

Pro-inflammatory modulation of the surface and cytokine phenotype of monocytes in patients with acute Charcot foot

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Short title: Pro-inflammatory monocyte changes in acute Charcot

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Objective: Despite increased information on the importance of an inappropriate inflammatory response in the acute Charcot process, there has been no previous attempt to define the specific pathways that mediate its pathogenesis. Here, the role played by monocytes was analyzed.

Research design and methods The immune phenotype of peripheral monocytes was studied by FACS analysis comparing patients with acute Charcot (n=10), in both the active and recovered phase, diabetic patients with neuropathy, with or without osteomyelitis and normal controls.

Results As compared to diabetic controls and healthy subjects, monocytes from acute Charcot patients showed a proinflammatory immune phenotype characterized by increased production of proinflammatory cytokines, reduced secretion of antiinflammatory cytokines, increased expression of surface costimulatory molecules, and increased resistance to serum withdrawal-induced apoptosis. In addition, the pattern of circulating cytokines confirmed activation of proinflammatory cytokines. No modulation of the monocyte phenotype was documented in diabetic controls and healthy subjects, thus indicating that the proinflammatory alterations of monocytes are specific and causative of acute Charcot.

Conclusions Together, these data provide evidence for the role of proinflammatory changes in the immune phenotype of monocytes in the pathogenesis of acute Charcot. These alterations may explain the abnormally intense and prolonged inflammatory response that characterizes this disorder and may represent a potential therapeutic target for specific pharmacological interventions.

Diabetes is probably the commonest cause of denervation-induced destruction of joints (Charcot foot) worldwide today. Painlessness and abnormal foot biomechanics play an important part in the pathogenesis of the disorder. Recent attention has, however, focused on several abnormalities, which together suggest a more complex cause. A pilot study by La Fontaine, et al. (1) suggests that abnormal calcitonin gene-related peptide and endothelial nitric oxide synthase activity may play a role in the development of Charcot foot. Also, nuclear factor-kappaB ligand (RANKL)-activated peripheral blood monocytes have been found to induce a significant increase in bone resorption in Charcot patients (2,3). Finally, Jeffcoat W (4), drew attention on the possible link between proinflammatory cytokines and neuroarthropathy in the context of an exaggerated inflammatory response to trauma. The inability of the Charcot patient to control the intensity and the length of the local inflammatory response would lead to increased expression of tumor necrosis factor- α (TNF- α) and interleukin 1 β (IL-1 β) which, in turn would trigger increased expression of RANKL, leading to maturation of osteoclast and subsequent bone changes (5-8). Despite increased information on the importance of an inappropriate inflammatory response in the acute Charcot process, there has been no previous attempt to define the specific pathways that mediate its pathogenesis. Here, the role played by monocytes was analyzed. The immune phenotype of monocytes was assessed by testing spontaneous and induced production of proinflammatory and antiinflammatory cytokines, by measuring the expression of surface molecules (CD40, CD80 and CD86), that enable monocytes to become competent costimulatory cells and to activate T lymphocytes responses (9-11), and by studying the ability of monocytes to undergo apoptosis, an important homeostatic

mechanism that contributes to regulate the intensity and length of the inflammatory response (12). Patients with acute Charcot, in both the active and recovered phase, were compared with diabetic patients with neuropathy, with or without osteomyelitis and normal controls.

RESEARCH DESIGN AND METHODS

Patients and controls. We studied ten consecutive diabetic patients (four type 1, six type 2 diabetes) without macrovascular complications referred to the diabetic foot clinic of the University of Rome for unexplained, relative painless, increasing swelling of a foot and ankle, absence of active foot ulceration and/or signs of soft tissue infection, no history of recent (6 months) immunosuppressive treatment, including corticosteroids and anti TNF- α compounds, fever, evidence of infectious diseases, inflammatory disorders or any kind of malignancy, presenting the magnetic resonance (MR) diagnostic characteristics of stress injuries swelling, local warmth, and clinical instability due to ligamentous injury/occult trauma, at this stage, radiographic changes are absent or minimal, but it is present the typical bone marrow oedema at MRI; Eight age and sex matched diabetic patients with polyneuropathy, without macrovascular complications and clinical and radiological evidence of history of Charcot foot; eight age and sex matched diabetic patients with polyneuropathy and clinical and radiological documented foot osteomyelitis, without clinical and radiological evidence of history of Charcot foot; eight age and sex matched healthy subjects. Blood samples by peripheral venipuncture from Charcot patients were first obtained within 24 hours of meeting enrolment criteria and then just after recovery (as determined by clinical and MRI evaluation). The blood was immediately separated from plasma by centrifugation, stored at +4 °C and

