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Cytokine signature of inflammation mediated y autoreactive Th-cells, in calf muscle of claudicating patients with Fontaine stage II peripheral artery disease

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Peripheral artery disease (PAD), a severe atherosclerotic condition primarily of the elderly, afflicts 200 million individuals, worldwide, and is associated with lower extremity myopathy. Circulating markers of inflammation have been linked to risk and severity of PAD but the contribution of local inflammation to myopathy remains unknown. We evaluated, by ELISA, calf muscle of PAD patients (N = 23) and control subjects (N = 18) for local expression of inflammatory cytokines including Granulocyte/Monocyte Colony-Stimulating Factor (GM-CSF), Interleukin 17A (IL-17A), Interferon Y (IFN-Y), tumor necrosis factor a (TNF-a), and Interleukin 6 (IL-6). One or more of these cytokines were expressed in nineteen patients and 2 controls and coordinated expression of GM-CSF, IL-17A, IFN-Y, and TNF-a, a signature of activated, MHC Class II dependent autoreactive Th-cells, was unique to 11 patients. GM-CSF is the central driver of tissue-damaging myeloid macrophages. Patients with this cytokine signature had a shorter (P= 0.017) Claudication Onset Distance (17 m) compared with patients lacking the signature (102 m). Transforming Growth Factor β1 (TGFβ1) and Chemokine Ligand 5 (CCL5) were expressed coordinately in all PAD and control muscles, independently of GM-CSF, IL-17A, IFN-Y, TNF-a, or IL-6. TGFβ1 and CCL5 and their gene transcripts were increased in PAD muscle, consistent with increased age-associated inflammation in these patients. Serum cytokines were not informative of muscle cytokine expression. We have identified a cytokine profile of autoimmune inflammation in calf muscles of a significant proportion of claudicating PAD patients, in association with decreased limb function, and a second independent profile consistent with increased “inflammaging” in all PAD patients. (Translational Research 2021; 228:94-108)

Abbreviations: COD = Claudication Onset Distance; dNTPs = Deoxynucleotide Triphosphates; DTT = Dithiothreitol; PWD = Peak Walking Distance; QoL = Quality of Life; TRIS = Tris(hydroxymethyl)aminomethane; vSMC = Vascular Smooth Muscle Cells; WIQ = Walking Impairment Questionnaire

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INTRODUCTION

Peripheral artery disease and calf muscle damage. Peripheral Artery Disease (PAD) is a severe atherosclerotic condition primarily of the elderly, with a prevalence of more than 200 million individuals, worldwide. The disease is diagnosed by an Ankle- Brachial Index (ABI) of less than 0.90 with limitation of lower leg function and carries an increased risk of coronary artery disease (CAD), carotid artery disease, and cardiovascular mortality.[1] During exercise, atherosclerotic plaques in the arteries supplying the lower legs, restrict blood flow causing a deficit in oxy- gen and nutrient delivery in relation to demand.[2] Chronic intermittent ischemia of the lower leg is associated with pathology of the lower leg muscles. Ischemia, *per se*, and myopathy primarily of the calf muscle, produce limb dysfunction,[3-5] and limit physical activity. Ischemia is regarded as the ultimate cause of pathological changes to skeletal muscle myofibers, nerves, and blood vessels of the lower legs of PAD patients.[6] Reactive oxygen species (ROS) generated in response to ischemia produce oxidative damage to the myofibers, which increases with advancing disease and correlates with reduced myofiber cross- sectional area, reduced ABI,[7] and selective loss of Type II myofibers.[8] In addition to impairing cellular function by damaging macromolecules, ROS may induce an inflammatory response that has myopathic effects.[1,9] Reduced calf muscle cross-sectional area and increased calf muscle fat content have been associated with increasing quartile of serum/plasma C- reactive protein (CRP).[10]

Inflammatory biomarkers and severity of atherosclerosis in patients With PAD. The severity of atherosclerosis in PAD patients is represented by (1) the higher rate of 1-year atherothrombotic events among PAD compared to CAD and cerebrovascular disease patients and (2) the high prevalence (up to 90%) of CAD among PAD patients compared to less than 25% PAD among CAD patients.[1] These observations together with the positive association of the prevalence of hypoechoic (inflamed) plaques in the carotid arteries with hypoechoic plaques in the femoral arterial bed, among PAD patients, suggested to some investigators that inflammation in the large atherosclerotic arterial beds of the affected PAD limbs is a principal contributor to systemic disease in PAD patients. Studies of inflammatory markers in the blood of PAD and CAD patients are consistent with this hypothesis. Relative to the aorta, blood from the femoral vein of the affected limb of PAD-plus-CAD patients contained increased numbers of activated neutrophils, in contrast to CAD- only patients who displayed no transfemoral increase.[11] PAD-plus-CAD patients whose transfemoral increases were greater than the median had a greater number of coronary stenoses, and an increased prevalence of 3- vessel CAD and myocardial infarct compared to PAD- plus-CAD patients whose transfemoral increases were less than the median. These findings are consistent with those of an earlier study[12] that documented an increased incidence of cardiac, cerebral and peripheral events, combined, in PAD patients with elevated leukocyte count. Moreover, the plasma concentration of soluble vascular cell adhesion molecule-1 was found to be a strong and independent predictor of risk of cardiovascular events, in PAD patients with intermittent claudication.[13]

Inflammatory biomarkers and risk of developing hemodynamic limitation and PAD. In addition to their association with cardiovascular events, inflammatory markers have been linked to risk of PAD and hemodynamic limitation in the lower legs of PAD patients. CRP was found to be a strong and independent predictor of the development of PAD among healthy men who participated in the Physicians' Health Study.[14] Baseline plasma CRP was higher ($P < 0.006$) among men who developed PAD (0.14 mg/dL) in the 9-year follow-up compared to men who did not develop PAD (0.10 mg/dL). The relative risk for

developing PAD was 2.5 times higher among men in the highest quartile of baseline CRP compared to men in the lowest quartile. Within the Rotterdam Study of the incidence and risk factors of disabling diseases among men and women aged 55 years and older, plasma markers of inflammation were investigated as potential predictors of ABI.[15] Both CRP and Interleukin-6 (IL-6) were positively associated with hemodynamic limitation in the lower leg as indicated by their correlation with decreasing ABI. As part of the Edinburgh Artery Study,[16] CRP in addition to IL-6 and soluble Intercellular Adhesion Molecule-1 were shown to be independent predictors of the decline of ABI, over 12 years of follow-up. In the same study, soluble vascular cell adhesion mole- cule-1 and E-selectin were not associated with changes in ABI.

