific Auto-Reactive T Cells in TAA/TAD

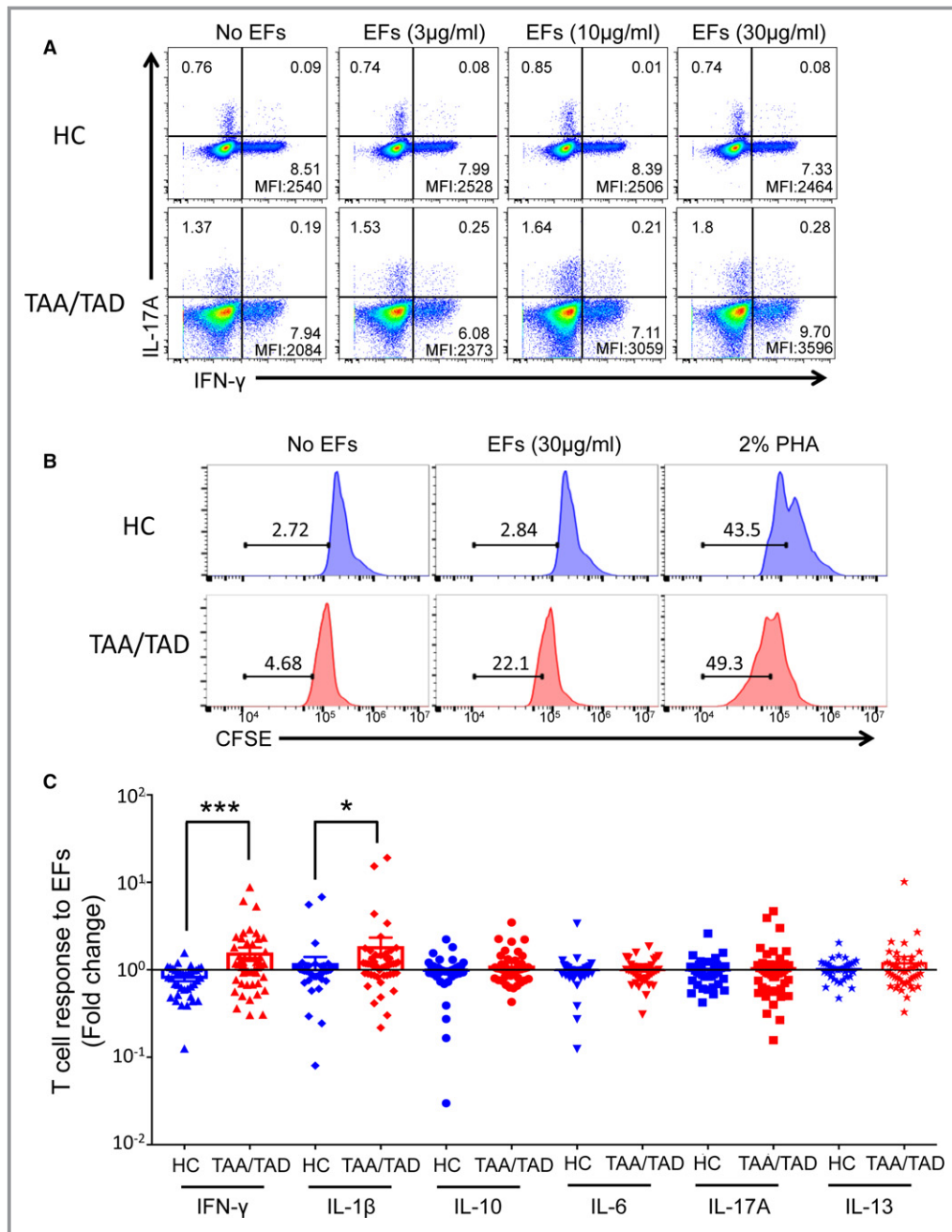
Previously, we had determined the optimal concentration of human EFs in T cell: APC co-culture assays in emphysema subjects.<sup>12,18</sup> Therefore, in our studies, we verified that 30 μg/mL of EFs induce elastin specific auto-reactivity in the TAA/TAD group compared with healthy controls as determined by >50% increase in mean fluorescent intensity (Figure 1A). Similarly, autoreactive CD4<sup>+</sup> T cells from TAA/TAD subjects proliferated in response to EFs, when compared with healthy controls (Figure 1B). At baseline, we did not find any significant differences between major cytokines in TAA/TAD patients and healthy control (Figure S2). However, as expected, we found wide variability in T cell cytokine production between individuals (Figure S3). Therefore, to normalize the data, we used fold-changes over nil-stimulation to identify T cell-specific cytokine responses in this population. In response to EFs stimulation, we found CD4<sup>+</sup> T cells isolated from TAA/TAD patients significantly increased interferon-γ and IL-1β secretion when compared with the healthy smoker or non-smoker controls (Figure 1C). Although there was a trend for an increase in IL-10 secretion in the TAA/TAD patients, it did not reach significance, and we failed to detect a significant difference in IL-17A, IL-13, and IL-6 secretion between the 2 groups (Figure 1C).

