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# Human cardiac fibroblasts isolated from patients with severe heart failure are immune-competent cells mediating an inflammatory response

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ABSTRACT

This study was aimed to elucidate the immunoregulatory properties of human cardiac fibroblasts cultured under pro-inflammatory and hypoxic conditions. Human heart tissue for isolating cardiac cells is generally hard to obtain, particularly from all four chambers of the same heart. Since different parts of the heart have different functions and therefore may have different immunoregulatory properties, ability to analyse cells from all chambers allows for a unique and comprehensive investigation.

Cells were isolated from all four chambers of the heart from patients undergoing cardiac transplantation surgery due to severe chronic heart failure (CHF) (n = 6). Cells isolated from one donor heart, were used for comparison with the experimental group. Primary cultured human cardiac fibroblasts were treated with Lipopolysaccharide (LPS) to induce an inflammatory response. Cells were also subjected to hypoxia. To determine immunoregulatory properties of the cells, cytokine and chemokine profiles were determined using multiplex ELISA.

*Results:* On average, the fibroblasts population constituted approximately 90% of the expanded non-myocytes. Levels of cytokines and chemokines were markedly increased in human cardiac fibroblasts cultured under inflammatory conditions, with a similar response in fibroblasts from all compartments of the heart. Unexpectedly, hypoxia did not further augment cytokine and chemokine secretion.

In conclusion, human cardiac fibroblasts are a robust source of pro-inflammatory mediators in the failing heart, independent of hypoxia, and might play a critical role in inflammation associated with the pathogenesis of CHF.

## 1. Introduction

Chronic heart failure (CHF) is still associated with high mortality and morbidity, despite increased treatment options [1]. Although heart failure has several aetiologies, a central theme in progression of the disease is adverse remodelling of the heart. This restructuring is characterized by increased accumulation of cardiac fibrosis caused by activated cardiac fibroblasts [2]. Cardiac fibroblasts are abundant in heart tissue and are involved in maintaining extracellular matrix homeostasis and repair, but also in adverse cardiac remodelling after myocardial infarction, which leads to loss of cardiac function and heart failure (HF) [3].

A relationship between ischaemia/hypoxia and HF exists, although the role of fibroblasts in myocardial ischaemia and CHF in the human remains largely unknown. However, it has previously been shown that hypoxia may stimulate pro-inflammatory cytokine production in rat fibroblasts [4]. Increased levels of inflammatory cytokines, such as tumour necrosis factor (TNF), interleukin (IL)-1 and IL-6, are found in the failing myocardium in HF patients, and low-grade systemic inflammation is shown in both acute and chronic HF [5]. Cytokines/chemokines associated with this low level inflammatory response may have several detrimental effects on cardiac function. Chemokines, such as MCP-1, IL-8 and eotaxins attract immune cells including monocytes/macrophages, neutrophils and eosinophils. These cells, in combination with the pro-inflammatory cytokine environment, may cause excessive fibrosis, reduced cardiomyocyte contractility, increased cardiomyocyte death and

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lack of cardiac repair [6–8]. In accordance with this, elevated levels of pro-inflammatory cytokines have been associated with worsening of HF symptoms and mortality [9].

In the human cardiac tissue, several genes are differentially expressed between the atrium and ventricle. Genes in the right ventricle show higher expression of genes related to the contractile network, whereas pathways related to immunity and defence are expressed in the right atrium [10]. The right ventricle shows resistance to myocardial injury, which could be a result of divergent fibroblast cell responses [11]. Taken together, these studies suggest that activated fibroblasts isolated from the atrium behave differently than fibroblasts isolated from the ventricle.

Thus, to increase our understanding of the immunoregulatory properties of human cardiac fibroblasts in chronic HF, effects of inflammatory and hypoxic activation on the cytokine and chemokine profile were measured in cultured human cardiac fibroblasts. Cells were isolated from all four chambers of the heart from patients suffering from severe HF as well as one control donor heart, not suitable for transplantation.