processed within 1h. Serum was obtained for circulating cytokine determination and immediately stored at -80 °C. A single blood sample was obtained from control diabetics and healthy controls. This study was approved by the Ethic Committee of the University of Rome "Policlinico Tor Vergata" hospital. Informed consent was obtained from all subjects.

Compounds. LPS from *Escherichia coli* 0111/B4 was purchased from Sigma Chemical Co. St. Louis, MO. Recombinant GM-CSF was obtained from Sandoz Research Institute (East Hanover, NJ) and contains 5.4×10^6 chronic myelogenous leukaemia units per milligram of glycoprotein.

Limulus amoebocyte lysate test. All the compounds and media used in this study were analyzed for endotoxin contamination by the limulus amoebocyte lysate test (QCL-1000, BioWhittaker, Inc, Walkersville, MD). All the samples analyzed were found free of endotoxin contamination (less than 0.1 EU/ml).

Antibodies. For FACS analysis, the following monoclonal antibodies were used: anti-CD40, anti-CD80, anti-CD86, anti-TNF- α , anti-IL-1 β , anti-IL-6, anti-IL4 and anti-IL-10 (all from PharMingen, San Diego CA). Staining was performed with FITC-, PE- and Quantum Red™ (QR)-conjugated antibodies.

ELISA immunoassay. Commercially available sandwich ELISA kits The analyses of TNF- α , IL-1 β , IL-6, IL-4 and IL-10 were done with commercially available ELISA test kits (R&D Systems, Minneapolis MN). According to the manufacturer specifications, these ELISAs are specific for the relative interleukin. All the samples were determined in duplicate, in a single analytical set. Intra-series variation coefficient was <20%.

Cell stimulation. Peripheral blood from controls or patients was enriched for PBMC by centrifugation over Ficoll Hypaque. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented

with 20% heat-inactivated foetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin, referred to as complete medium. The cells were kept at 37 °C in a humidified atmosphere of 5% CO₂ in air, in 96 wells V bottom plates (Corning Incorporated, Corning, NY) at a concentration of 5×10^5 cells/well/250 μ l. For the determination of intracellular cytokine production by FACS, PBMC from patients and controls were cultured at a concentration of 5×10^5 cells/well/250 μ l for 18 h in 96 wells V bottom plates (Corning Incorporated, Corning, NY) in complete medium in the presence or in the absence of 100 ng/ml LPS. Thirty minutes after stimulation, 1 μ g/ml of the protein transport inhibitor brefeldin A (Sigma Chemical Co. St. Louis, MO) was added. At the end of the incubation period, the cells were analysed by FACS for intracellular cytokine production.

Surface marker and intracellular cytokine Staining. After incubation, the cells were washed and stained for surface markers. Then, cells were either analyzed by FACS to determine cell surface antigen expression or suspended in Cytofix/Cytoperm solution (PharMingen), stained for intracellular cytokines and then analyzed by FACS.

FACS. Flow cytometry was performed using a FACScan flow cytometer and analyzed with Cell Quest software (Beckton Dickinson). For each analysis, 10^4 monocytes were gated according to scatter characteristics designed to include only viable cells.

Assessment of hypodiploid DNA formation. Hypodiploid DNA formation (a reliable marker of apoptosis) was assessed by propidium iodide assay as previously described (13). In brief, PBMC (5×10^5) were incubated in RPMI 1640 alone for 72 h. At the end of the incubation period, the cells were resuspended in 1.5 ml propidium iodide solution containing 250 μ g of DNase-free RNase A. The propidium iodide fluorescence of individual nuclei was measured by FACS.