### EFs-Specific Autoimmune Responses in TAA/TAD Smokers are Independent of COPD

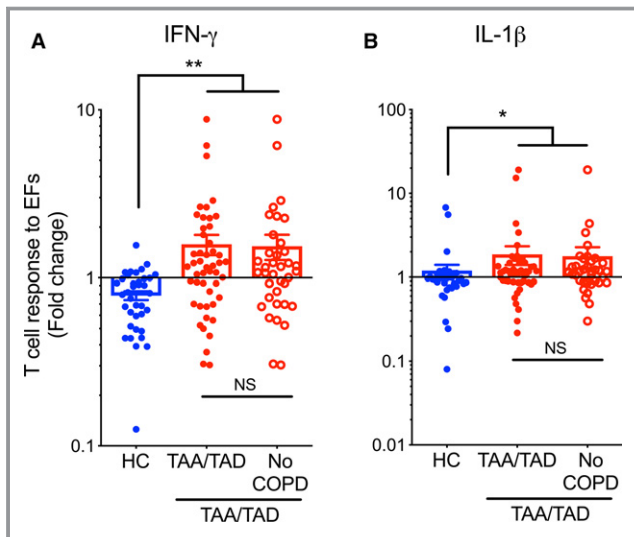
We next examined the association between EFs-specific T cell responses in TAA/TAD patients with or without airway obstruction. We found that compared with healthy controls (smokers and non-smokers), a significant immune response against EFs as assessed by interferon-γ or IL-1β measurements, persisted in TAA/TAD subjects without COPD (Figure 2A and 2B). Furthermore, there was no significant difference between TAA/TAD subjects without airway obstruction (open and closed circles Figure 2A and 2B). Because non-smokers in healthy control subjects and TAA/TAD groups were significantly younger than smokers in healthy control and TAA/TAD groups, we next examined the correlation between age and fold increase in interferon-γ or IL-1β in the same groups. We found no correlation between interferon-γ or IL-1β fold-increase in response to EFs and age (Figure S4).

We next examined whether current or former smoking status in TAA/TAD patients was associated with autoreactive CD4<sup>+</sup> T cells responses. We found that compared with healthy





**Figure 1.** EFs-specific CD4<sup>+</sup> T cell response in TAA/TAD. **A**, Representative intracytoplasmic cytokine of CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) isolated from peripheral blood of healthy controls (HC) or thoracic aortic aneurysm and dissection (TAA/TAD) patients were co-cultured with irradiated CD1a<sup>+</sup>/CD14<sup>+</sup> APCs ( $5 \times 10^4$ ) with EFs (3, 10, or 30  $\mu$ g/mL) or no EFs. After 3 days, interferon- $\gamma$  and IL-17A producing CD4<sup>+</sup> T cells were detected using flow cytometry. Relative abundance and mean fluorescent intensity (MFI) shown in the right lower quadrants. Data are representative of 4 to 5 subjects in each group. **B**, Representative histogram of EFs-specific CD4<sup>+</sup> T cell proliferation. CFSE-labeled CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) were cultured with APCs ( $5 \times 10^4$ ) in presence of EFs (30  $\mu$ g/mL) for 3 days. 2% phytohemagglutinin was used as positive control. Data are representative of 4 to 5 subjects in each group. **C**, Supernatants from the same co-culture conditions described in (A); concentration of interferon- $\gamma$ , IL-1 $\beta$ , IL-10, IL-17, and IL-13 were measured and plotted as fold change over nil stimulation. Each dot represents a data point from an individual subject. \* $P < 0.05$ , and \*\*\* $P < 0.001$  as determined by the Mann-Whitney test; HC: n=37; TAA/TAD n=49. APCs indicates antigen presenting cells; CFSE, carboxyfluorescein succinimidyl ester; EFs, elastin fragments; HC, healthy control; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; MFI, mean fluorescent intensity; PHA, phytohemagglutinin; TAA/TAD, thoracic aortic aneurysm and dissection.



**Figure 2.** EFs-specific CD4<sup>+</sup> T cell expression of interferon- $\gamma$  and IL-1 $\beta$  are independent of airway obstruction in TAA/TAD. Expression of interferon- $\gamma$  (A) and IL-1 $\beta$  (B) in response against EFs was assessed in healthy smoker and non-smoker HC (n=37), and smoker and non-smoker TAA/TAD patients (n=49) or TAA/TAD without chronic obstructive airway disease (COPD; n=37). Each dot represents a data point from an individual subject. \* $P$ <0.05, \*\* $P$ <0.01, as determined by the Kruskal–Wallis test with Dunn post-test for multiple comparisons. COPD indicates chronic obstructive airway disease; EFs, elastin fragments; HC, healthy control; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; NS, not significant; TAA/TAD, thoracic aortic aneurysm and dissection.

smokers and non-smokers, interferon- $\gamma$  was significantly increased in former and current TAA/TAD smokers (Figure 3A). In contrast, however, we found no significant differences in IL-1 $\beta$  expression in CD4<sup>+</sup> T cell responses to EFs in former or current smokers with or without TAA/TAD (Figure 3B). Further, as expected, smoking history was not associated with other cytokines measured (IL-17A, IL-13, IL-10, and IL-6) in this cohort (data not shown). Next to distinguish the role of TAA and TAD phenotypes, we examined T cell responses to elastin in TAA subjects (n=35) who did not have a concurrent diagnosis of TAD. We found that CD4<sup>+</sup> T cells in this group also responded to EFs as shown by increased interferon- $\gamma$  and IL-1 $\beta$  expression (Figure S5A). We also found that current and former smokers with TAA showed significant T cell responses, indicating that removal of the TAD subjects in this cohort did not change the results (Figure S5B and S5C). Furthermore, there were no differences between ascending and descending immune responses in the TAA/TAD cohort (Figure S6).

We have previously shown that CD4<sup>+</sup> T cells isolated from peripheral blood samples in ever-smokers with emphysema show enhanced elastin autoreactivity compared with control subjects.<sup>18</sup> We found that using a threshold for a positive

increase in T cell cytokine response (IL-6 or interferon- $\gamma$ ) to EFs at 1.5-fold over nil-stimulation had over 90% sensitivity to detect emphysema in smokers.<sup>18</sup> Therefore, we next assessed the association between CD4<sup>+</sup> T cell specific response to EFs in the TAA/TAD cohort, using over 1.5-fold increase in interferon- $\gamma$  as the threshold for the presence of autoreactive T cell responses to EFs. As expected, healthy smokers and non-smokers showed only between 0% to 7% positive response to EF stimulation (Table 2). Similarly, we found that 7% of never smokers with TAA/TAD showed autoreactive responses. Notably, this response was significantly increased to 38% in ever-smokers with TAA/TAD when compared with healthy control ever smokers ( $P=0.0018$ ) (Table 2). Together, these findings confirm that T cell-dependent acquired immune responses to EFs in TAA/TAD are associated with smoking history.