## 2. Material and methods

### 2.1. Human material and study design

Human primary cardiac fibroblasts were obtained from biopsies from explanted hearts from 6 patients undergoing heart transplantation due to severe heart failure. In addition, biopsy material was obtained from one organ donor heart that was not suitable for transplantation due to previous maze surgery. The organ donor did not have a history of HF. The medical data for the included research subjects are summarized in Table 1. Biopsy material was obtained from all four chambers of the heart when possible. For most research subjects however, it was only possible to obtain material from either left or right atria due to the surgical procedures performed. Fibroblasts were isolated from all biopsies as described in Section 2.2, then cultured and used in stimulation experiments with hypoxia or LPS. For some research subjects, due to limitations in the amount of biopsy material, either hypoxic or LPS treatment was performed. For a summary of obtained biopsies and conditions tested for individual research subjects, please see supplementary table 1.

Ethical approval was obtained for the human studies by the Ethical Committee of the University of Gothenburg. Written informed consent was obtained for patients undergoing cardiac transplantation prior to their inclusion in the study. The organ donor had approved of use of his tissue for research purposes. All experiments have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

### 2.2. Cell culture

To culture human primary cardiac fibroblasts, biopsies were collected in cold phosphate-buffered saline (PBS), then dissociated as described previously [12,13]. The samples were cut into small pieces and digested with Liberase TM 0.56 U/ml (Roche, Basel, Switzerland) and DNAse 0.002% (Sigma, St. Louis, MO, USA) in DMEM:F12 (Invitrogen, Carlsbad, CA, USA) at 37 °C for approximately 4.5 h with magnetic stirring. Then, the samples were washed once and further incubated for 10 min in Trypsin–EDTA 0.05% (Invitrogen). The cell suspension was washed once with DMEM:F12 and seeded into 6-well plates at a concentration of approximately 1 mg tissue/cm$^2$ in medium consisting of DMEM:F12 supplemented with 10% human serum, penicillin/streptomycin (PEST, PAA Laboratories, Pasching, Austria) and L-glutamine (Invitrogen). The cells were cultured for 10 days.

To induce an inflammatory response, cells were incubated with 1 μg/ml LPS derived from Salmonella typhimurium (Sigma-Aldrich, St Louis, MO, USA), which is a well-known inducer of inflammation mediated via interaction with the toll like receptor (TLR) 4 [14,15]. Control cells were incubated without LPS for 24 h. LPS. To mimic ischaemia, cells were incubated under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions for 24 h, then the medium was collected and immediately frozen at −80 °C before analysis. 1% oxygen concentration has been described as moderate hypoxia [16], and was chosen to mimic a chronic ischemic situation.

### 2.3. Characterization of cardiac fibroblasts by flow cytometry

To determine the composition of cells after monolayer culturing, flow cytometry was used. After 10 days, the cells were harvested by treatment with Trypsin-EDTA for 10 min, resuspended in DMEM:F12 supplemented with 10% foetal bovine serum (FBS) and washed in cold FACS buffer (PBS, 5% FBS, 1% BSA, 2 mM EDTA). The cell concentration was adjusted to 1.0–1.5 × 10$^7$ cells/ml, and the sample size was 100 μl. Antibodies used for flow cytometry analysis included mouse anti human CD45-PE-Cy7 (BD, USA), mouse anti-human CD31-APC (Biolegend, San Diego, Calif., USA), mouse anti-human CD34-Alexa 488 (Biolegend), mouse anti-human CD90-Brilliant Violet 421 and mouse anti-human PDGFRα-PE (BD, USA). Isotype controls IgG1-PE-Cy7 (eBioscience, San Diego, Calif., USA), IgG1-APC (BD), IgG1-Alexa 488 (Biolegend), IgG1-Brilliant Violet 421 (BD) and IgG2a-PE (Biolegend) were used. For all samples, 7-AAD (Invitrogen, Calif., USA) was added to identify dead cells. Cells were filtered through a 40-μm cell strainer, washed twice in cold FACS buffer and kept on ice in the dark until data acquisition. Analysis of cell origin was carried out using a FACSAria II cell sorter (BD) and data analysis was carried out using FACSDiva version 8.0.1 software. Gating was set using isotype controls with a range of false positives within 0.0–0.1% for all populations. CD45$^+$ cells were identified to exclude cells with haematopoietic origins.

