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Differences in Inflammatory Cytokine Levels between Patients with Varying Severity of Chronic Venous Insufficiency

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Abstract

Objective: Several studies have found that protein concentrations of inflammatory cytokines are significantly increased in tissue and serum from patients with chronic venous insufficiency (CVI) relative to that of healthy controls. We sought to determine whether inflammatory protein concentrations differ between patients with moderate clinical disease, classified as clinical, etiology, anatomy, pathophysiology (CEAP) Classes 2 and 3, and more severe clinical disease, classified as CEAP Class 4.

Methods: Twenty patients with abnormal venous function were included in the study. Blood from a competent leg vein and from an incompetent superficial vein was collected in addition to incompetent vein tissue extracted through a phlebectomy procedure. Cytokine levels of venous tissue lysate and serum samples were determined using a multiplex assay.

Results: Thirteen patients (65%) were classified as clinical CEAP Class 2 or 3, with seven patients (35%) falling into the more severe Class 4 category. Twenty-seven cytokines were measured. Serum isolated from normal veins had significantly higher levels of IFN-gamma in patients with Classes 2 and 3 than Class 4 disease (95.17 pg/mL vs. 71.97 pg/mL; p=0.036). In serum from incompetent veins, IFN-gamma concentrations averaged 95.47 pg/mL in Class 2 and 3 patients and 76.97 pg/mL in Class 4 patients (p=0.048). Eotaxin levels from diseased vein tissue averaged 3.37 pg/mL in Classes 2 and 3 patients, and 1.57 pg/mL in Class 4 patients (p=0.037). IP-10 levels in diseased vein tissue was also significantly less in Class 4 patients at 74.20 pg/mL in Class 2 and 3 patients versus 31.06 pg/mL in Class 4 patients (p=0.004).

Conclusion: Despite several studies documenting increased inflammatory cytokines in patients with CVI, our study shows that those patients with more severe disease have significantly lower levels of several inflammatory cytokines. Thus, particular inflammatory cytokines may function in a reparative capacity following tissue injury or as a control mechanism to inhibit further tissue destruction.

Keywords: Chronic venous insufficiency; Inflammation; Cytokines; CEAP class; Venous reflux

Introduction

Chronic venous insufficiency (CVI) affects 10-20% of the population and is clinically characterized by persistent lower extremity edema due to underlying venous hypertension [1,2]. The spectrum of clinical sequelae ranges from persistent swelling and venous stasis changes to venous ulcers. Venous ulcers and non-healing wounds affect 0.2-1% of the population in developed countries and represent the most severe form of CVI [3,4]. Chronic lower extremity wounds, of which approximately 75% are due to CVI, are estimated to cause more than 2 million lost work days per year [5]. Though venous ulcers may be induced by trauma, the vast majority of them occur spontaneously [6].

Despite the high prevalence of CVI, it is curious that venous ulcers are relatively uncommon and the triggering factors remain unknown. Several studies have found that protein concentrations of inflammatory cytokines are significantly increased in tissue and serum from patients with CVI when compared to that of healthy controls [7,8]. Furthermore, many of these cytokines decrease following treatment [7,8]. However, it is unclear if the inflammatory milieu present in CVI contributes to disease pathogenesis, or if it is a repair response to tissue injury [9].

Though levels of inflammatory cytokines have been well documented to differ between CVI patients and healthy controls, the variability among patients and correlation with disease progression have not been well studied. We hypothesize that not only are cytokine levels different between healthy controls and diseased patients, inflammatory cytokine concentrations in serum and venous tissue are also different between patients with moderate clinical disease, classified as clinical, etiology, anatomy, pathophysiology (CEAP) Class 2 and 3, and more severe clinical disease, classified as CEAP Class 4.

Materials and Methods

Clinical protocol

Sample, data collection, and analysis for this study was approved

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by the Scripps Health Institutional Review Board. Informed consent following the principles outlined in the Declaration of Helsinki was obtained from each patient prior to enrollment. Inclusion criteria for this study included patients 18 years of age or older with abnormal venous function of the great saphenous vein or small saphenous vein. Additionally, patients were included only if they had a superficial segment of an incompetent vein requiring ambulatory phlebectomy for treatment as the vein was too superficial to safely treat with endovenous laser or chemical ablation. A Duplex ultrasonography scan (Philips Ultrasound, HD15 Diagnostic Ultrasound System, Bothel, WA, USA) to evaluate for vein size and reflux was performed with patients in a supine position, and CVI in the great or small saphenous veins was defined as the presence of reflux of >0.5 seconds**.**

Upon initial consultation for each patient, a baseline assessment for clinical, etiology, anatomy, and pathophysiology classification was recorded. Patients were ranked as CEAP Class 2, corresponding to large varicose veins; Class 3, corresponding to lower extremity edema; or Class 4, corresponding to venous stasis skin changes without evidence of ulceration.