### Humoral Responses Against EFs in TAA/TAD

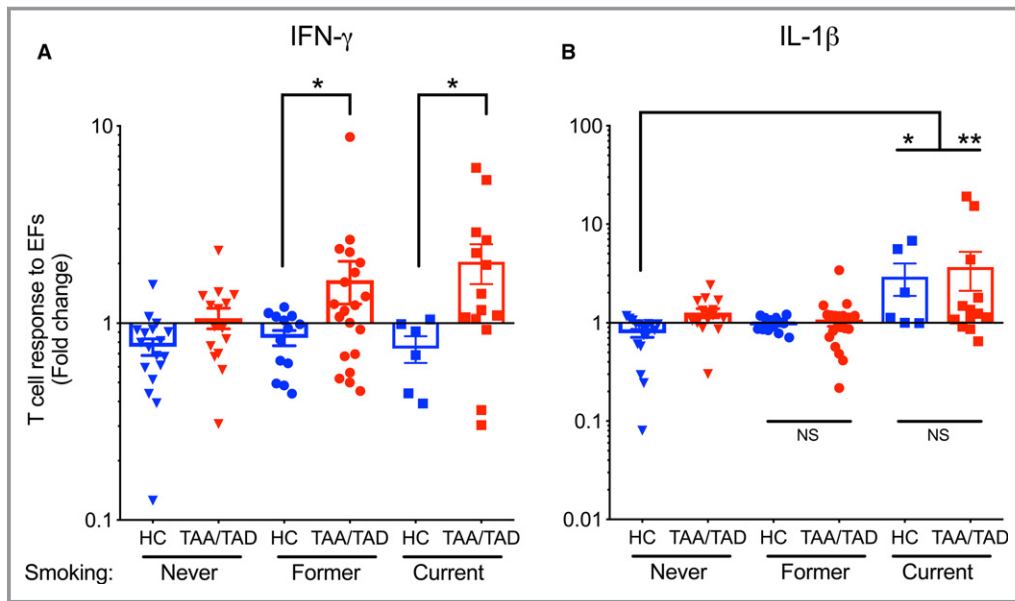
We next examined whether elastin-specific antibodies can be detected in the same TAA/TAD cohort and if they correlate with smoking status. We found that TAA/TAD subjects had significantly increased antibodies to EFs when compared with healthy smoker or non-smoker controls (Figure 4A). Similarly, when we stratified subjects based on their smoking status, we found that ever-smokers with TAA/TAD showed a relative increase in anti-elastin antibodies when compared with healthy smokers or non-smokers with TAA/TAD (Figure 4B).

### Circulating Plasma Elastin-Degrading Enzymes in TAA/TAD

We and others have previously shown that MMP-12, an endogenous proteinase that inhibits alpha-1 anti-trypsin and is highly associated with emphysema, can be directly induced through induction of interferon-inducible protein-10.<sup>12,19</sup> Therefore, we next examined whether members of the endogenous enzymes that can target elastin molecules are elevated in the plasma of TAA/TAD smokers, and if there was an association with airway obstruction. We found that circulating MMP-9, MMP-12, and neutrophil elastase (NE) concentrations were significantly elevated in TAA/TAD subjects when compared with healthy non-smokers and smokers (Figure 5A through 5C). A significant increase in plasma concentration of MMP-9, MMP-12, and NE persisted in TAA/TAD subjects without airway obstruction (Figure 5A through 5C).

### Discussion

The key hallmarks of the pathological changes in TAA/TAD, whether associated with any of the known heritable



**Figure 3.** EFs-specific CD4<sup>+</sup> T cell expression of interferon- $\gamma$ , but not IL-1 $\beta$  response is dependent on history of smoking in TAA/TAD. Expression of interferon- $\gamma$  (A) and IL-1 $\beta$  (B) in response against EFs was assessed in HC non-smoker (n=18), smoker (former n=13, current n=6), TAA/TAD non-smoker (n=15), TAA/TAD smokers (former n=20, current n=14). Each dot represents a data point from an individual subject. For 2 groups comparison, Mann–Whitney test was used. For multiple comparisons in >3 groups, the Kruskal–Wallis test with Dunn’s post-test for multiple comparisons was used. \* $P$ <0.05, \*\* $P$ <0.01. EFs indicates elastin fragments; HC, healthy control; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; NS, not significant; TAA/TAD, thoracic aortic aneurysm and dissection.

conditions or not, are the destruction of the elastic lamellae that manifests as fragmentation of elastic fibers, loss of smooth muscle cells, and progressive loss of normal aortic wall architecture.<sup>1</sup> In this report, we present a newly discovered association between activation of acquired immunity and TAA/TAD using well-characterized non-smokers and smokers. Of note, the association was independent of airway obstruction. Although immunohistochemical-based studies of the diseased aorta have consistently shown well-formed immune foci (eg, CD3 and CD68 cell surface markers) within

inflammatory lesions,<sup>11</sup> evidence for systemic immune responses to self-antigens (eg, elastin) in TAA/TAD has not been previously reported. Notably, we found no correlation between age and interferon- $\gamma$  signal in TAA/TAD patients, indicating that age is not a confounder in our studies. Furthermore, studies focusing on whether smoking status and/or a diagnosis of COPD play a role in autoreactive immune responses in TAA/TAD patients have not been reported. Therefore, to our knowledge, this study presents the first evidence for autoimmune T-cell activation associated with smoking in patients with TAA/TAD.