Statistics. The normality of variable distribution was assessed by the Kolmogorov-Smirnov goodness of fit test. Comparison between distribution of two variables for a single group was performed by either the Student's paired t test or Mann Whitney U test, as appropriate. All p values are 2-tailed. p values <0.05 were considered significant. Statistical analyses were performed using the SPSS (SPSS Inc., Version 10.0, Chicago, Illinois, USA) statistical package.

RESULTS

Patients. The mean age was similar between patients with acute Charcot (male/female: 6/4) and diabetic control patients with or without osteomyelitis (male/female: 5/3 and 6/2, respectively) (53 ± 2.8 versus 59 ± 2.9 and 59 ± 7 years, $p = >0.05$) as was the mean age between the former and healthy control participants (male/female: 4/4) (53 ± 2.8 versus 47 ± 2.7 years, $p >0.05$). The mean duration of diabetes (years, mean \pm SD) in acute Charcot cases and diabetic controls with or without osteomyelitis was 31 ± 5.1 , 27 ± 4.6 and 36 ± 2.9 , respectively. All diabetic patients, both acute Charcot and controls were on insulin therapy. The percentage (\pm SD) of HbA1c was 7.4 ± 1 in acute Charcot and 7.1 ± 0.5 and 7.6 ± 2.2 in diabetic controls with or without osteomyelitis, respectively.

Increased pro-inflammatory, and reduced anti-inflammatory cytokine production by monocytes from acute Charcot patients. Blood monocytes from cases and controls were evaluated for spontaneous (Fig. 1A) and inducible (Fig. 1B) TNF- α , IL-1 β , IL-6, IL-4 and IL-10 production. As shown by FACS analysis for intracellular cytokine production, monocytes from control diabetics and healthy controls did not spontaneously produce detectable amounts of TNF- α , IL-1 β , IL-6, IL-4 and IL-10. As opposed, a limited but detectable production of TNF- α , IL-1 β , IL-6, but not IL-4 and IL-10 was observed in cells from patients with acute

Charcot. When activated by LPS, monocytes from patients with acute Charcot produced significantly more TNF- α , IL-1 β and IL-6 but less IL-4 and IL-10 than monocytes from diabetic and healthy controls. Interestingly, both spontaneous and inducible cytokine production by monocytes from acute Charcot cases significantly decreased after recovery from the acute phase.

Circulating cytokines in patients with acute Charcot. Circulating cytokines in acute Charcot cases were analyzed during the acute phase of the process and after recovery. As shown in Table 1, the concentrations of TNF- α , IL-1 β and IL-6 were slightly but significantly higher than those found in control subjects and decreased after recovery to values similar to those found in controls.

Upmodulation of surface molecules in monocytes from Charcot patients. As shown in Fig 2, control diabetics and healthy controls did not statistically differ with respect to CD40, CD80 and CD86 expression ($p >0.05$). In contrast, monocytes from acute Charcot patients showed a statistically significant increase of both, percentage of positive cells and intensity of expression of these molecules, with respect to diabetic controls and healthy subjects. CD40, CD80 and CD86 expression markedly decreased in patients with acute Charcot after recovery, reaching values that were not different from those observed in control diabetics and healthy controls.

Increased resistance of monocytes from acute Charcot cases to serum withdrawal-induced apoptosis. As shown in Fig. 3A, just after separation, less than 1% of monocytes were nonviable as determined by trypan blue assay, and no hypodiploid DNA was detectable in cases and controls. The percentage of hypodiploid DNA increased over time in cultured cells, in both cases and controls. After 72 hours of culture, the percentage of hypodiploid DNA was significantly lower in cells from acute Charcot cases as compared with cells from diabetic and

healthy controls. Titration experiments (Fig. 3B) showed that addition of LPS at concentrations as low as 1 pg/ml reduced by more than 30% hypodiploid DNA formation in monocytes from acute Charcot patients but had a very limited effect in cells from recovered Charcot cases and controls. Moreover, more than 99% and less than 50% reduction of hypodiploid DNA formation could be obtained with 10 pg/ml LPS in monocytes from acute Charcot patients and recovered Charcot cases and controls, respectively.

CONCLUSIONS

We show here that in patients with acute Charcot, peripheral monocytes acquire a pro-inflammatory immune phenotype characterized by increased production of proinflammatory cytokines, reduced secretion of antiinflammatory cytokines, increased expression of costimulatory surface molecules and increased resistance to apoptosis.