Tissue collection

Incompetent superficial veins over the dorsal foot or ankle identified to have reflux were outlined with a tissue marker with the patient in the standing position. Local anesthesia was obtained through injection of 1% lidocaine and the vein was identified following subcutaneous incision. The vein was then ligated proximally and distally and the intervening vein was extracted and processed using the Bio-Plex cell lysis kit (Bio-PlexTM Cell Lysis Kit, Bio-Rad, Hercules, CA, USA).

Serum collection

Both control blood from a competent vein and blood from the incompetent vein were obtained from each patient at the time of their ambulatory phlebectomy procedure. Blood samples from the incompetent vein were acquired by puncturing the vein prior to ligation in the phlebectomy procedure described above. Control venous blood was taken from a competent vein in the lower leg, as demonstrated on Duplex ultrasonography as having no reflux.

Analysis of cytokines in venous tissue and serum

Cytokine levels of venous tissue lysate and serum samples were determined using the Bio-Plex Pro™ Human Cytokine 27-Plex Assay analyzed on a Bio-Plex® 200 System (Bio-Rad, Hercules, CA, USA). Total protein concentration of venous tissue lysate was determined by the Lowry method and normalized to 210 µg/ml per reaction. Antibody-coupled capture beads were incubated with antigen standards or samples and run in duplicate, as per assay protocol. This was followed with incubation of biotinylated detection antibodies and reporter streptavidin-phycoerythrin (S-P) conjugate. Beads were then passed through the Bio-Plex 200 suspension array reader according to the manufacturer's instructions. A 532 nm and 635 nm laser was used to excite the beads to determine the fluorescence intensity of bound S-P and the bead identity, respectively. The Bio-Plex Manager™ 6.0 software reported the concentration results in pg/mL.

Statistical analysis

Concentrations of inflammatory cytokines were not assumed to be a normal distribution. Differences between patient populations were analyzed using the Mann-Whitney U test. Significance was achieved when $p<0.05$.

Results

Patient demographics

Twenty patients were enrolled in the study. Fourteen (70%) were women and six (30%) were men. Ages ranged from 39 to 76 years of age, with an average of 60.2 years. The majority of the patients were Caucasian (80%) and the remaining 20% were of Hispanic origin (Table 1).

Each patient was evaluated for the number of endovenous ablative procedures performed. All patients underwent at least one endovenous laser ablation on either the great saphenous vein or small saphenous vein, and half of the patients had an endovenous laser ablation of both the great and small saphenous vein. The number of endovenous chemical ablations performed on each patient was also recorded. Patients ranged

Sex	Age	Race	Endovenous Laser Ablations	Endovenous Chemical Ablations	Total Procedure Number	CEAP Class
F	72	White	4	8	12	3
F	76	White	1	7	8	3
F	71	White	2	6	8	4
M	51	White	2	4	6	4
F	55	White	2	3	5	2
M	39	White	2	10	12	4
M	60	White	4	11	15	3
F	61	White	1	3	4	3
M	59	White	2	6	8	4
F	70	White	1	8	9	2
M	51	Hispanic	3	5	8	3
F	73	White	4	8	12	4
F	46	White	3	5	8	2
F	69	White	2	3	5	3
F	51	White	4	7	11	4
F	68	Hispanic	2	2	4	3
F	72	Hispanic	2	6	8	3
M	71	White	3	7	10	3
F	42	Hispanic	2	9	11	3
F	47	White	3	8	11	4

Table 1: Demographics.

from having a combined 4 endovenous laser and chemical ablations to 15 procedures, with an average of 8.75 procedures. Each patient also underwent at least one ambulatory phlebectomy procedure for a vein deemed too superficial to safely employ endovenous laser or chemical ablation.