Our analyses using T cell-specific responses to self-antigen in never, former, and current smokers with or without TAA/TAD showed a significant association between elastin-induced autoreactive T cells and expression of 2 proinflammatory cytokines: interferon- $\gamma$  and IL-1 $\beta$ . We show here that increased expression of these cytokines was specific in our assays, because quantification of several Th17 and Th2 related cytokines (ie, IL-17A, IL-6, IL-10, and IL-13), some of which have been associated with cigarette smoke-induced lung inflammation,<sup>17,18</sup> failed to show significant changes when compared with healthy smoker and non-smoker controls. Induction of T cell specific interferon- $\gamma$  in TAA/TAD patients was associated with smoking history, and we have previously shown that Th1 autoreactivity correlates with

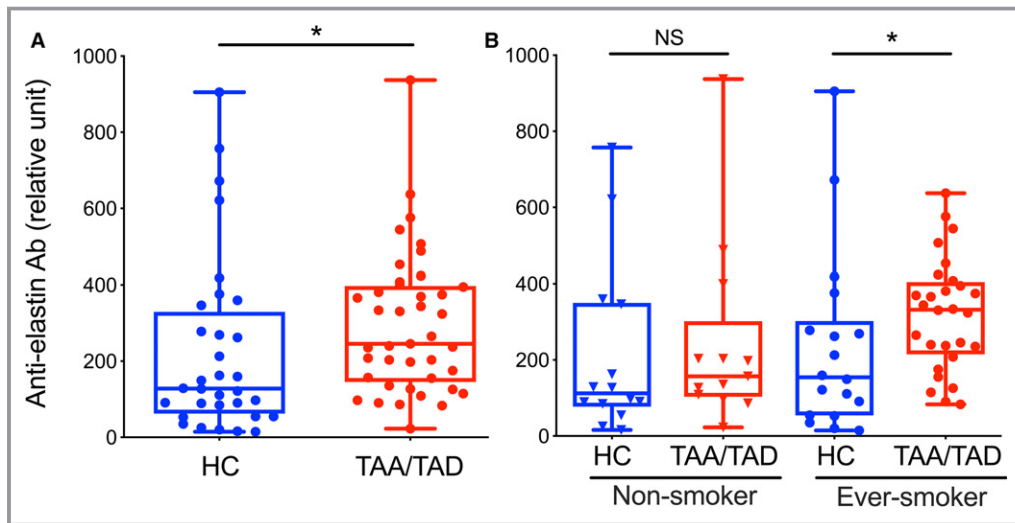
**Table 2.** Elastin-Specific T Cell Responses are Detected in TAA/TAD Patients

Group	HC		TAA/TAD		
	Never (n=18)	Ever (n=19)	Never (n=15)	Ever (n=34)*	
Autoimmune response (%)	+	1 (6%)	0 (0%)	1 (7%)	13 (38%)
	–	17 (94%)	19 (100%)	14 (93%)	21 (62%)

+ Autoimmune response (%) >1.5-fold induction of interferon- $\gamma$  T cell response to elastin fragments. – Autoimmune response (%) <1.5-fold induction of interferon- $\gamma$  T cell response to elastin fragments. HC indicates healthy control; TAA/TAD, thoracic aortic aneurysm and dissection.

\* $P$ =0.0018; Statistical analysis by the contingency test with 2-sided Fisher exact test comparing HC ever smokers vs TAA/TAD ever smokers.

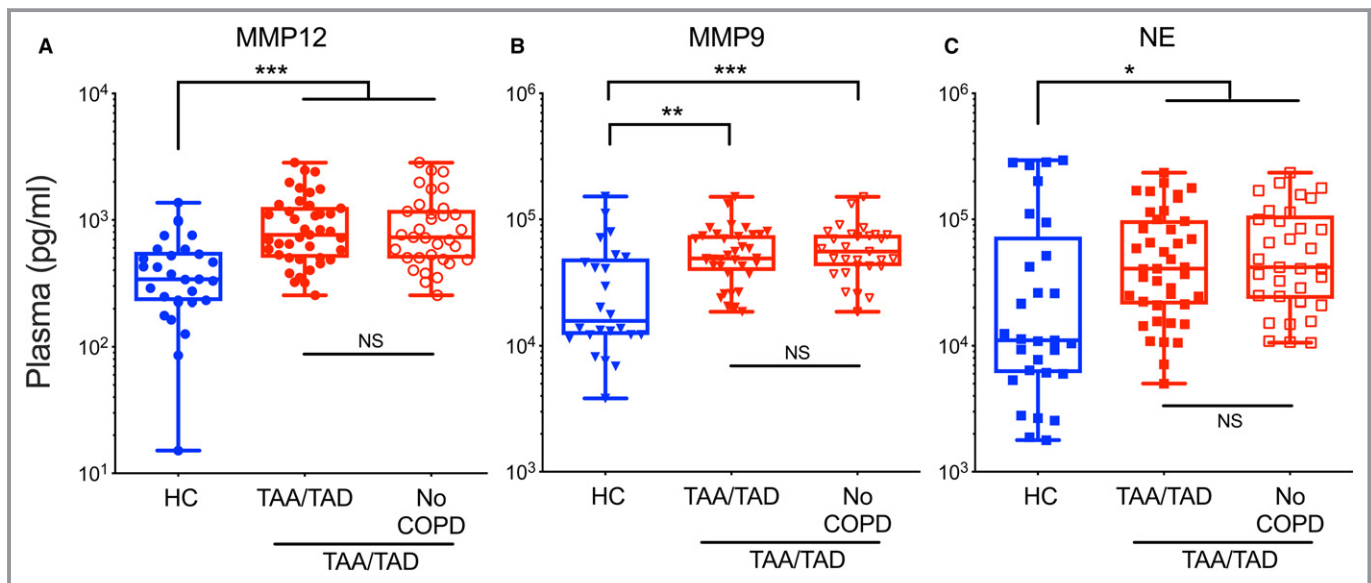




**Figure 4.** Elevated anti-EFs antibody in plasma of TAA/TAD. **A**, The relative concentration of anti-elastin antibody level in plasma was measured by ELISA in HC (n=31) and TAA/TAD (n=41). Each dot represents a data point from an individual subject. **B**, The relative concentration of anti-elastin antibodies was assessed in non-smoker HC (n=14), and non-smoker TAA/TAD (n=13), and HC ever-smoker (n=18), and TAA/TAD ever-smoker (n=28). \* $P$ <0.05 as determined by the Mann–Whitney test. Ab indicates antibody; COPD, chronic obstructive airway disease; HC, healthy control; NS, not significant; TAA/TAD, thoracic aortic aneurysm and dissection HC.

emphysema progression in active smokers.<sup>17</sup> However, IL1- $\beta$ , the canonical cytokine released in response to activation of the inflammasome pathway, was increased in current smokers with or without TAA/TAD. These findings suggest that active smoking may promote antigen-specific activation of

auto-inflammatory cytokines in smokers, potentially through an inflammasome-linked pathway that we previously showed was activated by carbon black, a highly proinflammatory substance found in incomplete combustion of organic matter.<sup>20</sup>