Monocytes play a pivotal role in the development and maintenance of the inflammatory response. These cells are the major source of proinflammatory (TNF α , IL-1 β , IL-6) as well as antiinflammatory cytokines (IL-4, IL-10) (14-16). Alterations in the correct timing, intensity and balance of expression of proinflammatory versus antiinflammatory cytokines by monocytes result in pathologic modulation of the inflammatory response. Thus, the activation of inflammatory and suppression of anti-inflammatory cytokines that we have found in patients with acute Charcot is consistent with the abnormally intense and prolonged inflammatory response that characterizes the acute phase of this disease. A growing body of evidences is now supporting the possibility that this inflammatory response plays a pivotal pathogenetic role in the changes in bone and joints that develop in this disorder (4). Indeed, TNF- α and IL-1 β , released during the inflammatory process, trigger increased expression of RANKL (5,6). This lead to

activation of NF- κ B and maturation of osteoclasts (7,8). The effect of IL-6 on bone formation/resorption is more controversial. Indeed, several reports support the possibility that IL-6 could in fact induce an osteocytic phenotype (17). As opposed, there are evidences that IL-6 can stimulate osteoclasts differentiation and bone resorption by an indirect mechanism, increasing interactions between osteoblasts and osteoclasts (18). At variance with Devaraj S, et al (19) and Kulseng B et al, (20) who reported increased pro-inflammatory cytokine production by monocytes from diabetic patients with respect to healthy subjects we did not find differences between diabetic controls and healthy subjects. It is possible that these seemingly conflicting data are due to variations related to the experimental procedures used to detect cytokine production in cultured monocytes. Both Devaraj S, et al and Kulseng B et al, measured cytokine production by ELISA testing of supernatants from unfractionated cell cultures. Here we employed a FACS-based techniques that allows to identify producer cells by cell surface markers and by cytokine production simultaneously (13). It is likely that this technique is more suitable to study a primarily cell-based phenomenon than simple measurement of cytokine production by a bulk-release method such as ELISA testing of supernatants from unfractionated cell cultures.

CD40, CD80 and CD86 are involved in antigen presentation to T cells, CD4⁺ T cell activation (e.g. cytokine expression) and proliferation/differentiation (9-11). Thus, the enhanced expression of these molecules on Charcot-derived monocytes suggests that increased costimulatory signals were received by T cells, which might alter the balance between regulation and inflammation and further increase bone loss. Indeed, activated T cells are able to produce RANKL and to initiate the differentiation programme that leads to the formation of mature osteoclasts (21).

Unless activated, monocytes undergo apoptosis. Pro-inflammatory cytokines, such as IL-1 β or TNF- α , protect monocytes from apoptosis (22). This suggests that monocytes recruited to a site of inflammation receive stimuli allowing for survival through activation. Inversely, when inflammation abates, the rate of monocyte apoptosis increases markedly. Thus, apoptosis represents an important homeostatic mechanism that regulates the duration and intensity of inflammation (23). As we show here, monocytes from patients with acute Charcot were significantly more resistant to apoptosis than monocytes from controls. Nonetheless, we cannot exclude that contaminating lymphocytes contributed to the measured apoptosis in the monocyte cultures. However, since lymphocytes survive for weeks, a major contribution from this cell type is unlikely (22,23). It is possible that this increased resistance to apoptosis, which may be related to increased release of IL-1 β and TNF- α , may contribute, at least in part, to the inability of Charcot patients to terminate the inflammation in the affected limb.

The proinflammatory alterations we have found in the phenotype of monocytes from acute Charcot patients appear to be specific to this condition. Indeed, both the phenotype of monocytes from diabetic patients with uncomplicated neuropathy and that of monocytes from diabetic patients with neuropathy and osteomyelitis-associated foot inflammation was not different from that of cells from healthy controls. This indicates that

neither diabetes nor neuropathy or inflammation, per se, is associated with any modulation of the inflammatory response of monocytes. Interestingly, we found that in patients with acute Charcot all the modification of the immune phenotype of monocytes disappeared after recovery. This suggests that the initiating cause that triggers the inflammatory response in patients with acute Charcot acts in an environment where mechanisms that physiologically control the intensity and duration of inflammation are lacking. Calcitonin gene-related peptide (CGRP), a 37-amino acid peptide widely distributed in the central and peripheral nervous systems, mainly in sensory nerves (24) has been shown to inhibit proinflammatory cytokine production and to augment the release of IL-10 by monocytes (25). Thus, local reduction of CGRP in the denervated Charcot foot may affect monocytes activation with increased production of proinflammatory cytokines and reduced release of antiinflammatory cytokines.