**Table 1**
Medical background of included patients and donors (*).

| Subject | Sex | Age | IHD | HF | LVAD | NYHA | LVEF (%) | Cause of HF | Other diseases |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 60 | No | Yes | No | IIIA | 20 | Idopathic DCM | Diabetes type II, renal insufficiency |
| 2 | M | 67 | Yes | Yes | Yes | IIIA | N/A | Ischemic DCM | Renal insufficiency |
| 3 | M | 39 | Yes | Yes | No | III | 30 | Ischemic DCM | previous MI, Diabetes type I, PAH |
| 4 | F | 64 | No | Yes | No | IIIB | 20–30 | Idopathic DCM | Atrial fibrillation, Diabetes type II, renal insufficiency, hypertension, previous malignancy, hypothyroidism |
| 5 | M | 50 | Yes | Yes | No | IIIA | 30–40 | Ischemic DCM | Previous CABG, Diabetes type II, hypertension, previous stroke |
| 6 | M | 61 | No | Yes | No | | 55 | HCM | Atrial fibrillation, previous Maze surgery, Chronic obstructive pulmonary disease, renal insufficiency, previous stroke |
| 7* | M | 62 | No | Un-known | No | Un-known | N/A | N/A | Atrial fibrillation |

Table 1 summarizes the information about the research subjects. Subject 1–6 patients undergoing cardiac transplantation. Subject 7* an organ donor which heart was not suitable for transplantation due to previous Maze surgery. Cause of death Subarachnoid hemorrhage. IHD = Ischemic heart disease, HF = Heart failure, NYHA = New York Heart Association Functional Classification, LVEF = Left Ventricular Ejection Fraction, DCM = Dilated cardiomyopathy, HCM = Hypertrophic cardiomyopathy, MI = Myocardial infarction, CABG = Coronary artery bypass surgery.

Because there is no universal cell surface marker for fibroblasts, a combination of markers was used. Cells were considered fibroblasts if they were negative for the endothelial marker CD31 and positive for the fibroblast marker THY1/CD90 [17] or if they were negative for the endothelial marker CD31 and positive for PDGFRα, which has emerged as a promising marker for cardiac fibroblasts in adult tissue [18,19]. The total number of cardiac fibroblasts were determined by the sum of the CD31-CD90+ and CD90-CD31-PDGFRα+ populations. The CD31+ CD90+ and CD31-CD90- populations were not considered fibroblasts.

### 2.4. Analysis of protein expression

Levels of cytokines and chemokines were measured in cell culture medium using multiplex electrochemiluminescence immunoassays (ELISA) (Meso Scale Diagnostics (MSD), Rockville, MD, USA). The human V-PLEX Pro-inflammatory Panel 1 Human Kit for the quantitative determination of interferon gamma (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13 and tumour necrosis factor (TNF)-α, and V-PLEX Plus Chemokine Panel 1 Human Kit for the quantitative determination of eight CC chemokines, including eotaxin, MIP-1β, eotaxin-3, thymus- and activation-regulated chemokine (TARC), macrophage inflammatory protein (MIP)-1α, monocyte chemoattractant protein (MCP)-1, macrophage-derived chemokine (MDC), and MCP-4, as well as two CXC chemokine assays for interferon gamma-induced protein (IP)-10 and IL-8 were applied. Intensity of the emitted light was measured according to the manufacturer's instructions on an MSD QuickPlex SQ120 plate reader (Meso Scale Diagnostics).

### 2.5. Statistics

Data were plotted as the mean and standard error of the mean (SEM) unless stated otherwise. To obtain a normal distribution, data were log transformed prior to statistical analysis. Multivariate analyses were carried out by principal component analysis (PCA) using SIMCA v. 14.1 (Umetrics, Umeå, Sweden). Goodness of fit was used to assess explained variation (R2X), while reproducibility of the model was assessed by cross validation (Q2X). A R2X value of 1 represents a model that perfectly explains all variation while a Q2X value of 1 represents a model that is perfectly reproducible. To determine which factors that were most important to segregate the observed clusters in the PCA analysis, an orthogonal partial least square discriminant analysis (OPLS-DA) model was applied. Levels of significance for differences between group means were determined with two-way ANOVA followed by Tukeýs multiple comparison tests. These calculations were carried out using SPSS v. 24 (IBM, New York, NY, USA). Graphs were created using GraphPad Prism version 6 (GraphPad Software, San Diego California USA, www.graphpad.com).