**Figure 5.** Circulating plasma elastin-degrading enzymes in TAA/TAD and HC. Levels of MMP-12 (**A**), MMP-9 (**B**), and NE (**C**) were measured in plasma using Luminex in smoker and non-smoker HC (n=30), and TAA/TAD (n=41) and TAA/TAD patients without COPD (n=33). \*\*\* $P$ <0.001, \*\* $P$ <0.01, \* $P$ <0.05 as determined by the Kruskal–Wallis test with Dunn post-test for multiple comparisons. COPD indicates chronic obstructive airway disease; HC, healthy control; MMP, matrix metalloproteinase; NE, neutrophil elastase; NS, not significant; TAA/TAD, thoracic aortic aneurysm and dissection.

Although T-cell specific response to elastin fragments was seen in current smokers, irrespective of TAA/TAD, nonetheless, increased expression of this proinflammatory cytokine to self-antigen could potentially contribute to disease in smokers. Consistently, increased expression of IL-1 $\beta$  in macrophages has been associated with atherosclerosis and vascular disease.<sup>21</sup> The CANTOS trial (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) showed that inhibition of IL-1 $\beta$  could reduce the risk for subsequent thrombotic events disease in patients with existing cardiovascular disease.<sup>22</sup> Mechanistically, IL-1 $\beta$  has been shown to promote the expression of tumor necrosis factor in dendritic cells to activate antigen-specific T cells.<sup>23</sup> Although a direct causative role for IL-1 $\beta$  in TAA/TAD pathogenesis has not been shown in humans, TAA samples showed higher concentrations of IL-1 $\beta$  when compared with normal human aortas isolated from organ transplant donors.<sup>24</sup> Notably, IL-1 $\beta$  was also found to be required in the elastase-induced TAA model, because mice with deficiency in this cytokine or its receptor showed decreases in proinflammatory cytokines and reduced disease progression.<sup>24</sup> Here we found an association between smoking history and an increase in IL-1 $\beta$  expression in our studies.

MMPs have been extensively examined as potential biomarkers in several diseases, although their diagnostic and/or prognostic role has not been widely accepted.<sup>25–27</sup> We examined baseline concentrations of elastolytic MMPs and NE in our cohort and found a significantly increased plasma concentrations of MMP-9, MMP-12, and NE in TAA/TAD subjects regardless of a concurrent diagnosis of COPD. These findings suggest that a proinflammatory state in TAA/TAD patients persists and whether they return to lower levels after TAA/TAD repair warrants future study. A caveat of our study includes sex differences among some subgroups although it was not statistically different across all groups; specifically, fewer men were enrolled in our healthy control non-smoking group when compared with TAA/TAD non-smoker group.

To date, 30 distinct genetic mutations have been associated with increased susceptibility to development of TAA/TAD.<sup>2</sup> Several of these genes involve structural proteins within the extracellular matrix (eg, fibrillin, fibulin, elastin, collagen I & III). Discovery of these heritable conditions that display variable penetrance has shed new light on the pathophysiological mechanisms of vascular damage in TAA/TAD.<sup>28</sup> Our findings here raise the possibility of a new paradigm for understanding the interaction between genetic susceptibility to TAA/TAD and environmental factors such as smoking-induced activation of the acquired immunity that could further promote disease progression. Specifically, given that 20% of TAA/TAD patients have a family history of aneurysms,<sup>29,30</sup> the combination of gene loci associated with an aortic aneurysm and risk associated with autoimmunity in smokers, may

provide new pathways that promote disease progression in this group. Further studies are warranted to explore this hypothesis.

In conclusion, we and others have shown that multiple endogenous proteinases can cleave elastin to create fragments that are highly immunogenic.<sup>18,31–33</sup> Our new findings here point to the increased presence of autoreactive T cells in TAA/TAD smokers. Future studies validating these findings, including those that prospectively evaluate the role of autoinflammation and disease progression in TAA/TAD patients, may provide novel strategies to harness the immune system for treatment options and reduce morbidity in this population.

## Acknowledgments

The Baylor College of Medicine section of Epidemiology and Biostatistics had full access to all of the data.

## Author Contributions

Ms Gu, and Drs Scheurer, Kheradmand, and LeMaire take responsibility for the integrity of the data, and the accuracy of the data analysis. Drs Kheradmand and LeMaire conceptualized and designed the study. Data acquisition: Ms Gu, Drs Choi, and Shen; Analysis and interpretation of data: Ms Gu, Drs Scheurer, Kheradmand, LeMaire, Koth, Woodruff; Administrative, technical, and material support: Drs Luong, Rodriguez, Song.

## Sources of Funding

This work was supported by R01 HL110883 to Kheradmand and Corry; VA Merit CX000104 to Kheradmand; HL140398 to Rodriguez, Corry; Baylor College of Medicine Pilot Award from Cardiovascular Research Institute to Gu, LeMaire, and Kheradmand; NHLBI contract HHSN268200900014C to Woodruff. Dr LeMaire's work is supported in part by the Jimmy and Roberta Howell Professorship in Cardiovascular Surgery.

## Disclosures

None.