Inflammatory biomarkers and limb dysfunction in PAD patients. Inflammation has been linked to limb dysfunction in PAD patients. A study[17] of 127 patients with PAD verified by arteriography, found that plasma IL-6 exhibited an inverse correlation with maximum tread- mill walking distance. In contrast, tumor necrosis fac- tor-a (TNF-a), monocyte chemoattractant protein-1, and CD40 ligand were not associated with treadmill walking distance. A more extensive study of the association of inflammation with limb function in PAD patients was completed by McDermott et al[18] who determined the associations of serum CRP, fibrinogen, and amyloid A with 6-minute walking distance, 4-m walking velocity, and a summary performance score. CRP but not fibrinogen or amyloid A was associated with 6-minute walking distance and summary performance score. Higher CRP concentrations were associated independently with poorer 6-minute walking distance and lower summary performance score. In a prospective study of PAD patients,[19] greater annual increases in serum CRP were found to be associated with greater annual decline in 6-minute walking distance, over a period of 3 years

Local inflammation in calf muscle of PAD patients. The association of inflammatory markers in circulating blood with risk and severity of PAD is well established. This association, however, is based on circulating markers that likely originate from active atherosclerotic plaques in these patients and does not address local inflammation in calf muscle or other lower leg muscles. Myofiber pathology in the lower legs of PAD patients is characterized by mitochondrial dysfunction,[6,20] oxidative damage,[7,8] intra- cellular accumulation of damaged proteins,[3,7] abnormal morphology,[4,21] degeneration, and replacement with fibrotic tissue.[22] These features are consistent with myo- fiber necrosis, a process that releases both damaged and intact molecules normally sequestered in the cell and, consequently, may be recognized as danger-associated molecular patterns (DAMPs),[23] autoantigens, or neoantigens. Recognition of DAMPs by pattern recognition receptors on macrophages and dendritic cells increases transcription of genes that code for inflammatory cytokines and chemo- kines.[24] In contrast, sequestered self-proteins released during necrosis may be immunogenic and in association with MHC class II molecules on antigen presenting cells activate autoreactive Th-cells,[25] thereby driving chronic auto- immune inflammation.[26] On the other hand, lipid peroxidation generates oxidatively damaged proteins that are highly immunogenic neoantigens.[27] In association with MHC class I alleles[28] these neoantigens drive chronic T-cell activation and inflammation. We evaluated local expression of inflammatory cytokines including GM-CSF, IL-17A, IFN-Y, TNF-a, and IL-6, as well as TGFb1[29] and CCL5[30] and now present data supporting inflammation mediated by autoreactive Th-cells,[31] in calf muscle of claudicating patients with PAD, as well as increased age- associated inflammation in all PAD patients.

METHODS

Human subjects. This study conforms to the ethical principles for medical research

involving human sub- jects, as set forth by the World Medical Association (Declaration of Helsinki). The Institutional Review Boards of the VA Nebraska Western Iowa Medical Center and University of Nebraska Medical Center approved the experimental protocol and all subjects gave informed consent. We recruited 23 consecutive patients scheduled for lower extremity operations for the treatment of symptomatic PAD (Table I). Diagnosis of PAD was based on medical history, physical examination, decreased ABI (<0.9), and computerized or standard arteriography showing stenosis and/or occlusions in the arteries supplying the legs. We selected patients with Fontaine Stage II disease characterized by intermittent claudication in the absence of rest pain or tissue loss. Anatomic pattern of disease and planned treatment for each PAD patient enrolled are presented in Table II. We recruited 18 control patients scheduled for lower extremity operations for indications other than PAD (Table I). These patients had no history of PAD symptoms, normal blood flow to their legs and normal pulses in their lower extremities. Medical condition and planned treatment for each control subject enrolled are presented in Table III. All controls had normal ABIs at rest and after stress and all led sedentary lifestyles.

Biopsy of the calf muscle. Calf muscle biopsies weighing approximately 250 mg were obtained with a 6 mm Bergstrom needle, from the anteromedial aspect of the muscle belly, 10 cm distal to the tibial tuberosity. A biopsy was taken from the more diseased leg (lower ABI) of each PAD patient. Each biopsy was divided and immediately processed. Some pieces were frozen for ELISA and biochemical analyses and some were fixed in cold methacarn for future microscopic study.

Quantification of cytokines in calf muscles of control subjects and patients with PAD, by sandwich ELISA. We analyzed expression of 7 inflammatory cytokines in the calf muscle of both PAD patients and control subjects. These included Tumor Necrosis Factor alpha (TNF-a), Interferon gamma (IFN-g), Interleukin 17A (IL-17A), Interleuklin 6 (IL-6), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Chemokine Ligand 5 (CCL5), and Transforming Growth Factor beta 1 (TGFb1). The lower limit of detection for these cytokines was 21.4, 12.6, 28.4, 14.0, 48.3, 8.0, and 19.7 pg/mL, respectively.

Calf muscle homogenates. One day prior to analysis, an 80 mg piece of calf muscle (stored in liquid nitrogen vapor) was washed with ice-cold Tris-buffered saline (50 mM Tris and 150 mM NaCl at pH 7.5) containing 30% v/v protease inhibitor mix (Sigma P-8340), blot- ted and then placed in 660 mL of the same buffer with protease inhibitor. The tissue was homogenized, on ice, with a Dounce homogenizer and then centrifuged at 14,000 x g for 20 minutes, at 4˚C. Supernatant (570 mL), beneath the fat layer, was collected and stored at –80˚C.

ELISA procedure. On the day of assay, the samples were thawed and diluted 1:1 (v:v) in sample dilution buffer supplied with the Multi-Analyte ELISA kit (Qiagen, Germantown, MD; product # 336111). Diluted samples and standards were added to an ELISA plate consisting of 8-well columns. Wells in each column were coated with capture antibody specific for 1 cytokine. After the appropriate incubations and washes according to the Qiagen protocol, biotinylated detection antibody wash added to the wells. This was followed by washes and then incubation for 30 minutes with streptavidin-HRP. Finally, the plate was washed, incubated for 15 minutes with development solution and then treated with stop solution. The plate was read with a microplate reader, at 450 nm and at 570 nm. The 450 absorbances were corrected for any plate imperfections by subtracting the corresponding 570 absorbances. Absorbances of samples and standards, in addition, were corrected for negative controls (sample dilution buffer instead of sample or standard per well). Cytokine concentrations (picograms/mL) of the sample dilutions

added to the wells were computed from sample absorbance and absorbance of the corresponding cytokine standard. Cytokine concentrations in the original muscle specimen were computed from the sample dilutions, with the original addition of 80-mg muscle to 660 mL tris-buffered saline considered to be a dilution of 80 mL in a total of 740 mL (ie, a dilution of 4/37).