In conclusion, these data provide evidence for a pathogenetic role of pro-inflammatory changes in the immune phenotype of monocyte in acute Charcot. These alterations may explain the abnormally intense and prolonged inflammatory response that characterizes this disorder and may represent a potential therapeutic target for specific pharmacological interventions.

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Table 1. Circulating cytokines in acute Charcot cases

Cytokine	Cytokine concentrations (pg/ml) in sera from				
	Acute Charcot (onset)	Acute Charcot (recovered)	Diabetic controls with osteomyelitis	Diabetic controls without osteomyelitis	Healthy subjects
TNF- α	5.2 \pm 3.2*	1.5 \pm 0.9	2.8 \pm 2.3	2.1 \pm 1	2.6 \pm 1.2
IL-1 β	0.6 \pm 0.3*	<0.125	<0.125	<0.125	<0.125
IL-6	15.3 \pm 7.4*	7.4 \pm 4.2	3 \pm 2.9	5.2 \pm 2.7	6.7 \pm 3.5
IL-4	<0.25	<0.25	<0.25	<0.25	<0.25
IL-10	<0.78	<0.78	<0.78	<0.78	<0.78

Note. TNF- α and IL-6 were detectable in 100% of the samples; IL-1 β was detectable in 3 out of 9 acute Charcot patients at the onset time point. The lowest standards were as follows: TNF- α , 0.5 pg/ml; IL-1 β , 0.125 pg/ml; IL-6, 0.156 pg/ml; IL-4, 0.25 pg/ml; IL-10 0.78 pg/ml. Data are mean \pm SD. The asterisk indicates $p < 0.05$, with respect to controls.

Legend to figures

Figure 1. Spontaneous cytokine production by monocytes from acute Charcot cases and controls. Spontaneous (panel A) and inducible (monocytes stimulated for 18h by 100 ng/ml LPS) (panel B) cytokine production was assessed by FACS, as intracellular accumulation on a single cell basis. Appropriate controls with isotype matched irrelevant mAbs were carried out and consistently showed <1% of positive cells. For each analysis, 10^4 monocytes were gated according to scatter characteristics designed to include only viable cells. Fluorescence data were expressed as percentage of positive cells after subtraction of background isotype-matched values. The data represent the mean \pm SD (error bars). The asterisk indicates $p < 0.05$, with respect to recovered acute cases and controls.

Figure 2. Upmodulation of surface molecules in monocyte-macrophages from Charcot patients. The expression of CD40, CD80 and CD86 was analyzed by FACS on CD14⁺ gated cells in fresh explanted PBMC. Appropriate controls with isotype matched irrelevant mAbs were carried out and consistently showed <1% of positive cells. For each analysis, 10^4 monocytes were gated according to scatter characteristics designed to include only viable cells. Fluorescence data were expressed as mean channel fluorescence (MCF) (panel A) and as percentage of positive cells (panel B) after subtraction of background isotype-matched values. The data represent the mean \pm SD (error bars). The single asterisk indicates $p < 0.05$, with respect to controls; the double asterisk indicate $p > 0.05$, with respect to recovered acute cases and controls.

Figure 3. Kinetics of Hypodiploid DNA formation in monocyte-macrophages. PBMC were cultured in medium with no added serum and hypodiploid DNA formation was determined at indicated intervals (panel A). PBMC were cultured in medium with no added serum with different LPS concentrations and hypodiploid DNA formation was determined at 72h (panel B). The red fluorescence due to propidium iodide staining of the DNA was registered on a logarithmic scale at >620nm. The forward and side scatter of particles were simultaneously measured. Cell debris was excluded from analysis by appropriately raising the forward scatter threshold. The residual cell debris had a very low DNA fluorescence emission and a low side scatter signal. At least 10^4 cells of each sample were analysed. The asterisk indicates $p < 0.05$, with respect to recovered acute cases and controls.