## 3. Results

### 3.1. Increased levels of cytokines and chemokines in human primary cardiac fibroblasts cultured under inflammatory conditions

To determine cellular composition after expansion, cells from one donor heart (control) were expanded and analysed by FACS. On average, the fibroblasts population constituted approximately 90% of the expanded non-myocytes (Suppl. Fig. 1).

Human cardiac fibroblasts were subjected to hypoxic and LPS treatment respectively. Levels of cytokines and chemokines were then measured in the culture media. Principal component analysis (PCA) showed a clear separation of the LPS-treated samples from untreated samples and hypoxic samples (Fig. 1a, Suppl. Fig. 2a, d). LPS treatment resulted in an increase in all cytokines/chemokines with the exception of IL-4 and MDC (Fig. 1b). Interestingly, a further separation of the LPS group into two subgroups was seen (Fig. 1a). No clear separation of the hypoxia-treated and untreated samples was observed. To confirm these findings, two-way ANOVA was carried out for each of the included cytokines/chemokines (Fig. 2). Taken together, the LPS group had a significantly higher production of most of the analysed cytokines/chemokines compared to control samples and hypoxia-treated samples. Only production of IL-4 was significantly lower in the LPS group compared to the two other groups. No significant differences were observed between the control samples (no LPS, normoxic conditions) and hypoxia-treated samples.

### 3.2. Similar response in cytokine and chemokine production in fibroblasts from all compartments of the heart with a high degree of inter-patient variation

To investigate whether the two observed subgroups of LPS-treated samples (Fig. 1a) represented different compartments of the heart, the PCA analysis was colour coded according to compartment. However, no clear separation was observed (Fig. 3a, Suppl. Fig 2b). In contrast, when samples were colour coded according to patient, a clear clustering was observed both in the LPS samples as well as the control/hypoxic samples (Fig. 3b, Suppl. Fig. 2c).

### 3.3. Patients suffering from idiopathic dilated cardiomyopathy may have a distinct cytokine profile in response to LPS stimulation

To further investigate whether the two LPS subgroups might represent different groups of patients, patients were colour coded according to cause of heart failure (Fig. 3c). Interestingly, the two patients in group 1 were both suffering from idiopathic dilated cardiomyopathy (DCM), whereas the three subjects in group 2 suffered from ischaemic DCM, hypertrophic cardiomyopathy (HCM) or had no known history of HF (donor heart). To illustrate which cytokines/chemokines differed the most between the two LPS subgroups, an OPLS DA model was tested using only samples from the two groups. The model showed a high degree of explained variation (R2X) as well as reproducibility as measured by cross validation (Q2X) (Fig. 3d). Concentrations of several cytokines/chemokines were higher in Group 1 with significant coefficients for MCP-4, IFN-γ, IL-12 p70, TARC, MDC, IL-2, IL-13, and IL-10 (Fig. 3d). For group 2, the concentrations of IL-6, MCP-1 and MIP-1β were higher compared to group 1 with significant coefficients (Fig. 3d). Taken together, this indicates that Group 1 and Group 2 respectively, are quite homogenous in terms of cytokine expression profile. Several cytokines with distinct differences in expression levels between the two groups could be observed. Observational data suggest that the group subdivision may be a result of different clinical background, with fibroblasts from patients suffering from idiopathic DCM having a distinct cytokine profile.

## 4. Discussion

In this study, we demonstrated that inflammatory activation of human cardiac fibroblasts by LPS leads to increased secretion of several cytokines and chemokines. Furthermore, we observed that patients suffering from idiopathic dilated cardiomyopathy seem to have a cytokine response profile that is distinct from other groups of research subjects. Our results also show that cytokine and chemokine secretion from human cardiac fibroblasts were unaffected by hypoxia.