Table I. Demographics of claudicating patients with peripheral artery disease and control subjects

	CTRL	PAD-II	*P* value
Number of subjects	18	23	_
Median age (years)	63.8 § 1.7	63.8 § 7.6	*0.991
Height (m)	1.76 § 0.02	1.79 § 0.01	*0.422
Weight (kg)	90.9 § 3.7	90.6 § 4.2	*0.958
Body mass index (BMI)	29.9 § 1.2	28.6 § 1.2	*0.438
Ankle brachial index	1.08 § 0.01	0.57 § 0.04	*0.0001
Gender % (male/ female) Smoking	97.6/2.4	100/0	y0.901
% (N)			
Never	33.3 (6)	4.3 (1)	y0.042
Former	33.3 (6)	47.8 (11)	y0.538
Current	33.3 (6)	47.8 (11)	y0.538
Coronary artery dis- ease %	27.8 (5)	34.8 (8)	y0.889
Obesity %	27.8 (5)	26.1 (6)	y1.000
Dyslipidemia %	77.8 (14)	82.6 (19)	y1.000
Diabetes mellitus %	22.2 (4)	30.4 (7)	y0.815
COPD %	22.2 (4)	26.1 (6)	y1.000
Renal insufficiency %	5.6 (1)	17.4 (4)	y0.504
Statins %	77.8 (14)	91.3 (21)	y0.441
Family history % car- diovascular diseases	27.8 (5)	26.1 (6)	y1.000
Hypertension %	72.2 (13)	78.3 (18)	y0.936

Abbreviations: CTRL, control subjects; PAD-II, claudicating patients with Fontaine Stage IIPeripheral Artery Disease; COPD, Chronic Obstructive Pulmonary Disease.
Obesity: Body mass index >30. Renal Insufficiency: Creatinine Clearance <60 mL/min/1.73m^2. Continuous variables are presented as mean § S.E.M.
*Randomization *t* test was used for continuous variables.
yYates' Continuity Corrected Chi-Square test was used for cate- gorical variables.

Quantification of cytokine message in calf muscles of PAD patients and controls subjects. Total RNA was extracted from frozen muscle (»20 mg), with Trizol Reagent (Invitrogen, Austin, TX) per the manufacturer's instructions. RNA extracts were treated with DNAse and then purity and quantity of RNA were determined by Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). About 200 ng per sample were used for the synthesis of cDNA in a 20 mL reaction volume containing RNA, random hexamer (Promega, Madison, WI), 0.5 mM dNTPs, 1 unit of RNAsin (Promega, Madison, WI), and 1 unit of Improm-II Reverse Transcriptase (Promega, Madison, WI) in a buffer containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 2 mM DTT. Reverse Transcriptase (RT) reactions were incubated at 42˚C for 1 hour, followed by inactivation at 75˚C for 15 minutes. After briefly chilling on ice, RNase H (Promega, Madison, WI) was added to remove RNA strand from RT reactions, which subsequently were diluted 5- fold with nuclease-free water. The synthesized cDNA's were aliquoted and stored at –84˚C, and subsequently amplified by qPCR (CFX Connect, Bio-Rad Laboratories, Hercules, CA). For qPCR, 10% of each DNA aliquot was used with 200 pmol of each primer pair (Table IV) in DyNAmo SYBR Green qPCR mix (Fisher Scientific, Pittsburgh PA) according to the manufacturer's instructions. qPCR was implemented with a CFX96 connect (Bio-Rad, Hercules, CA). Cycling times were once at 95˚C for 10 minutes followed by 40 cycles at 95˚C for 15s and 60˚C for 1 minute. Melting curves were analyzed for specificity.

Table II. Patients with peripheral artery disease: anatomic pattern of disease and planned treatment

Patient number	Segment of arterial tree involved			Treatment[y]	Ankle-brachial index	
	Aorto-Iliac	Femoral-popliteal	Tibial	Planned	Left	Right
P1	Positive	Positive	Negative	AF2	0.64	0.10
P2	Negative	Positive	Negative	CFA-BKPop (L)	0.66	1.05
P3	Negative	Positive	Negative	SFA-BKPop (L)	0.54	1.16
P4	Negative	Positive	Negative	CFA-AKPop (L)	0.66	0.78
P5	Positive	Positive	Negative	AF2	0.16	0.10
P6	Positive	Positive	Negative	TF2	0.10	0.10
P7	Negative	Positive	Negative	CFA-BKPop (L)	0.55	1.03
P8	Negative	Positive	Negative	SFA-TP (L)	0.43	0.74
P9	Positive	Negative	Negative	SET	0.66	0.68
P10	Positive	Positive	Negative	AF2	0.62	0.28
P11	Positive	Positive	Negative	HB-Exer	0.40	0.43
P12	Negative	Positive	Negative	SET	0.56	0.68
P13	Positive	Positive	Negative	SET	0.68	0.66
P14	Positive	Negative	Negative	HB-Exer	0.96	0.58
P15	Positive	Negative	Negative	HB-Exer	1.03	0.66
P16	Positive	Negative	Negative	HB-Exer	0.80	1.00
P17	Negative	Positive	Negative	HB-Exer	0.56	0.70
P18	Negative	Positive	Negative	HB-Exer	0.08	0.87
P19	Positive	Positive	Negative	HB-Exer	0.57	0.51
P20	Positive	Positive	Negative	HB-Exer	0.48	0.66
P21	Positive	Negative	Negative	Stent-AI(Bi)	0.84	0.64
P22	Negative	Positive	Negative	HB-Exer	0.34	0.76
P23	Negative	Positive	Negative	HB-Exer	0.58	0.88

[y]Treatment Abbreviations: AF2, aorto-bifemoral bypass grafting; TF2, thoracobifemoral bypass grafting; CFA-BKPop (L), left common femoral artery to below-Knee popliteal artery bypass graft; SFA-BKPop (L), left superficial femoral artery to below-knee popliteal artery bypass graft; CFA-AKPop (L), left common femoral artery to above-knee popliteal artery bypass graft; SFA-TP (L), left superficial femoral artery to tibiopero- neal trunk bypass graft; SET, supervised treadmill exercise therapy; HB-Exer, home-based exercise; Stent-AI(BI), stent-aortoIliac bilateral

Table III. Control subjects: medical condition and treatment

Control number	Medical condition		Ankle-brachial index	
	Diagnosis	Treatment[y]	Left	Right
C1	Carotid artery disease	CEA	1.02	1.02
C2	Abdominal aortic aneurysm	EVAR	1.10	1.10
C3	Carotid artery disease	CEA	1.13	1.14
C4	Iliac artery aneurysm	EVAR	1.10	1.10
C5	Varicose veins	Vein stripping	1.10	1.10
C6	Abdominal aortic aneurysm	OAR	1.22	0.95
C7	Carotid artery disease	CEA	1.06	1.06
C8	Popliteal aneurysm	SFA-Pop RSVG	1.10	1.10
C9	Abdominal aortic aneurysm	EVAR	1.11	1.03
C10	Varicose veins	Vein stripping	1.12	1.10
C11	Abdominal aortic aneurysm	EVAR	1.09	1.05
C12	Varicose veins	Vein stripping	1.10	1.10
C13	Carotid artery disease	CEA	1.03	1.01
C14	Varicose veins	Vein stripping	1.17	1.14
C15	Abdominal aortic aneurysm	EVAR	0.97	1.01
C16	Carotid artery disease	CEA	1.08	1.08
C17	Carotid artery disease	CEA	1.10	1.10
C18	Abdominal aortic aneurysm	EVAR	1.01	1.11

[y]Treatment Abbreviations: CEA, carotid endarterectomy; EVAR, endovascular abdominal aortic aneurysm repair; vein stripping for varicose veins; OAR, open abdominal aortic aneurysm repair.Each Biopsy was collected during the surgical procedure.