Thirteen patients (65%) were classified as Class 2 or 3, with seven patients (35%) falling into the more severe Class 4 category. No patient fell into Class 5 or 6, corresponding to a healed or present venous ulcer, respectively. The average age of Class 2 and 3 patients was not significantly different that the average age of Class 4 patients, 62.5 years vs. 55.9 years, respectively (p=0.23). Additionally, the percentage of female patients in each group was not significantly different, 0.77% vs. 0.57% (p=0.96).

Cytokine concentrations

Twenty-seven cytokines were measured via a multiplex assay**.** In serum, nine cytokines (IL-2, IL-5, IL-6, IL-7, IL-10, IL-13, IL-15, MCP-1, and RANTES) were too low to be quantified accurately on a standard curve. In the vein tissue samples, ten cytokines' concentrations (IL-1Ra, IL-4, IL-5, IL-8, IL-13, IL-15, G-CSF, PDGF, TNF-a, and VEGF) were too low to be measured. The data was queried for differences in protein concentrations found in serum obtained from normal veins and serum from diseased veins. Protein levels of eotaxin were found to be higher in serum taken from varicose veins, when compared to serum taken from a normal vein (88.7 pg/mL vs. 78.7 pg/mL; p=0.001). MIP-1B levels were also higher in serum from varicose veins, 133 pg/mL vs. 128 pg/mL (p=0.02), and RANTES was found in higher concentration in serum from varicose veins, 12650 pg/mL vs. 12301 pg/mL (p=0.02). No statistically significant difference was found in any of the other cytokines measured (Table 2).

Patients were then divided into groups with more moderate disease, CEAP Class 2 and 3, versus those patients with more severe disease, CEAP Class 4. Cytokine levels measured from serum obtained via healthy and diseased veins in each group are summarized in Tables 3 and 4, respectively. Serum taken from normal veins had significantly higher protein levels of IFN-gamma in patients with Class 2 and 3 disease than Class 4 disease (95.17 pg/mL vs. 71.97 pg/mL; p=0.036) (Figure 1). This finding was replicated in serum taken from a diseased varicose vein. IFN-gamma concentrations averaged 95.47 pg/mL in Class 2 and 3 patients and 76.97 pg/mL in Class 4 patients (p=0.048) (Figure 2).

Cytokine concentrations in diseased vein tissue lysates divided into CEAP classes are summarized in Table 5. Eotaxin protein levels averaged 3.37 pg/mL in Class 2 and 3 patients, and 1.57 pg/mL in Class 4 patients (p=0.037) (Figure 3). The difference in protein concentration of IP-10 was also significantly less in Class 4 patients, measuring 74.20 pg/mL in Class 2 and 3 patients, but only 31.06 pg/mL in Class 4 patients (p=0.004) (Figure 4).

Discussion

Tissue injury in CVI has been attributed to inflammatory processes responding to chronically elevated venous pressure. However, despite several studies documenting the presence of elevated inflammatory cytokines, the mechanism by which inflammation mediates tissue destruction has not been elucidated [6-10].

Mean (SD) (n=20)					Mean Paired Differences	
Cytokine	Varicose Serum		Normal Serum		Varicose Serum – Normal Serum	Protein Concentration (p*)
IL-1b	3.3	0.9	3.5	1	-0.1	0.46
IL-1ra	115	114	115	105	0.9	0.26
IL-2	0.1	0.6	0.005	0	0.1	0.99
IL-4	7.9	1	8.2	1.4	-0.3	0.25
IL-5	0.6	2.6	0.9	2.4	-0.3	0.75
IL-6	2.5	3.1	2.1	2.2	0.4	0.71
IL-7	0.7	2.1	0.9	2	-0.2	0.2
IL-8	41	32.4	50.2	67.3	-9.2	0.62
IL-9	138	38	132	36	6.5	0.28
IL-10	0.8	1.8	0.6	1.6	0.2	0.81
IL-12	44.5	59.3	43	46.1	1.5	0.99
IL-13	1.9	2.7	1.9	1.5	-0.01	0.74
IL-15	0.005	0	0.005	0	0	---
IL-17	90	87.9	86	82.7	4	0.33
Eotaxin	88.7	37.9	78.7	33.6	10	0.001
Basic FGF	102	65	96.5	54.4	5.5	0.89
G-CSF	75.8	51.4	69.8	41.9	6.1	0.31
GMCSF	70.5	104.4	55.6	79.5	14.8	0.53
IFN-g	89	19.8	87.1	25.1	1.9	0.62
IP-10	592	242	580	272	11.3	0.22
MCP-1	1.6	6.9	4.1	12.7	-2.6	0.75
MIP-1a	7.5	6	7.7	5.4	-0.2	0.73
MIP-1b	133	64	128	60	5.8	0.02
PDGF-BB	2707	648	2693	1059	13.4	0.7
RANTES	12650	648	12301	841	349	0.02
TNF-a	44.5	23.2	48.9	22.8	-4.4	0.13
VEGF	144	84	142	82	2.4	0.9