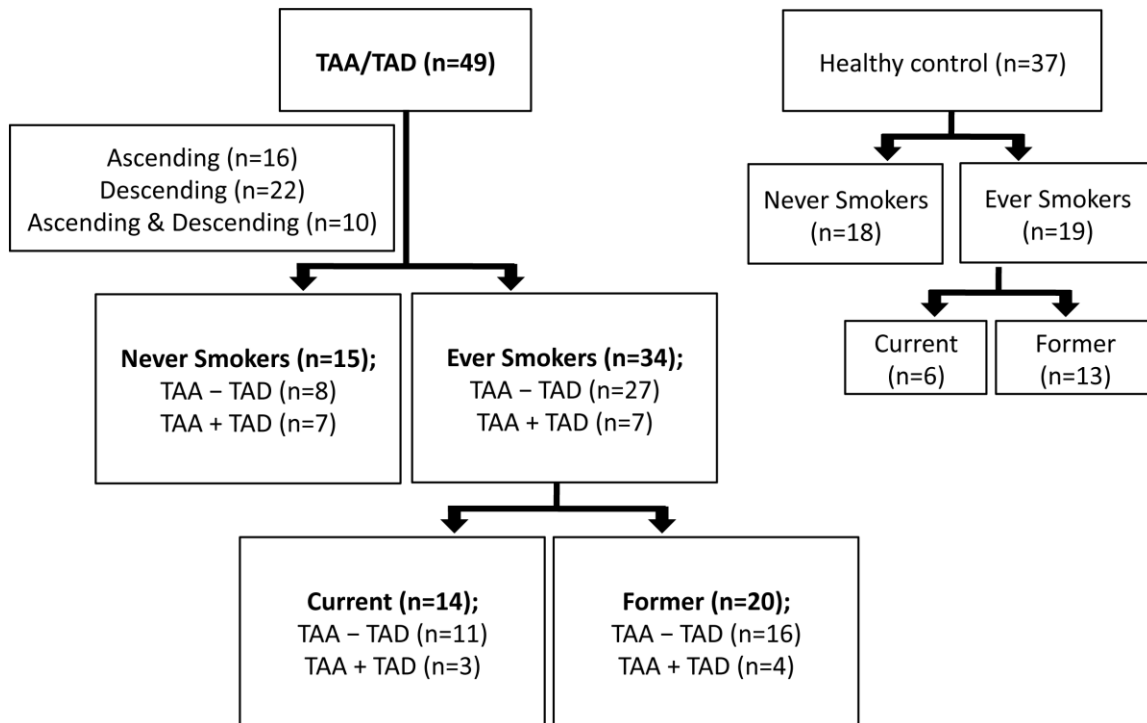
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# **Supplemental Material**

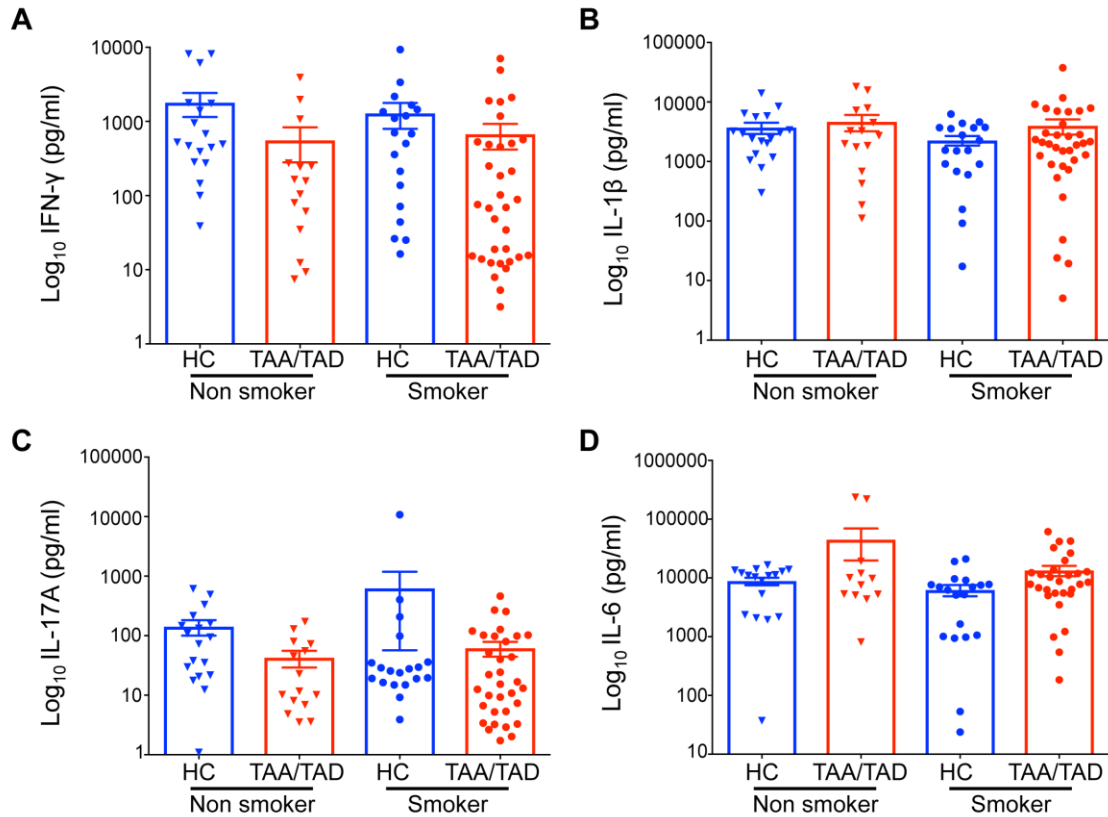
**Figure S1. Schematic representation of study subjects.**



Patients who were newly diagnosed with an aortic aneurysm or dissection were enrolled in the Molecular Mechanisms of Thoracic Aortic Aneurysm and Dissection (MMTAD) study at Baylor College of Medicine. Healthy controls non-smokers with no known vascular disease were recruited from the HSC-MS-13-0443 study at the McGovern Medical School of the University of Texas Health Science Center. Healthy control ever-smokers with no known vascular disease were recruited from the SPIROMICS cohort at the University of California San Francisco). Definition of abbreviations: HC = healthy control; TAA/TAD = thoracic aortic aneurysm and dissection; TAA-TAD = thoracic aortic aneurysm (TAA) without dissection; TAA+TAD = TAA with dissection.