Graded treadmill test and 6-minute walking distance. Walking performance of each PAD patient was evaluated as Peak Walking Distance (PWD) and Claudication Onset Distance (COD) with the Gardner-Skinner treadmill test[32] and 6-minute walking distance[33] administered indoors in a 20-m hallway.

Quality of life assessments. Physical function and health-related quality-of-life (QoL) were assessed with the Walking Impairment Questionnaire and Short Form 36 Health Survey,[34] on the same day that walking performance was assessed.

Statistics. Baseline characteristics for PAD and control subjects were compared with the Randomization *t* test for continuous variables and Yate's Continuity Corrected Chi Squared Test for categorical variables. Differences in the prevalence of detectable cytokine in calf muscles of PAD patients and Control subjects were determined with the Yates' Continuity Corrected Chi Square and differences in cytokine concentrations were determined with the Randomization *t* test. Correlations and their significance among cytokine measurements were determined as the Spearman Rank or Kendall's Tau correlation coefficient. Differences between PAD patients with and those without the signature cytokine profile of autoreactive Th cells were determined with the Randomization *t* test for continuous variables and Fisher's Exact Test for categorical variables. All analyses were completed with NCSS statistical software version 19.0.1 (NCSS, LLC, Kaysville, UT) Statistical significance was held at 0.05.

RESULTS

Inflammatory cytokines in the calf muscles of patients with PAD. Calf muscle homogenates and serum samples from PAD patients (N = 23) and control subjects (N = 18) were assayed by ELISA, for the 5 inflammatory cytokines IL-6, IL-17A, IFN-g, TNF-a, and GM-CSF which were detected at picogram to nanogram per mL in calf muscle of PAD patients (Table V). Cytokine was detected in biopsy specimens from 19 PAD patients and specimens from 15 of these patients co- expressed 2 or more of these inflammatory cytokines. IL-17A, IFN-g, TNF-a, and GM-CSF were co- expressed in the calf muscles of 11 PAD patients and muscles of 7 of these patients expressed, in addition, IL-6. Co-expressed inflammatory cytokines were strongly correlated (Table VI), establishing coordinated expression of these cytokines in the affected muscles of 65% of PAD patients. IL-17A, IFN-g, and GM-CSF were not detected in the calf muscles of control subjects, whereas IL-6 was detected in the calf muscle of one control subject and TNF-a in that of another (Table V). Neither PAD nor control serum contained detectable IL-6, IFN-γ, TNF-α, or GM-CSF while 5 PAD sera and 4 control sera contained detect- able IL-17A (Table VII). IL-17A in PAD serum was not correlated with PAD muscle IL-17A. Cytokines in the serum of PAD patients did not reflect the inflammatory environment within calf muscle. These data establish local inflammatory activity in the affected muscles of a substantial proportion of PAD patients.

Table V. Cytokine concentrations in calf muscle biopsies of PAD patients and control subjects, determined by enzyme-linked immunosorbent assay (ELISA)

	PAD Muscle				Control Muscle				
Cytokine	Positive (total) patients	Value for single positive (pg/mL)	Median (pg/mL)	Inter quartile range (pg/mL)	Positive (total) controls	Value for single positive (pg/mL)	Median (pg/mL)	Inter quartile range (pg/mL)	*P* value (PAD vs control)
IL-6	14 (23)	NA	1694	2484	1 (18)	2184	NA	NA	*0.0009
IFN-γ	14 (23)	NA	1533	6226	0 (18)	NA	NA	NA	*0.0002
TNF-α	13 (23)	NA	9962	15,368	1 (18)	4092	NA	NA	*0.0020
IL-17A	11 (23)	NA	3175	5276	0 (18)	NA	NA	NA	*0.0021
GM-CSF	11 (23)	NA	2444	2716	0 (18)	NA	NA	NA	*0.0021
TGFβ1	23 (23)	NA	35,019	53,234	18 (18)	NA	8882	12,239	†0.0011
CCL5	23 (23)	NA	21,267	40,968	18 (18)	NA	3560	2538	†0.0001

Abbreviations: IL-6, Interleukin 6; IFN-Y, interferon gamma; TNF-α, tumor necrosis factor alpha; IL-17A, Interleukin 17A; GM-CSF, Granulocyte Monocyte Colony Stimulating Factor; TGFβ1, transforming growth factor beta1; CCL5, chemokine ligand 5 also known as RANTES.
*Yates' Continuity Corrected Chi-Square.
†Randomization *t* test.

Table IV. Primers for quantification of cytokine mRNAs by the quantitative polymerase chain reaction

mRNA	5'————————————3'	ForwardReverse	GenBank
Interleukin 6 (IL 6)	ACAAATGCCAGCCTGCTGACGAAG	Forward	NM_000600
	TCTGACCAGAAGAAGGAATGCCCA	Reverse	
Interleukin 17A (IL 7A)	CCGGCTGGAGAAGATACTGGTG	Forward	NM_002190
	GAACTCTAATGAGTTTAGTCCG	Reverse	
Interferon-g (IFN-g)	ACTAGGCAGCCAAC TAAGCAAGA	Forward	NM_000619
	CATCAGGGTCACCTGACACATTCA	Reverse	
Tumor Necrosis Factor-a (TNF-a)	CTGACAAGCTGCCAGGCAGGTTCTCTT	Forward	NM_000594
	ATCAGGAAGGAGAAGAGGCTGAGGAAC	Reverse	
Transforming Growth Factor-b1 (TGF-b1)	CCGCAAAGACTTTTCCCCAGACC	Forward	NM_000660
	ACCTAGATGGGCGCGATCTGGTA	Reverse	
Chemokine Ligand 5 (CCL5)	AGTGTGTGCCAACCCAGAGAAGAA	Forward	NM_002985
	TGCCTGTTTCTGCTTGCTCTTGTCCT	Reverse	
Human Myosin Heavy Chain	TCGAAAATCAGAAAAGGAGCGGAT	Forward	NM_005963
	GAGGGTTCATGGGGAAGACTTGGT	Reverse	

Human Myosin Heavy Chain: Reference mRNA for quantification of cytokine message.