Figure 1

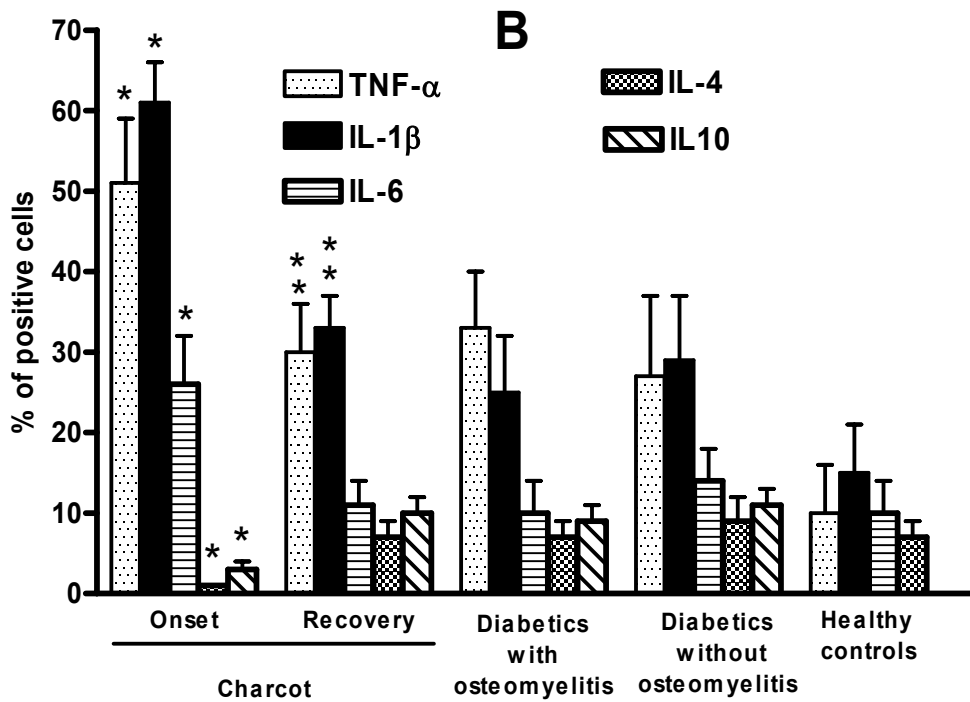
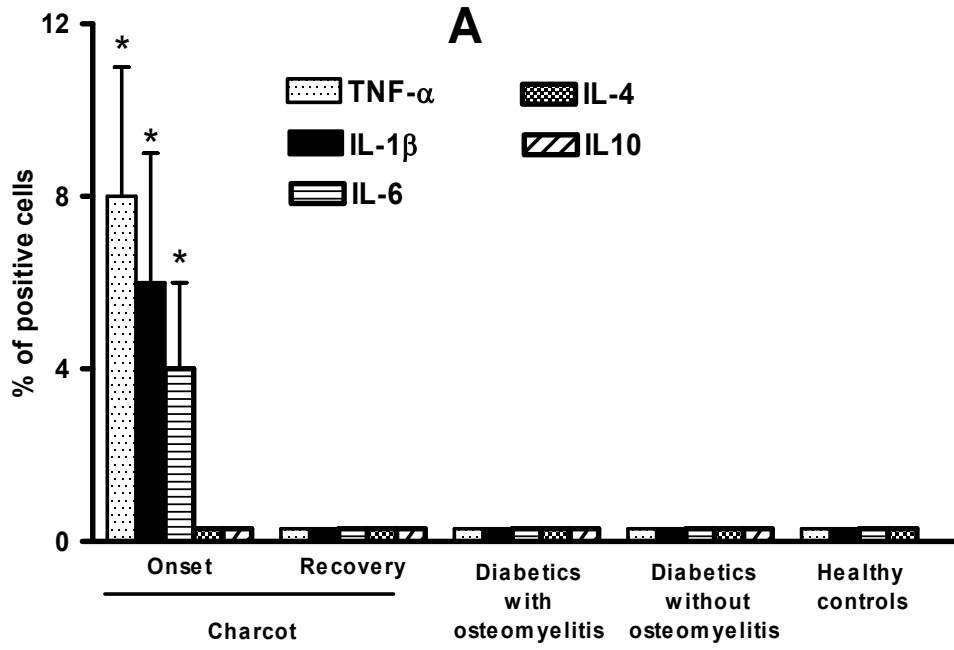