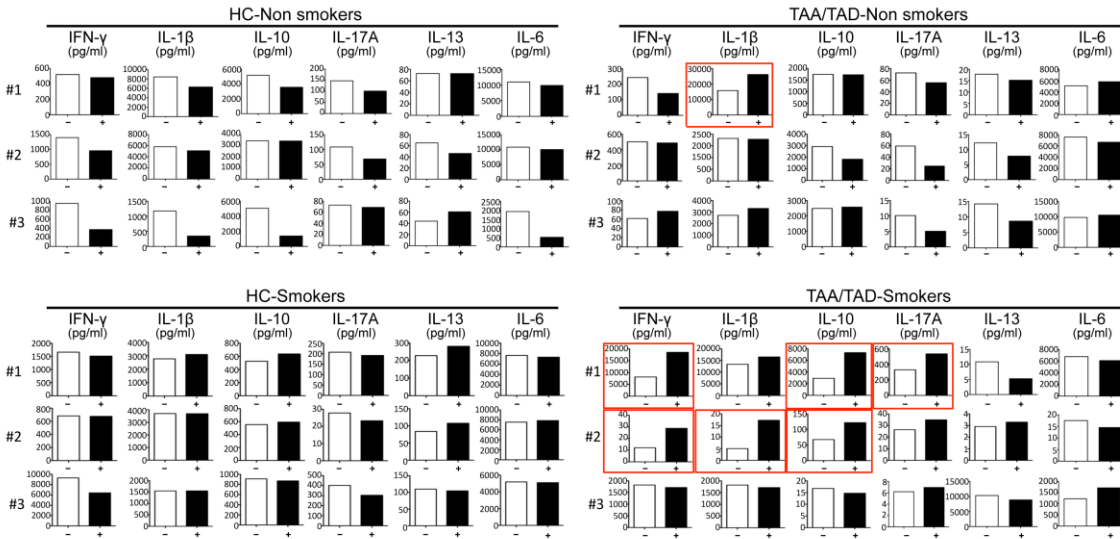


**Figure S2. No significant differences in baseline cytokine expression in CD4 T cells in patients.**



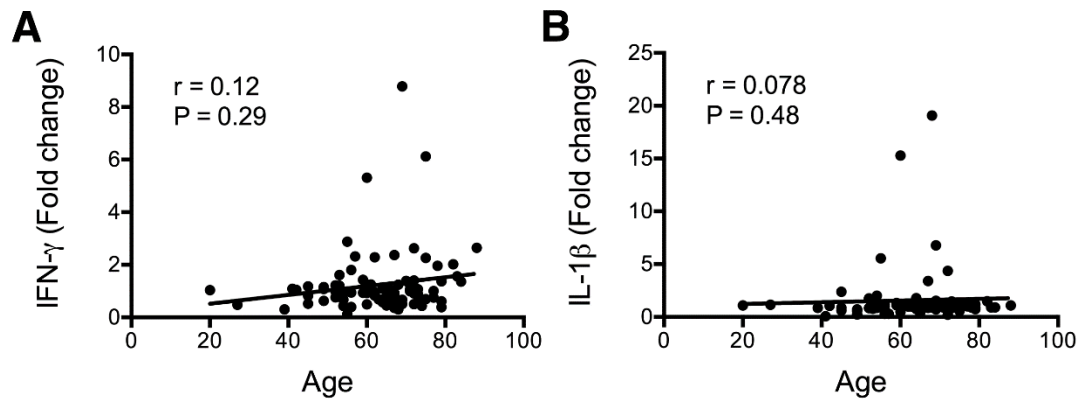
CD4<sup>+</sup>T cells ( $5 \times 10^5$ ) isolated from peripheral blood of healthy controls (HC), never (N=18), ever-smokers (N=19) or thoracic aortic aneurysm and dissection (TAA/TAD) patients never, (N=15) or ever-smokers (N=34) were co-cultured with irradiated CD1a<sup>+</sup>/CD14<sup>+</sup> APCs ( $5 \times 10^4$ ). After 3 days, representative cytokines (**A**) IFN-γ, (**B**) IL-1β, (**C**) IL-17A, and (**D**) IL-6 were measured using ELISA in the supernatants. Each dot represents a concentration from each subject. There were no significant differences among the groups. Definition of abbreviations: HC = healthy control; TAA/TAD = thoracic aortic aneurysm and dissection; APCs = antigen presenting cells.

**Figure S3. Human T cells express large variations in cytokine response to EFs.**



CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) isolated from peripheral blood of healthy controls (HC; never, current or former smokers) or thoracic aortic aneurysm and dissection (TAA/TAD) patients (never, current or former smokers) were co-cultured with irradiated CD1a<sup>+</sup>/CD14<sup>+</sup> APCs ( $5 \times 10^4$ ) with EFs (30  $\mu$ g/ml, marked with +) or no EFs (nil-stimulation, marked with -). After 3 days, cytokines (IFN- $\gamma$ , IL-1 $\beta$ , IL-10, IL-17A, IL-13, and IL-6) were measured using ELISA in the supernatants. Actual concentration from three representative subjects is shown. The red box indicates >1.5 increases over nil stimulation in each case. Definition of abbreviations: HC = healthy control; TAA/TAD = thoracic aortic aneurysm and dissection; APCs = antigen presenting cells.

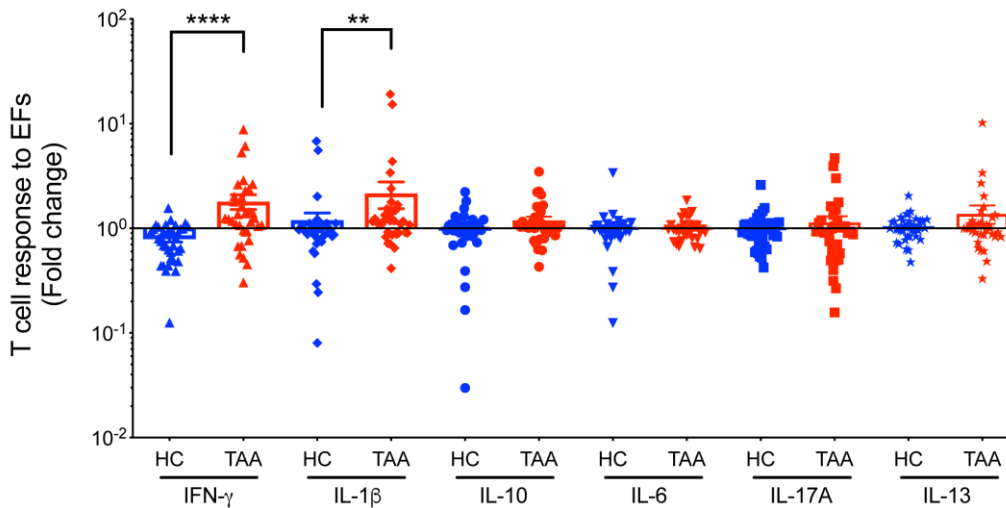
Figure S4. No correlation between cytokine induction and age in TAA/TAD and HC.