Walking performance and QoL of PAD of patients who co-expressed IL-17A, IFN-γ, TNF-α, and GM-CSF in their calf muscle and PAD patients who did not co-express these cytokines. We identified 11 PAD patients who co-expressed IL-17A, IFN-g, TNF-a, and GM-CSF in calf muscle. Walking performance and QoL were compared (Table VIII) with the remaining 12 PAD patients of whom 2 co-expressed IFN-g and TNF-a, 1 expressed TNF-a, only, and the remainder did not express any of the 4 inflammatory cytokines. These 2 groups did not differ in any of the demographic parameters identified in Table I or in anatomic pattern of disease Table IX. If there were a strong and direct association of local inflammation with leg function parameters, we would expect reduced walking performance and/or decreased QoL in those patients who co-expressed the 4 inflammatory cytokines. We found a significantly (P= 0.017) lower median COD (17 vs 102 m) in patients who co-expressed all 4 cytokines, and, although median PWD was substantially lower (55 vs 147 m) in these patients, it did not reach statistical significance (P= 0.169). Six-minute walking distance was nearly the same in both groups of patients with a median of 324 m for the 11 patients who co-expressed all 4 cytokines and 305 m for the remaining 12 patients. None of the QoL measures were significantly different between these 2 groups of patients.

TGFβ1and CCL5 in calf muscle biopsies and serum samples of PAD patients and control subjects. Calf muscle biopsies and serum samples collected from PAD patients (N = 23) and control subjects (N = 18) were evaluated for 2 more cytokines with inflammatory properties: TGFb1, a pro-fibrotic inflammatory cytokine,35 and CCL5, a chemokine with monocyte chemotactic activity. TGFb1 and CCL5 were detected at high picogram per mL of calf muscle of all PAD patients and control subjects, however, median concentrations were 3.94 and 5.67 times greater, respectively, in PAD compared to control muscle (Table V). The greater concentrations of TGF b1 (P < 0.002) and CCL5 (P <0.001) in PAD muscle are consistent with increased gene expression in the affected calf muscles of PAD patients. It is well known that megakaryocytes synthesize these and other growth factors, cytokines, and chemokines that are sequestered in their fragmentation products, that is, blood platelets.[36,37] Since platelets are expected to be present in muscle biopsies and their contents released in muscle homogenates, we extended our analysis of TGFb1 and CCL5 to evaluate potential contributions from contaminating platelets. Median concentrations of TGFb1 and CCL5 in PAD and control sera (Table VII) were similar with PAD/control ratios of 1.20 and 0.92, respectively. If platelets were a principal source of these cytokines in muscle, we would expect these ratios to be close to 1.0 reflecting the platelet cytokine complement released during serum preparation. As noted, PAD/control ratios for muscle were 3.94 and 5.67 for TGFb1 and CCL5, respectively. More

conclusively, these cytokines exhibited strong correlations across PAD muscles (N = 23; Kendall's Tau = 0.834; *P*< 0.0001) and across control muscles (N = 18; Kendall's Tau = 0.779; *P*< 0.0001; Fig 1, *A* and *B*) but were not correlated across PAD or control sera (Fig 1, *C* and *D*). If contaminating platelets accounted for TGFb1 and CCL5 in muscle, we would expect the correlations in muscle to occur in serum. We conclude that coordinated expression of these 2 inflammatory cytokines in muscle biopsy specimens is a local event in both PAD and control muscle.

Table VI. Correlations among cytokines detected by ELISA in calf muscle of PAD Patients.

CYTOKINE	IL-6	IL-17A	IFN-g	TNF-a	GM-CSF	TGFb1	CCL5
IL-6	1 --- N=14	[*]0.929 [†]0.0025 N=7	0.800 0.0096 N=9	0.857 0.0065 N=8	0.929 0.0025 N=7	0.371 0.1910 N=14	0.147 0.6154 N=14
IL-17A	---	1 --- N=11	0.900 0.0002 N=11	0.855 0.0008 N=11	0.946 0.0001 N=11	0.082 0.8110 N=11	0.064 0.8525 N=11
IFN-γ	-----		1 --- N=14	0.879 0.0001 N=13	0.955 0.0001 N=11	0.073 0.8054 N=14	0.121 0.6806 N=14
TNF-α	----			1 --- N=13	0.891 0.0002 N=11	-0.220 0.4706 N=13	-0.220 0.4706 N=13
GM-CSF	-----				1 --- N=11	0.046 0.8944 N=11	0.046 0.8944 N=11
TGFβ1	-----					1 --- N=23	0.959 0.0001 N=23

N: The number of patients positive for the cytokine or cytokine pair.
[*]Spearman Rho correlation coefficient.
[†]p-value.

Table VII. Cytokine concentrations in sera of PAD patients and control subjects, determined by ELISA

	PAD SERUM			Control SERUM			
Cytokine	Positive (total) patients	Median (pg/mL)	Inter quartile range (pg/mL)	Positive (Total) controls	Median (pg/mL)	Inter quartile range (pg/mL)	*P* value (PAD vs control)
IL-6	0 (23)	NA	NA	0 (18)	NA	NA	ND
IFN-g	0 (23)	NA	NA	0 (18)	NA	NA	ND
TNF-a	0 (23)	NA	NA	0 (18)	NA	NA	ND
IL-17A	5 (23)	763	712	4 (18)	1496	2071	[*]0.1970
GM-CSF	0 (23)	NA	NA	0 (18)	NA	NA	ND
TGFb1	23 (23)	17365	6155	18 (18)	14481	3428	[*]0.0006
CCL5	23 (23)	8247	4330	18 (18)	8920	468	[*]0.1750

[*]Randomization *t* test.

TGFβ1 and CCL5 message in calf muscles of PAD patients and control subjects. In another test for local gene expression, we quantified TGFb1 and CCL5 transcripts in calf muscles of PAD patients and control sub- jects, by RT qPCR. Total RNA was extracted from frozen muscle biopsies of a subset of the PAD patients (N = 12) and control subjects (N = 12) and cDNAs were prepared by reverse transcriptase reactions. Subsequently, cDNAs for TGFb1 and the reference protein myosin heavy chain (MyHC) were amplified by qPCR. The median transcript ratio for TGFb1 (TGFb1/ MyHC) was nearly 3.5 times greater (P= 0.0260) in PAD muscle (ratio, LCL -> UCL: 0.00893, 0.00020 -> 0.02714) compared to control muscle (ratio, LCL -> UCL: 0.00259, 0.00055 –> 0.00694), suggesting greater inflammatory activity in the affected leg muscles of PAD patients. The median transcript ratio for CCL5 (CCL5/MyHC) was 43 times greater (P= 0.0005) in PAD muscle (0.00729, 0.00014 –> 0.01828) compared to control muscle (0.00017, 0.00011 –> 0.00037). The greater concentrations of TGFb1 and CCL5 transcripts in PAD compared to control muscle support increased gene expression in the affected muscle of PAD patients. This interpretation was strengthened by analysis of the correlations between RNA transcripts for TGFb1 and CCL5 and the corresponding proteins determined by ELISA. Transcripts for both TGFb1 and CCL5 and their corresponding proteins in PAD muscle, were strongly correlated (Kendall's Tau = 0.833, P< 0.0001 and 0.778, P< 0.0001, respectively) (Fig 2, *A* and *B*), how- ever, cytokine transcripts and protein in control muscle were not correlated (Fig 2, *C* and *D*). The absence of correlation in control muscle is consistent with low- level, steady-state gene expression.