Table 2: Mean paired differences in serum cytokines in healthy vs. diseased veins.

	Mean (SD) (n=20)				Mean Difference	
	CEAP Class 2, 3		CEAP Class 4		CEAP 2,3-CEAP 4	Protein Concentration (p*)
IL-1b	3.4	0.9	3.7	1.2	-0.3	0.522
IL-1a	129.6	92.3	196.5	143.3	-66.9	0.417
IL-4	8.2	1.6	8.3	1.1	-0.1	0.918
IL-8	41.5	40.3	66.2	103.2	-24.6	0.563
IL-9	130.4	43.6	133.6	19.0	-3.2	0.822
IL-12	55.2	47.6	61.7	47.2	-6.5	0.799
IL-17	103.6	95.3	92.3	64.7	11.3	0.769
Eotaxin	84.0	40.1	68.8	13.3	15.2	0.230
Basic FGF	109.8	65.8	99.5	37.0	10.2	0.648
G-CSF	77.0	49.1	56.3	20.3	20.8	0.201
GM-CSF	77.7	52.5	103.2	120.3	-25.4	0.762
IFN-g	95.2	24.5	72.0	19.6	23.2	0.036
IP-10	561.2	259.8	615.8	311.7	-54.6	0.700
MIP-1a	7.1	5.0	8.7	6.3	-1.6	0.576
MIP-1b	118.6	45.8	144.0	82.0	-25.4	0.469
PDGF-BB	2692.7	910.2	2693.9	1376.8	-1.2	0.998
TNF-a	48.6	26.4	49.6	15.7	-1.0	0.917
VEGF	141.2	78.9	163.0	91.7	-21.8	0.600

Table 3: Mean differences in serum cytokines from a healthy vein between CEAP classes.

	Mean (SD) (n=20)				Mean Difference	
	CEAP Class 2, 3		CEAP Class 4		CEAP 2,3-CEAP 4	Protein Concentration (p*)
IL-1b	3.3	0.7	3.4	1.1	-0.2	0.752
IL-1a	148.3	118.2	149.8	125.5	-1.5	0.983
IL-4	8.0	1.4	7.7	2.3	0.3	0.775
IL-8	38.9	21.1	45.0	49.1	-6.1	0.764
IL-9	142.9	41.3	128.8	30.9	14.1	0.401
IL-12	75.9	62.3	71.8	57.3	4.1	0.911
IL-17	97.3	96.8	105.4	75.7	-8.1	0.844
Eotaxin	97.8	42.3	71.7	21.4	26.1	0.083
Basic FGF	113.4	70.6	125.7	65.1	-12.3	0.658
G-CSF	82.9	59.6	62.7	30.5	20.2	0.331
GM-CSF	94.6	62.6	182.2	157.8	-87.6	0.397
IFN-g	95.5	18.2	77.0	17.9	18.5	0.048
IP-10	618.3	236.2	542.2	264.0	76.1	0.536
MIP-1a	7.1	6.2	8.2	6.0	-1.1	0.717
MIP-1b	124.9	50.1	149.0	87.1	-24.1	0.518
PDGF-BB	2750.4	946.6	2625.1	1823.1	125.4	0.869
TNF-a	45.3	26.4	48.6	18.6	-3.3	0.744
VEGF	142.7	79.6	167.3	94.2	-24.6	0.563

Table 4: Mean differences in serum cytokines from a diseased vein between CEAP classes.