Inflammation has been described in the setting of acute myocardial infarction and the early healing process. However, much less is known regarding inflammatory mechanisms in the setting of established cardiac heart disease [7,20]. Cardiac fibroblasts have traditionally been regarded as responsible for production of extracellular matrix proteins [21]. More recently, they have also been shown to play an important role in cardiac inflammation by secreting various pro-inflammatory cytokines and chemokines [2,22]. Most studies, however, have been focused on inflammatory signalling in cardiomyocytes [7,23]. To the

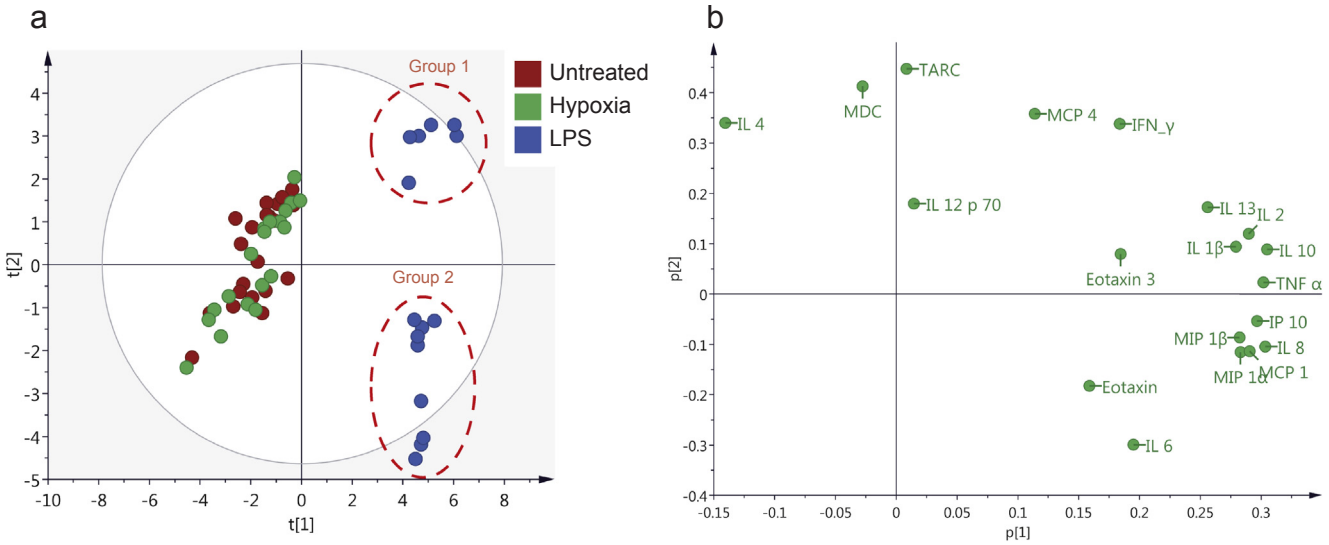


**Fig. 1.** Principal Component Analysis (PCA) analysis of cytokine and chemokine levels. (a) PCA Score plot of the first two components showing a clear separation of LPS-treated samples. Furthermore, the LPS-treated samples were further subdivided into two distinct groups (indicated in the figure as group 1 and group 2). (b) PCA loading plot showing the distribution of variables.

best of our knowledge, this study is the first in which a cytokine/chemokine profile of primary cultured fibroblasts from all four locations in the human heart have been investigated in patients suffering from CHF. In addition, we were able to include tissue from one organ donor with no known history of HF. After inflammatory induction by LPS, we observed a marked up-regulation of most of the analysed cytokines/chemokines with the exception of IL-4 and, to a lesser extent, MDC.