Table VIII. Walking performance and quality-of-life of patients co-expressing IL-17A, IFN-g, TNF-a, and GM-CSF compared to patients who did not co-express these cytokines

	Group 1: Patients co-expressing IL-17A, IFN-g, TNF-a, and GM-CSF		Group 2: Patients non co-expressing IL-17A, IFN-g, TNF-a, and GM-CSF		
Walking performance / Quality-of-Life	Median (N)	LCL -> UCL	Median (N)	LCL -> UCL	*P value
Six-minute walk distance (meters)	324 (11)	210 -> 353	305 (11)	204 -> 326	0.498
Claudication onset distance (meters)	17 (7)	5 -> 99	102 (8)	21 -> 117	0.017
Peak walking distance (meters)	55 (10)	23 -> 215	147 (11)	55 -> 190	0.169
WIQ-1	50 (11)	25 -> 50	50 (11)	25 -> 63	1.000
WIQ-2	68 (11)	29 -> 83	50 (11)	25 -> 64	0.164
WIQ-3	67 (11)	38 -> 75	44 (11)	17 -> 81	0.486
WIQ-4	50 (11)	8 -> 75	67 (11)	33 -> 75	0.187
SF-physical function	50 (11)	22 -> 65	45 (11)	20 -> 70	0.814
SF-pain	45 (11)	23 -> 60	45 (11)	23 -> 55	0.511
SF-general health	60 (11)	35 -> 69	50 (11)	30 -> 70	0.714

WIQ, Walking Impairment Questionnaire; SF, short form 36. LCL, lower confidence limit; UCL, upper confidence limit.
Of the patients in Group 2, 3 expressed IFN-g, or IFN-g plus TNF-a, and the remainder expressed none of the 4 inflammatory cytokines.
*Randomization *t* test.

Table IX. The relationship between the level of arterial tree blockages of claudicating patients with peripheral artery disease and the autoimmune inflammatory cytokine profile in their calf muscle

[*]Anatomic disease pattern	PAD patients with the autoimmune cytokine profile (N = 11)	PAD patients without the autoimmune cytokine profile (N = 12)	[¶]P value
[†]Aorto-Iliac	8 yes / 3 no	5 yes / 7 no	0.2138
[‡]Femoro-Popliteal	6 yes / 5 no	11 yes / 1 no	0.0686
Aorto-Iliac and Femoro-Popliteal	4 yes / 7 no	4 yes / 8 no	1.0000
Aorto-Iliac only	4 yes / 7 no	2 yes / 10 no	0.3707
Femoro-Popliteal only	3 yes / 8 no	7 yes / 5 no	0.2138

[*]Segment of the arterial tree with hemodynamically significant blockages.
[†]Aorto-Iliac involvement with or without a concurrent Femoro-Popliteal involvement.
[‡]Femoro-Popliteal involvement with or without an Aorto-Iliac involvement.
[¶]Fisher's Exact Test: The test is for the null hypothesis that the distribution of blockages is independent of the autoimmune cytokine profile. The null hypothesis is rejected at a P value of 0.05 or less.

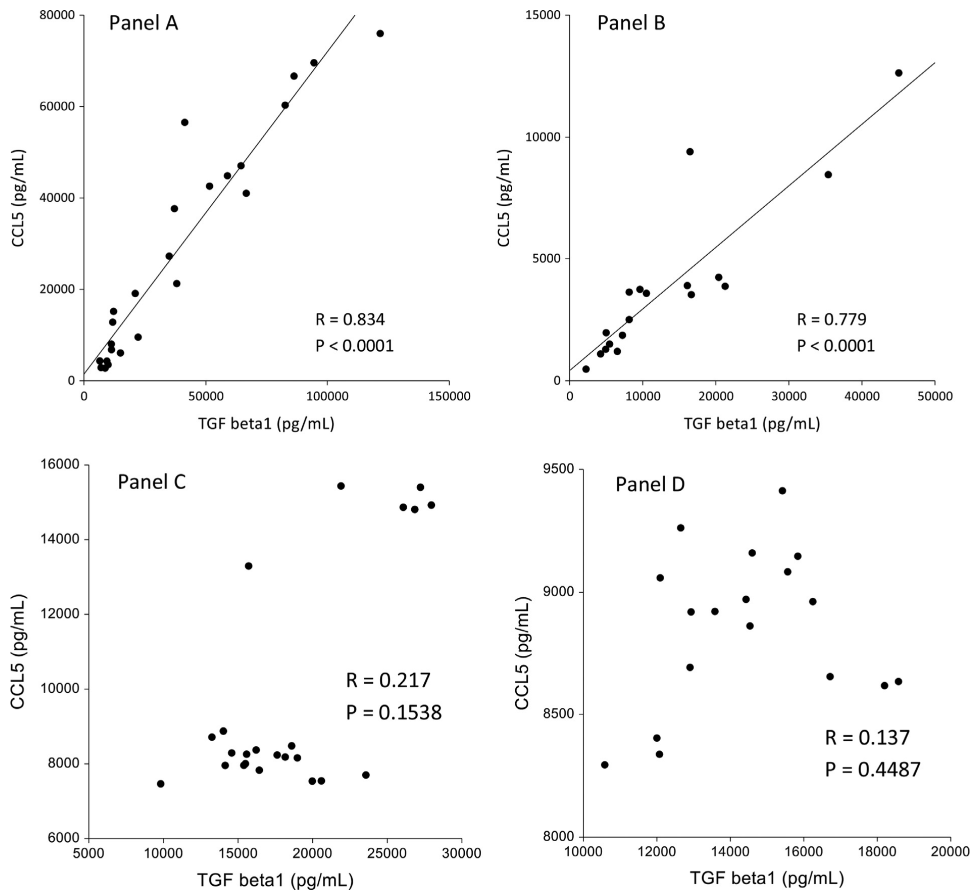

Fig 1. TGFb1 and CCL5 proteins were correlated in calf muscle across PAD patients (Panel A) and control subjects (Panel B) but were not correlated in serum across PAD patients (Panel C) or control subjects (Panel D). Muscle homogenates were prepared from biopsies of calf muscle of the more affected leg (lower ABI) of 23 patients and from calf muscle of 18 controls. Serum was collected at the time of biopsy. Cytokines were quantified by sandwich ELISA (Qiagen Kit; Germantown, MD) and their concentrations expressed as picograms per mL (pg/mL) muscle. Correlations are expressed as Kendall's Tau (R) with P values.

