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Description of human AAA by cytokine and immune cell aberrations compared to risk-factor matched controls☆

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ABSTRACT

Background: The pathogenesis driving the formation of abdominal aortic aneurysms continues to be poorly understood. Therefore, we systemically define the cytokine and circulating immune cell environment observed in human abdominal aortic aneurysm compared with risk-factor matched controls.

Methods: From 2015 to 2017, a total of 274 patients donated blood to the Indiana University Center for Aortic Disease. Absolute concentrations of circulating cytokines were determined, using enzyme-linked immunosorbent assays while the expression of circulating immune cell phenotypes were assayed via flow cytometric analysis.

Results: Human abdominal aortic aneurysm is characterized by a significant depletion of the antigen-specific, $CD4^+$ Tr1 regulatory lymphocyte that corresponds to an upregulation of the antigen-specific, inflammatory Th17 cell. We found no differences in the incidence of Treg, B10, and myeloid-derived suppressor regulatory cells. Similarly, no disparities were noted in the following inflammatory cytokines: IL-1β, C-reactive protein, tumor necrosis factor α, interferon γ, and IL-23. However, significant upregulation of the inflammatory cytokines osteopontin, IL-6, and IL-17 were noted. Additionally, no changes were observed in the regulatory cytokines IL-2, IL-4, IL-13, TNF-stimulated gene 6 protein, and prostaglandin E2, but we did observe a significant decrease in the essential regulatory cytokine IL-10.

Conclusion: In this investigation, we systematically characterize the abdominal aortic aneurysm–immune environment and present preliminary evidence that faulty immune regulation may also contribute to aneurysm formation and growth.

Background

Abdominal aortic aneurysm (AAA) is a chronic inflammatory condition associated with male gender, advanced age, and tobacco use which continues to be a major source of morbidity and mortality in the Western Hemisphere.[1,2] If left untreated, progressive aortic dilation results in increasing wall tension, rupture, hemorrhagic shock, and probable death.[3] Currently, the accepted treatment paradigm consists of regimented imaging until a cross-sectional aortic diameter of 5.5 cm is reached—at this point, the risk of rupture and death exceeds the high morbidity associated with surgical reconstruction.[4] Recent research has focused heavily on elucidating the pathogenesis and mechanism of AAA formation, with hopes of developing a new pharmaceutical option to arrest aneurysm growth and prevent the need for surgical intervention.[5–9]

Previous studies have established that the histologic environment of the native aorta undergoing the transition to an aneurysmal state is characterized by an infiltration of mononuclear cells such as T and B lymphocytes, neutrophils, macrophages (Mϕs), and mast cells.[10–12] Unfortunately, the inciting event and mechanism that drives ectatic transformation remains a mystery. In this report, we present a descriptive analysis of the inflammatory and regulatory circulating immune environments of a large cohort of AAA patients and compare it with risk-factor matched (RFM) controls.

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Methods

Separation of whole blood

The protocols presented herein are HIPAA compliant and were approved by the Indiana University Institutional Review Board (IRB #1408881234). After we acquired written informed consent, we collected whole blood from 274 subjects during a span of 2 years to be banked at the Indiana University Center for Aortic Disease (IUCAD). Potential subjects were obtained from individuals presenting for their US Preventative Task Force-recommended AAA duplex screening appointments or from previously diagnosed patients presenting for follow-up in the vascular surgery clinic.[13] Patients who screened negative by ultrasound (diameter <30 mm) at their vascular lab appointments were deemed a RFM control for the purposes of this study.

All whole blood samples were processed within 24 hours of collection, using previously described Ficoll (GE Healthcare, Little Chalfont, UK) density centrifugation protocols with the assistance of Accuspin gradient tubes (Sigma, St. Louis, MO, USA) to isolate both peripheral blood mononuclear cells (PBMC) and plasma.[14] Plasma samples were stored in small aliquots at –80°C to minimize freeze-thaw cycles; PBMCs were stowed in liquid nitrogen suspended in fetal bovine serum (Sigma) plus 20% dimethyl sulfoxide (Sigma) in units of 10^6 cells.

Inflammatory and regulatory cell phenotyping

Cell staining for flow cytometric analysis using antibodies specific to identifying cell markers was performed per manufacturer's instructions (1:10, Miltenyi Biotec, Bergisch Gladbach, Germany) unless otherwise noted. Tr1: CD4-FITC, CD49b-PE (1:20), LAG3-APC (1:20); Treg: CD4-FITC, CD25-PE, FOXP3-APC (alternate: CD4-FITC, CD25-PE, CD127-APC); Th17: CD4-FITC, CD194-PE, CD196-APC; B10: CD1d-APC, CD5-FITC, CD19-PE, IL10-PerCP; MDSC: CD11b-PerCP, CD33-PE, CD66abce-FITC, HLA-DR-APC; Activated Mϕ: CD14-PE, CD16-FITC, CD45-APC; Inflammatory Mϕ: CD14-PE, CD16-FITC, CD45-APC; Resident Mϕ: CD14-PE, CD16-FITC, CD45-APC. Flow cytometric analysis was performed on an Accuri C6 (BD Biosciences, San Jose, CA, USA) flow cytometer and compiled using CellQuest software (BD).