In addition of secreting pro-inflammatory markers such as Il-1 beta, TNF-alfa and IL-6, activated cardiac fibroblasts also secreted several markers for recruitment of immune cells. For example, levels of macrophage chemoattractant protein (MCP)-1 and macrophage inflammatory proteins MIP-1α and MIP-1β were increased after LPS stimulation. These molecules are known to attract and activate human mononuclear immune cells, which are an important part of the inflammatory tissue response [24]. Furthermore, levels of eotaxins were increased after LPS stimulation. These chemokines are potent chemoattractants for eosinophils and increase eosinophil recruitment. Interestingly, it has previously been shown that both macrophages and fibroblasts produce eotaxins and the signalling pathway controlling eosinophil trafficking was identified in the inflamed heart [25]. The pathogenesis of HF may be associated with inflammatory fibroblast activation and eosinophil trafficking within heart tissue. In addition, IL-4 is a well-known inducer of M2 polarization of macrophages that is associated with tissue repair. The decrease in IL-4 expression after inflammatory activation of cardiac fibroblasts in our study could thus be interpreted as further evidence of a pro-inflammatory cytokine profile associated with adverse tissue remodelling.

Notably, in the present study, LPS treatment was used as a model to induce an inflammatory response. LPS is present on gram negative bacteria, and its signalling is mainly mediated by binding to the TLR4 receptor [14,15]. To this date, ten different TLRs have been identified in the human [26]. These are all important for inducing an inflammatory response, by recognition of either pathogen associated molecular patterns (PAMPs) such as LPS or damage-associated molecular patterns (DAMPs) resulting from damage of host cells. Increased levels of DAMPs have been observed in CHF [26], and it could be speculated that these would elicit a fibroblast cytokine response similar to what has been observed in the present study. For future studies, it would thus be of great interest to further study the effects of DAMPs on cardiac fibroblasts. This has unfortunately been beyond the scope of the present study.

Unexpectedly, our data show that fibroblasts from different compartments of the human heart produce similar levels of both cytokines and chemokines. This was regardless of LPS treatment. In contrast, it has previously been suggested that fibroblasts from different anatomical locations have different properties [27,28]. In addition, several differentially expressed genes were observed when ventricular tissue was compared with atrial tissue along with higher expression of pathways related to immunity and defences in the right atrium [10]. Based on our data, it could be speculated that these differences are caused by different cellular compositions between different compartments of the heart rather than differences in fibroblast properties. This might include compartment-specific cross-talk between fibroblasts and other cell types, a property that naturally would be lost when separating the fibroblasts from other cell types in cell culture.

In our study, hypoxic stimulation of human cardiac fibroblasts did not result in any clear changes in the cytokine profile. Few previous studies have investigated the direct effects of hypoxia on human cardiac fibroblasts. In one study, however, hypoxic stimulation was found to increase the production of IL-1β and TGF-β, but only when combined with mechanical stretch. Notably, no increase was observed in the levels of pro-inflammatory cytokines, such as TNF-α and IL-6 [29]. It could thus be speculated that hypoxia alone is not enough to elicit an inflammatory cytokine response in fibroblasts in, for example, a myocardial infarction event. Instead, other factors, such as mechanical stimulation and signalling via DAMPs, may be necessary.

PCA analysis revealed clustering of the LPS-treated samples into two distinct groups. Based on colour coding of the samples according to different factors, it could be speculated that the basis for this clustering may be disease background, as patients suffering from idiopathic DCM clustered separately from all other patients and the control donor heart. Although the statistical model showed both high degree of explained variation as well as reproducibility as measured by cross validation, it should be noted that due to the very small number of patients, this has to be verified in future larger studies.

It should be noted that in the PCA analysis, the control donor heart was not distinctly separated from patients suffering from HF. With the exception of clustering of LPS treated samples, described above, the donor heart responded similar to LPS and hypoxic stimuli compared to HF patients. Although it should be acknowledged that it is hard to draw general conclusions based on only one donor heart, these results indicate that cultured fibroblasts from healthy hearts respond with a