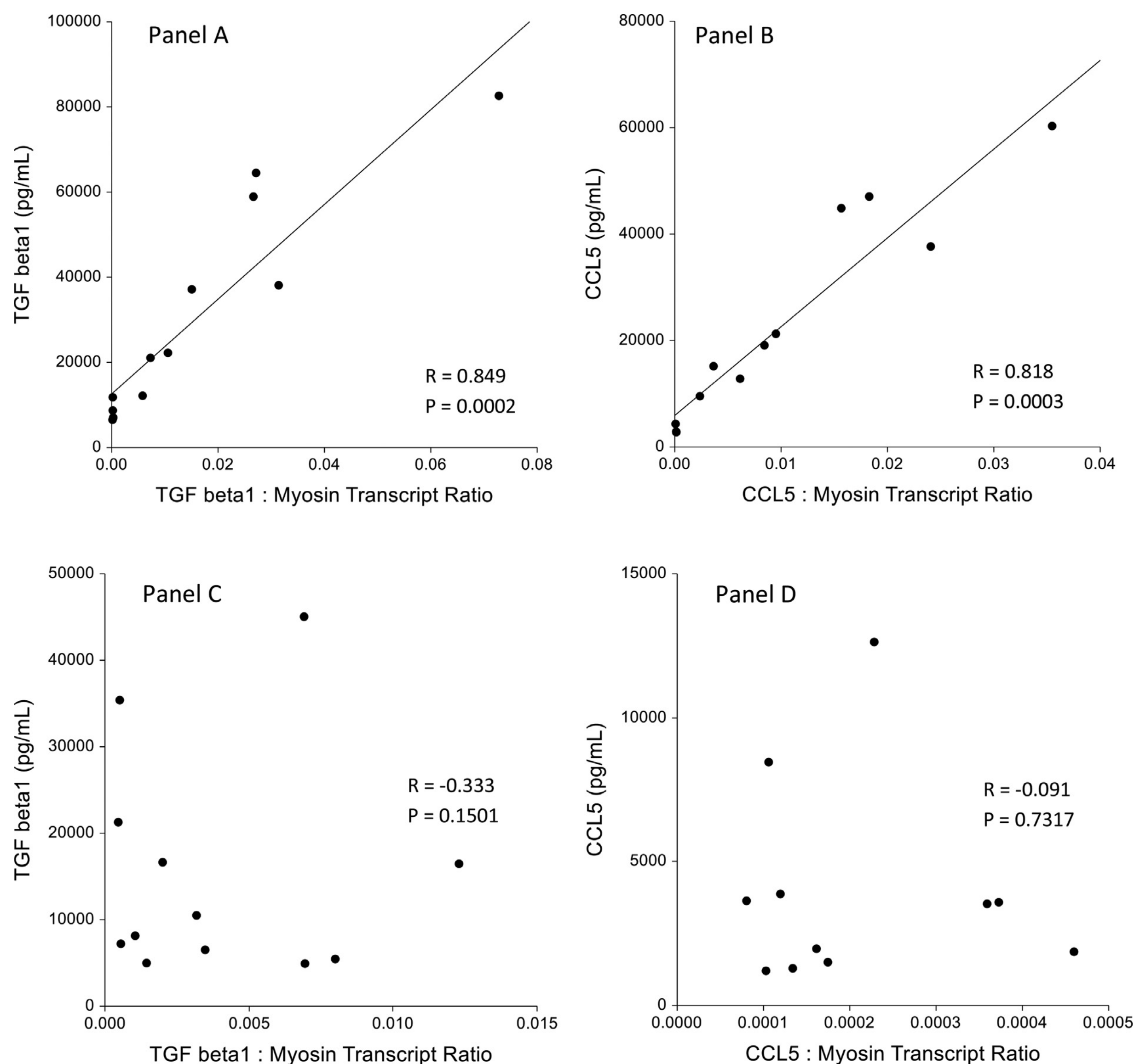

Fig 2. TGFb1 and CCL5 proteins correlated with their corresponding gene transcripts in calf muscle of PAD patients (Panels A and B) but did not correlate in calf muscle of control subjects (Panels C and D). Muscle homogenates were prepared from biopsies of 12 PAD patients and 12 control subjects. Cytokines were quanti- fied by sandwich ELISA (Qiagen; Germantown, MD) and their concentrations expressed as picograms per mL (pg/mL) muscle. Gene transcripts were quantified by RT qPCR and abundances expressed as a ratio to tran- scripts of the human myosin heavy chain gene. Correlations are expressed as Kendall's Tau (R) with P values.

DISCUSSION

Studies by many investigators have documented associations between circulating inflammatory cytokines and limb function, walking performance, and QoL as well as risk for PAD.[1,10,11,14,16-18] However, none of these studies evaluated local inflammation in lower extremity muscles, and the outcomes of all these studies are readily interpreted as representative of the burden of inflamed atherosclerotic plaques in these patients. Atherosclerotic disease in PAD patients is severe[1] and the burden of plaques is expected to be directly related to both limb dysfunction and serum concentrations of inflammatory cytokines and acute- phase proteins. We identified 2 groups of PAD patients at the extremes of cytokine expression in the calf muscle. One group of 11 patients co- expressed 4 inflammatory cytokines (IL-17A, IFN-g, TNF-a, and GM-CSF) and another group of 12 patients did not co- expressed these 4 cytokines. If local inflammation has a strong, direct effect on walking performance or QoL, we would expect significant

differences between the 2 groups. Mean COD was significantly lower (83% lower; *P*= 0.017) in co-expressors (17 m) compared to patients who did not co-express these 4 cytokines (102 m). PWD also was reduced in co-expressors (55 m vs 147 m) but did not reach significance (*P*= 0.169). Given the small population size, these differences suggest a strong effect of autoimmune inflammation on walking performance.

Detection of GM-CSF in calf muscle of PAD patients but not in calf muscle of control subjects is a significant finding of the present study. Until recently, GM-CSF was considered primarily a hemopoietic growth factor, but now it is clear that the cytokine is for the most part not required for development of macrophages and granulocytes.[26] GM-CSF is a central and essential mediator of tissue inflammation.[31] The cytokine is absent from the circulation and is produced and active locally at sites of tissue inflammation.[26,38] GM-CSF is increased at diverse sites of inflammation and is essential for disease progression in models of arthritis and multiple sclerosis (MS).[26,31] The central mechanism of chronic inflammation across diverse inflammatory diseases including rheumatoid arthritis (RA), psoriasis (PS), MS, and diabetes is activation of tissue damaging myeloid (vs embryonically derived resident) macrophages by GM-CSF secreted from activated autoreactive Th-cells.[31] The ability of these Th- cells to initiate tissue inflammation requires their pro- duction of GM-CSF under the influence of IL-23 which is essential for development of many inflammatory dis- eases.[39,40] Clinical treatment of PS patients with blocking antibody to IL-23 has produced improvement in these patients and targeting GM-CSF with blocking antibody has produced improvement in patients with RA.[26,41] GM-CSF$^+$ Th-cells are part of a functionally integrated Th-cell subset that secretes GM-CSF, IFN- Y, TNF-a and IL-17A, a multi-cytokine signature.[31] Though not part of this Th-cell subset signature, IL-6 expression is upregulated along with Toll-Like receptors in GM-CSF stimulated myeloid macrophages.[40] The presence of GM-CSF together with strong correlations (Spearman coefficients of 0.855-0.955) among these 4 inflammatory cytokines in the calf muscles of 11/23 claudicating PAD patients' supports a local auto- immune inflammation in their calf muscles. Since activation of autoreactive Th-cells is MHC Class II restricted,[25,31,42] we suggest that MHC Class II haplo- type is a significant risk factor for autoimmune inflammation in calf muscle of PAD patients. Moreover, the availability of therapeutic, blocking antibodies to GM- CSF and IL-23 and their documented safety and efficacy in clinical trials, offer the opportunity for specific intervention in PAD.[26,31,41]