To explore the differences in cytokine levels between normal and elevated venous pressure environments, Lattimer et al. compared blood taken from the arm and the ankle in patients with CVI, as well as blood taken from both sites in healthy controls [10]. In their study, IL-6, IL-8, and MCP-1 all increased in concentration in the patients' legs when compared to the arms. However, the study found that IL-6 was also increased in legs of healthy controls when compared to arm blood. Thus, the authors hypothesized that IL-6 may reflect a normal physiologic response to high venous pressures rather than represent a biomarker of CVI or act as a mediator of disease. To better control for normal physiologic differences between upper and lower extremity cytokines, serum obtained from a non-diseased vein in our study was taken from a vein in the lower extremity which had been shown on Duplex ultrasonography to be of normal size without reflux.

	Mean (SD) (n=20)				Mean Difference	
	CEAP Class 2, 3		CEAP Class 4		CEAP 2,3-CEAP 4	Protein Concentration (p*)
IL-1b	0.1	0.1	0.1	0	0	0.332
IL-6	0.1	0.1	0.2	0.1	0	0.504
IL-7	2.2	1.1	2.7	2.1	-0.6	0.527
IL-9	11.7	8.6	9	5.7	2.7	0.41
IL-10	3.1	1.7	4.1	2.6	-1	0.359
IL-12	0.1	0.1	0.1	0.1	0	0.617
IL-17	4.7	5.1	6.2	4.1	-1.5	0.492
Eotaxin	3.4	2.6	1.6	0.8	1.8	0.037
Basic FGF	2.1	1.1	3.1	2.2	-1	0.303
GM-CSF	0.9	0.6	1.5	1	-0.6	0.157
IFN-g	2.6	2	2.4	1.5	0.1	0.889
IP-10	74.2	37.1	31.1	21.7	43.1	0.004
MCP-1	5.4	4.3	3.9	3.3	1.6	0.432
MIP-1a	0.5	0.2	0.5	0.3	0	0.861
MIP-1b	8.9	7	6.3	4.4	2.6	0.317
RANTES	711.8	756	330.4	237.1	381.5	0.114

Table 5: Mean differences in vein tissue cytokines from a diseased vein between CEAP classes.

An additional study by Tisato et al. used multiplexed beads to measure differences in 31 cytokines between plasma taken from within a normal vein and a diseased varicose vein, as well as plasma from healthy controls. The vast majority of cytokines were no different in CVI patients' plasma regardless of its source; only EGF, PDGF, and RANTES were noted to be increased at the varicose vein site [8]. Similarly, our data show very little difference in protein concentrations found in serum from a healthy versus a diseased vein, with only eotaxin, MIP-1B, and RANTES showing minimally increased concentrations. Thus, though cytokines are likely changed by the inflammatory process occurring in diseased veins, the circulating flow distributes inflammatory cytokines systemically.

When comparing CVI patients to healthy controls in the same study, 14 cytokines in blood samples from CVI patients showed increased levels. However, 6 months following a minimally invasive surgery to restore physiologic venous drainage (ambulatory conservative hemodynamic correction of venous insufficiency (CHIVA) procedure), several of the cytokine levels decreased to amounts similar to those seen in healthy controls [8]. A return to normal levels of inflammatory cytokines 6 months following a CHIVA procedure was also shown in an investigation by Zamboni et al. [11].

However, our study shows that among patients with CVI, those patients with more severe disease have significantly lower levels of several inflammatory cytokines, including IFN-gamma, eotaxin, and IP-10. Of note, no patients with venous ulceration were included in our study so the inflammatory changes involved in epidermal wound healing would not confound the results.

Thus, rather than a direct mediator of venous damage, particular inflammatory cytokines may function in a reparative capacity following tissue injury or as a control mechanism to inhibit further tissue destruction. Their decreased involvement may then allow for progression of CVI. Though numerous inflammatory mediators have been shown to be increased significantly in the peripheral blood of patients with CVI, levels do not necessarily correlate with clinical

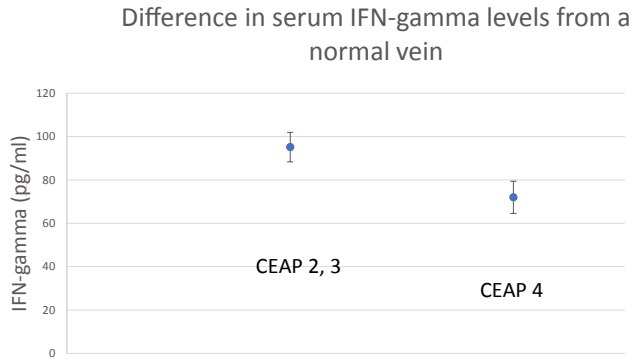

Figure 1: Difference in serum IFN-gamma levels from a healthy vein between CEAP classes.