Figure 2

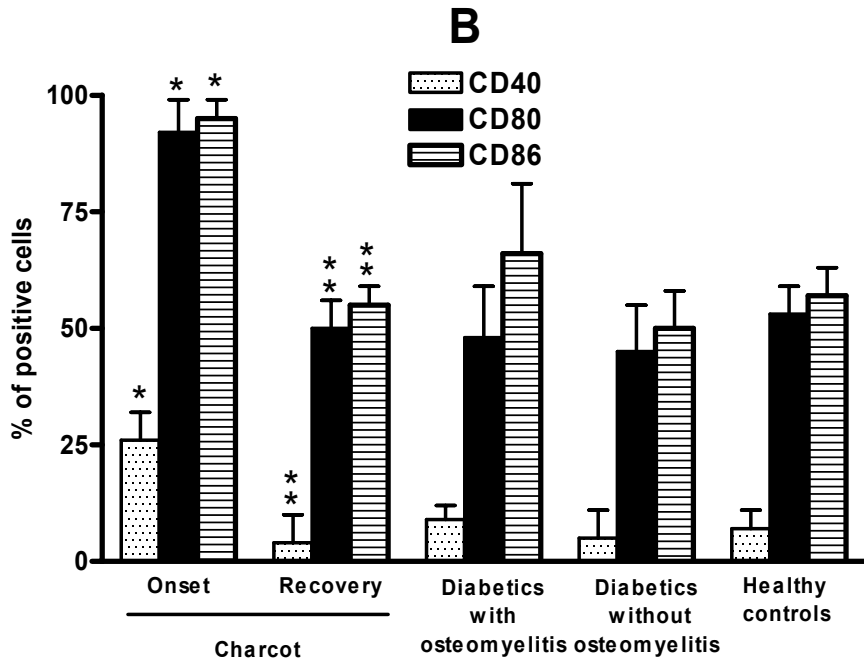
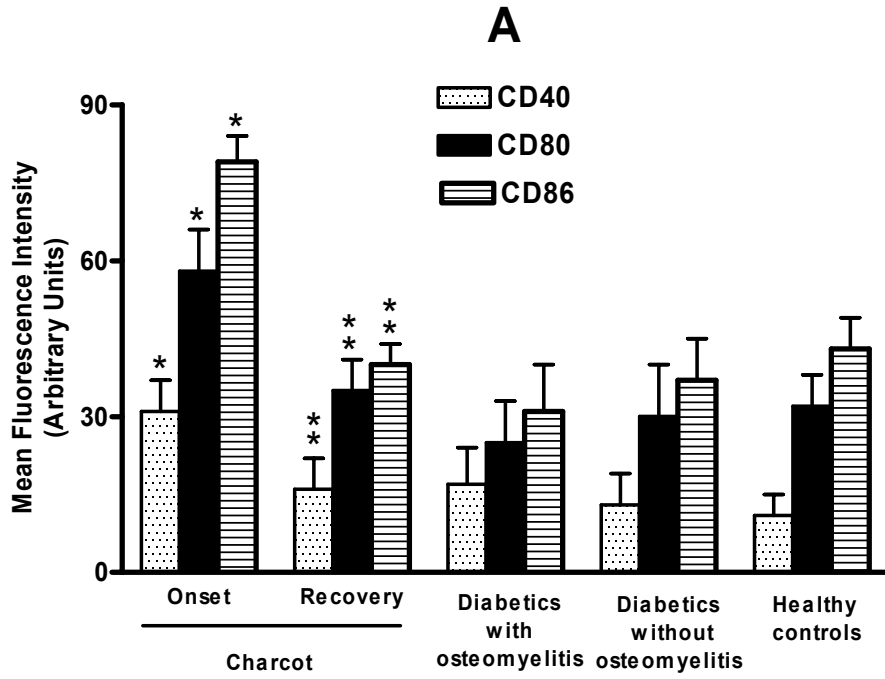


Figure 3