Tissue inflammation has been reported as profibrotic and injurious to tissue, often leading to organ failure, and, conversely, as essential for tissue repair and maintenance of organ function. Addressing this problem, Lavine et al.[43] studied macrophage populations in a model of cardiomyocyte injury in neonatal and adult mice. In neonatal mice, cardiac recovery occurred in association with cardiomyocyte proliferation, angio- genesis, and minimal inflammation. In adult mice, cardiac repair was limited and was associated with a robust inflammatory response and tissue damage. Cardiac recovery in neonatal mice was linked to the presence of resident (embryonically derived) macrophages in the injured heart, while inflammation and tissue damage in the injured adult heart was linked to replacement of resident macrophages with monocyte-derived (myeloid) macrophages. In the present study, we documented expression of GM-CSF, a central communicator between activated autoreactive Th-cells and tissue damaging myeloid macrophages,[26] as part of an inflammatory cytokine signature of autoreactive Th- cells[31] in calf muscle of a significant proportion (11/ 23) of claudicating patients with PAD. Consequently, we propose that myeloid macrophages predominate in the inflamed calf muscles of these PAD patients and are the immediate effectors of muscle damage and fibrosis. Our future studies will address this proposal.

In this study, we found that TGFb1 and CCL5 were co-expressed in all calf muscle specimens but were present at higher concentrations in the calf muscles of PAD patients compared to control subjects. These cytokines exhibited strong correlations (R = 0.957 and 0.913, respectively) across PAD muscles and across control muscles, suggesting their origin from a com- mon process in each group. For both TGFb1 and CCL5, the strong correlation between mRNA and protein in PAD muscle indicated upregulated gene expression. In contrast, the absence of detectable correlation in control muscle indicated low-level, steady-state gene expression, consistent with the observed lower concentrations of these cytokines in control muscle. Our findings with control specimens are consistent with chronic, low-grade inflammation that develops with age, a process that has been termed "inflammaging."[44] Several mechanisms have been pro- posed for inflammaging, including a heightened acute- phase response, reduced capacity to resolve immune responses, increased oxidative stress, and innate and/or adaptive immune responses to cellular self-debris including both damaged proteins and intact immuno- privileged molecules.[44,45] Currently, increased accumulation of cellular debris, misfolded proteins, and exposed immunoprivileged molecules are considered the predominant stimuli for inflammaging, which is consistent with an imbalance of cell damage and repair and the decline of autophagy with age.[44] Oxidatively modified proteins accumulate with advancing age and should be included among these stimuli.[27,45,46] The precise mechanism of inflammaging is not known but it is possible that the condition is mediated by activation of pattern recognition receptors on macrophages, dendritic cells and/or neutrophils, by DAMPs[24] that may be expressed on oxidatively damaged proteins,[47] nonoxidatively damaged proteins[44,48] and unaltered immunoprivileged proteins, and other molecules[23,49] released from damaged or dying cells. Alternatively, inflammaging may be mediated by Th-cells that are not autoreactive but react with neoantigens generated by oxidative damage to self-proteins.[27,28] We propose that upregulated inflammaging and Th-cell mediated auto- immune inflammation occur concurrently in damaged muscle of the lower legs of claudicating patients with PAD.

Limitations. A limitation of the present study is the relatively small number of PAD patients and control subjects included in the study. Despite this limitation, we observed a statistically significant (P= 0.017) and markedly lower COD for patients who expressed the cytokine signature of activated autoreactive Th-cells (17 m) compared to those patients who did not express this signature in their calf muscles (102 m). Moreover, PWD was markedly lower in patients who expressed this cytokine signature (55 m) compared to those who did not (147 m) but did not reach statistical significance (P= 0.169).

Serum cytokine measurements were not informative of muscle cytokine concentrations and did not serve as markers of local inflammation. Our assay was sensitive, specific, and reproducible. Adjusting for serum dilution, lower limits of detection for our ELISA were 28.0, 56.8, 19.6, 42.8, and 96.6 pg/mL for IL-6, IL-17A, IFN-g, TNF-a, and GM-CSF, respectively. It is possible that these cytokines are present at lower concentrations and may be informative of muscle cytokines. However, it has been reported that blood values of inflammatory cytokines are not informative of local inflammation.[44] Another limitation of the manuscript is our inability to determine precisely the contribution of locally produced TGFb1 and CCL5 to our measurements of calf muscle, because of the potential contribution of these cytokines from possible platelet contamination as well as TGFb1 production by hyper- plastic vSMC in small veins and arteries of PAD muscle.[22] Strong correlations of these cytokines in PAD and control muscle and the absence of correlation in serum, however, support the conclusion that our cytokine measurements were largely unaffected by platelets or vSMC. Instead, our measurements likely represent low-grade

inflammation in calf muscles of PAD patients and control subjects.

CONCLUSION

The principal finding of the present study was the presence of a signature cytokine profile of inflammation mediated by autoreactive Th-cells, in calf muscle of nearly 50% of claudicating patients with PAD. This signature of autoimmune inflammation comprised highly coordinated expression of 4 inflammatory cytokines (GM-CSF, IL-17A, IFN-Y, and TNF- a), was associated with decreased leg function and was not found in control subjects. Patient serum was not informative of this signature in muscle. GM-CSF is the central and essential driver of tissue damage mediated by myeloid macrophages. Clinically tested antibodies against GM-CSF are relatively safe therapeutic agents that have been effective in treating RA, an autoimmune inflammatory disease, and offer the potential for effective therapeutic intervention for *ca.*50% of claudicating patients with PAD. Since Th- cell mediated autoimmune inflammation is MHC Class II restricted, it may be possible to identify PAD patients at high risk of autoimmune inflammation based on their MHC Class II haplotype, thus, substituting an invasive biopsy-based diagnostic procedure with a much less invasive procedure based on a blood sample. More research is needed to assess (1) MHC Class II haplotype as a biomarker of autoimmune inflammation in claudicating patients with PAD and
(2) the effectiveness of anti-GM-CSF therapy for PAD patients with inflamed myopathy.

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