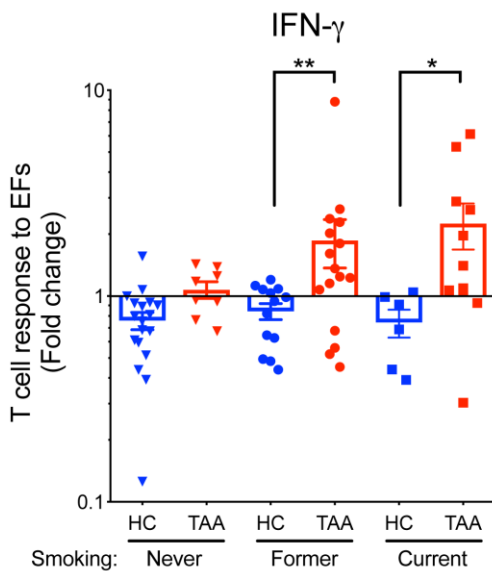
Correlation between age and IFN- $\gamma$  (A), and IL-1 $\beta$  (B) in TAA/TAD patients (N=86) were examined using the Spearman test. There were no significant correlations found between IFN- $\gamma$  ( $p=0.29$ ) and IL-1 $\beta$  ( $p=0.48$ ) and age in TAA/TAD patients and HC. Definition of abbreviations: HC = healthy control; TAA/TAD = thoracic aortic aneurysm and dissection.

**Figure S5. EFs-specific CD4<sup>+</sup> T cell response in patients with only TAA.**

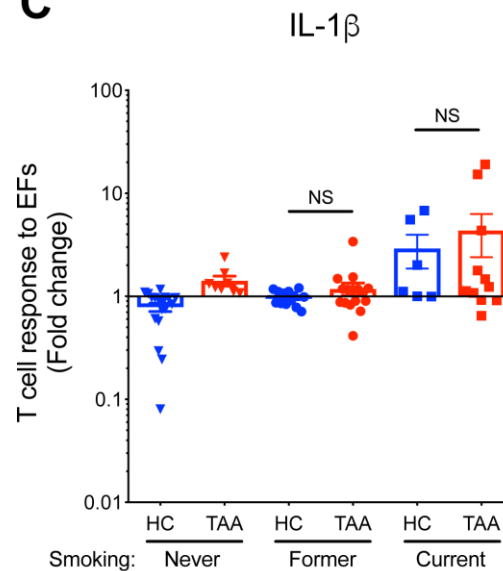
**A**



**B**

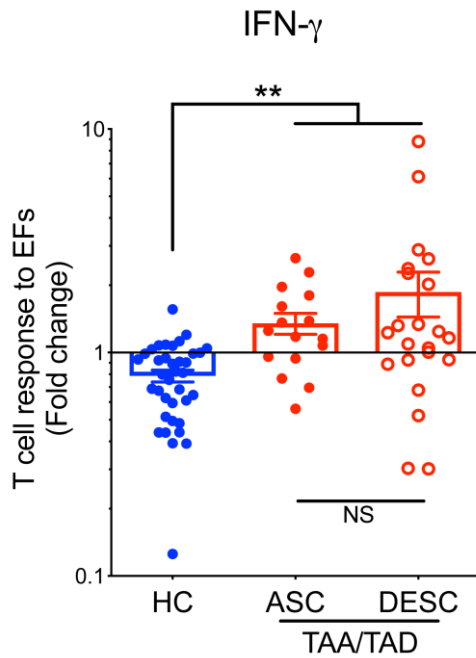


**C**



(A) CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) isolated from peripheral blood of healthy controls (HC, N=37) or thoracic aortic aneurysm without dissection (TAA, N=35) patients were co-cultured with irradiated CD1a<sup>+</sup>/CD14<sup>+</sup> APCs ( $5 \times 10^4$ ) with EFs ( $30 \mu$ /ml) or no EFs. Supernatants from the same co-culture conditions were used to measure concentration of IFN-γ, IL-1β, IL-10, IL-17, and IL-13 were plotted as fold change over nil stimulation. Expression of IFN-γ (B) and IL-1β (C) in response against EFs was assessed in HC non-smokers (N=18), smokers (former N=13, current N=6), TAA non-smokers (N=8), TAA smokers (former N=16, current N=10). Each dot represents a data point from an individual subject. \*\*\*\*P<0.0001, \*\*P<0.01, \*P<0.05 as determined by the Mann-Whitney test. Definition of abbreviations: HC = healthy control; TAA = thoracic aortic aneurysm without dissection; EFs = elastin fragments; APCs = antigen presenting cells; NS = not significant.

Figure S6. EFs-specific CD4<sup>+</sup> T cell IFN- $\gamma$  response in ASC and DESC patients with TAA/TAD.



CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) isolated from peripheral blood of healthy controls (HC, N=37) or ascending (ASC, N=16) or descending (DESC, N=22) in TAA/TAD patients were co-cultured with irradiated CD1a<sup>+</sup>/CD14<sup>+</sup> APCs ( $5 \times 10^4$ ) with EFs ( $30 \mu$  /ml) or no EFs. Supernatants from the same co-culture conditions were used to measure concentration of IFN- $\gamma$ . \*\*P<0.01 as determined by the Kruskal-Wallis test with Dunn's post-test for multiple comparisons. Definition of abbreviations: HC = healthy control; TAA = thoracic aortic aneurysm without dissection; EFs = elastin fragments; APCs = antigen presenting cells; ASC = ascending; DESC = descending; NS = not significant.