Determination of circulating cytokine concentrations

Circulating inflammatory and regulatory cytokine concentrations were determined using commercially available enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems, Minneapolis, MN, USA) and performed per manufacturer's instructions. For all protocols, absorbance was measured at a wavelength of 450 nm and absolute concentrations were calculated with the assistance of a 4-parameter standard curve.

Determination of plasma antibody concentrations

Human elastin fragments (ELNf) were generated by in vitro digestion with MMP-2 and MMP-9 (Sigma) per manufacturer's instructions. Relative antibody concentrations specific to human collagen V (COLV) and ELNf were assayed via a previous described modified ELISA protocol.[15] In short, respective antigenic peptides were dissolved in phosphate-buffered saline (PBS) to a stock working solution of 25 µg/mL. This stock solution was used to coat a high-protein binding 96-well polystyrene plate (Sigma) for 2 hours at 37°C or overnight at 4°C. Copious washings were performed between all steps with PBS-T (Tween 20, Sigma). The 96-well plate was blocked with 1% bovine serum albumin (BSA, Sigma) for 2 hours at 37°C or overnight at 4°C. Plasma samples were then sequentially diluted up to 1:1000 to determine optimal concentrations and incubated for 2 hours at 37°C or overnight at 4°C. A goat antihuman IgG Fc antibody conjugated to horseradish peroxidase (HRP, Sigma) was utilized as a secondary antibody per manufacturer's recommended dilution for a duration of 1 hour at 37°C. Reactions were performed using a 1-step TMB turbo substrate (Sigma) for 30 minutes before a 1 M sulfuric acid stop solution was added. Absorbance at 450 nm was measured within 30 minutes to calculate relative self-antibody concentrations.

Table 1
Comorbidities.

	RFM ($n=121$)	AAA ($n=153$)	*P* value
Age, y	68.9 ± 4.9	69.4 ± 6.4	.48
Male	99.2%	96.7%	.23
HLD	76.7%	85.2%	.12
HTN	68.3%	83.2%	<.01
BMI>30	48.3%	43.0%	.46
DM	37.5%	28.2%	.12
Active smoker	30.0%	57.0%	<.01
CAD	24.2%	39.6%	<.01
COPD	20.0%	30.2%	.07
PAD	8.3%	14.8%	.10
CKD	7.5%	8.7%	.82
FHx	4.2%	4.7%	1.0
Framingham score	35.6 ± 15.8%	40.5% ± 18.5%	.02

Depiction of the comorbidities of the blood donors to the IUCAD biorepository by cohort.
HLD, hyperlipidemia; *HTN*, hypertension; *BMI*, body mass index; *DM*, diabetes mellitus; *CAD*, coronary artery disease; *PAD*, peripheral arterial disease; *CKD*, chronic kidney disease; *FHx*, family history of AAA.

Table 2
Medication use.

	RFM ($n=121$)	AAA ($n=153$)	*P* value
Statin	66.7%	72.5%	.29
Aspirin	43.3%	55.7%	.05
Beta blocker	35.0%	49.0%	.02
ACEi	32.5%	47.0%	.01
Metformin	24.2%	16.1%	.13
ARB	15.8%	10.7%	.21
Systemic steroids	11.7%	16.1%	.38
Clopidogrel	6.7%	6.8%	1.0
Nitrates	2.5%	8.0%	.06

Baseline medications of the blood donors to the IUCAD biorepository by cohort.
ACEi, angiotensin-converting enzyme inhibitor; *ARB*, angiotensin receptor blocker.

Results

Demographics and comorbidities

A total of 274 patients with AAA ($n=153$) or deemed RFM controls ($n=121$) from January 2015 to September of 2017 donated blood samples to the IUCAD biorepository (Table 1). The mean aneurysm size at the time of sample collection for the AAA cohort was 49.4 mm (median = 50 mm). In the RFM cohort, 52.9% of the patients had an aortic diameter of less than 20 mm at the time of blood collection. AAA patents had more comorbidities as demonstrated by significantly higher incidences of hypertension, active smoking, and coronary artery disease. These findings were corroborated by a higher Framingham risk score (35.6% vs 40.5%, $P=.02$). Of note, a trend toward a decrease in diabetes mellitus was noted in the AAA group compared with the RFM controls. Baseline medications prescribed for the blood donors at the time of collection are noted in Table 2.

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