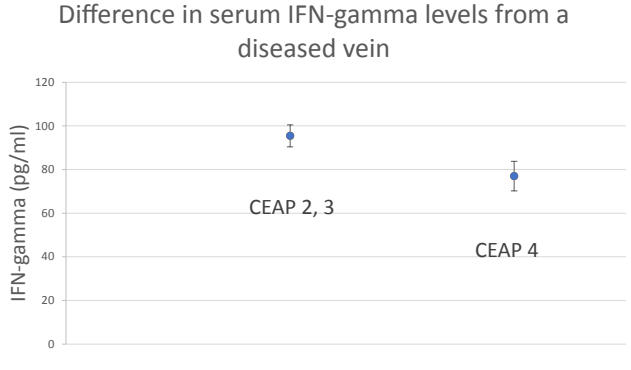

Figure 2: Difference in serum IFN-gamma levels from a diseased vein between CEAP classes.

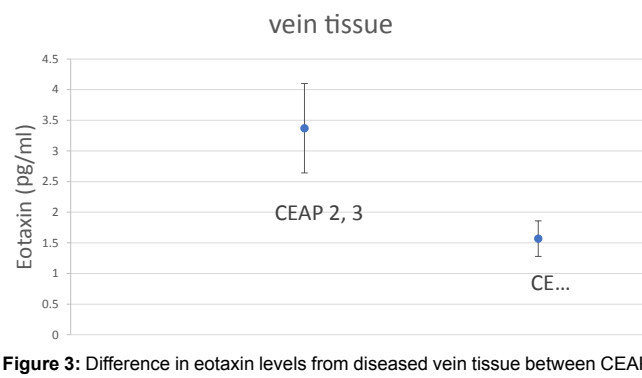

Figure 3: Difference in eotaxin levels from diseased vein tissue between CEAP classes.

symptoms. One study investigated cytokine levels in patients with CVI and found that though symptoms were worse in warmer weather, certain cytokines were much higher in autumn [12].

Limitations of our study include a small sample size of C2 patients as the difference in cytokine levels between C2 and C3 patients could not be evaluated in our study. Additionally, it would be interesting to investigate changes in cytokine levels 6 months following patients' treatment.

Rather than purely mediating destruction in venous disease, several previous studies have indicated that certain pro-inflammatory

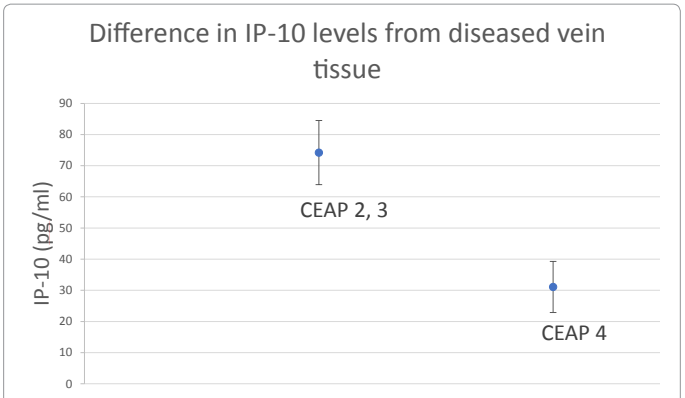

Figure 4: Difference in IP-10 levels from diseased vein tissue between CEAP classes.

cytokines may promote healing in the proper context. In patients with venous stasis ulcers, healing within 4 weeks of treatment with compression therapy was more likely in those patients with higher levels of IL-1a, IL-1b, IFN-gamma, IL-12p40, and GM-CSF [7]. In particular, total change in IFN-gamma levels was predictive of healing, further supporting IFN-gamma as a critical component of wound healing. In fact, several studies have found that lacking pro-inflammatory cells in acute wounds results in delayed healing [13,14].

Conclusion

Despite the observation that inflammatory cytokines are increased in patients with CVI, the mechanism of tissue destruction has not been defined. Until now, it has been hypothesized that leukocytes are mediators of inflammatory tissue destruction. However, our study shows that several inflammatory markers, rather than continuing to increase with worsening clinical disease, actually decrease. Thus, it is possible that a component of the inflammatory milieu in CVI actually promotes healing and tissue repair.

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