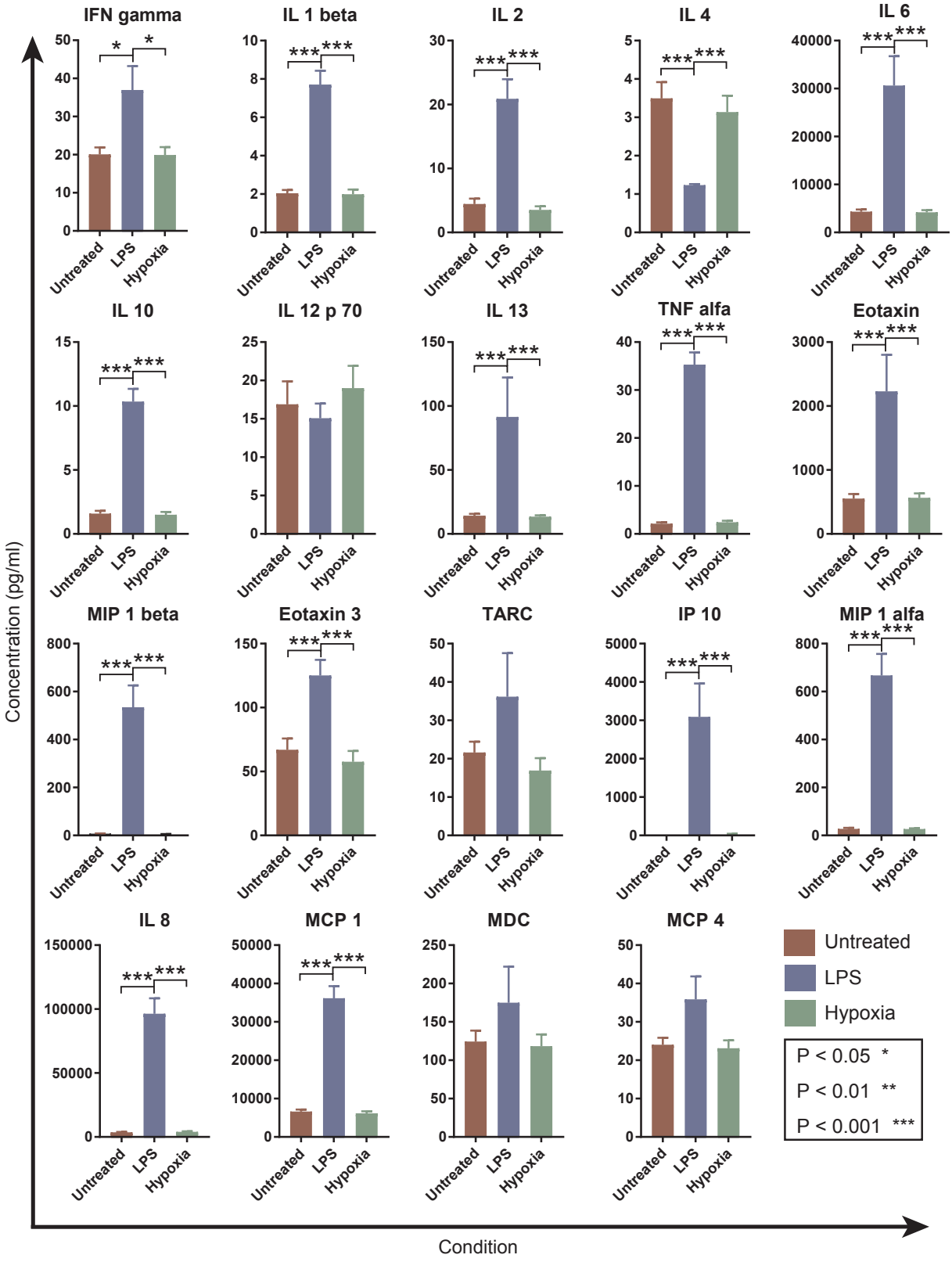


**Fig. 2.** Graphs showing the mean expression levels for different conditions. Significance was determined using two-way ANOVA for chemokine and cytokine levels with condition and compartment of the heart as fixed factors and research subject as the random block factor. For several of the analysed cytokines/chemokines, the condition was determined to be a significant factor and post hoc analysis showed a significant difference between the LPS group and the two other groups. The compartment of the heart was not found to be a significant factor for any of the analysed cytokines/chemokines.

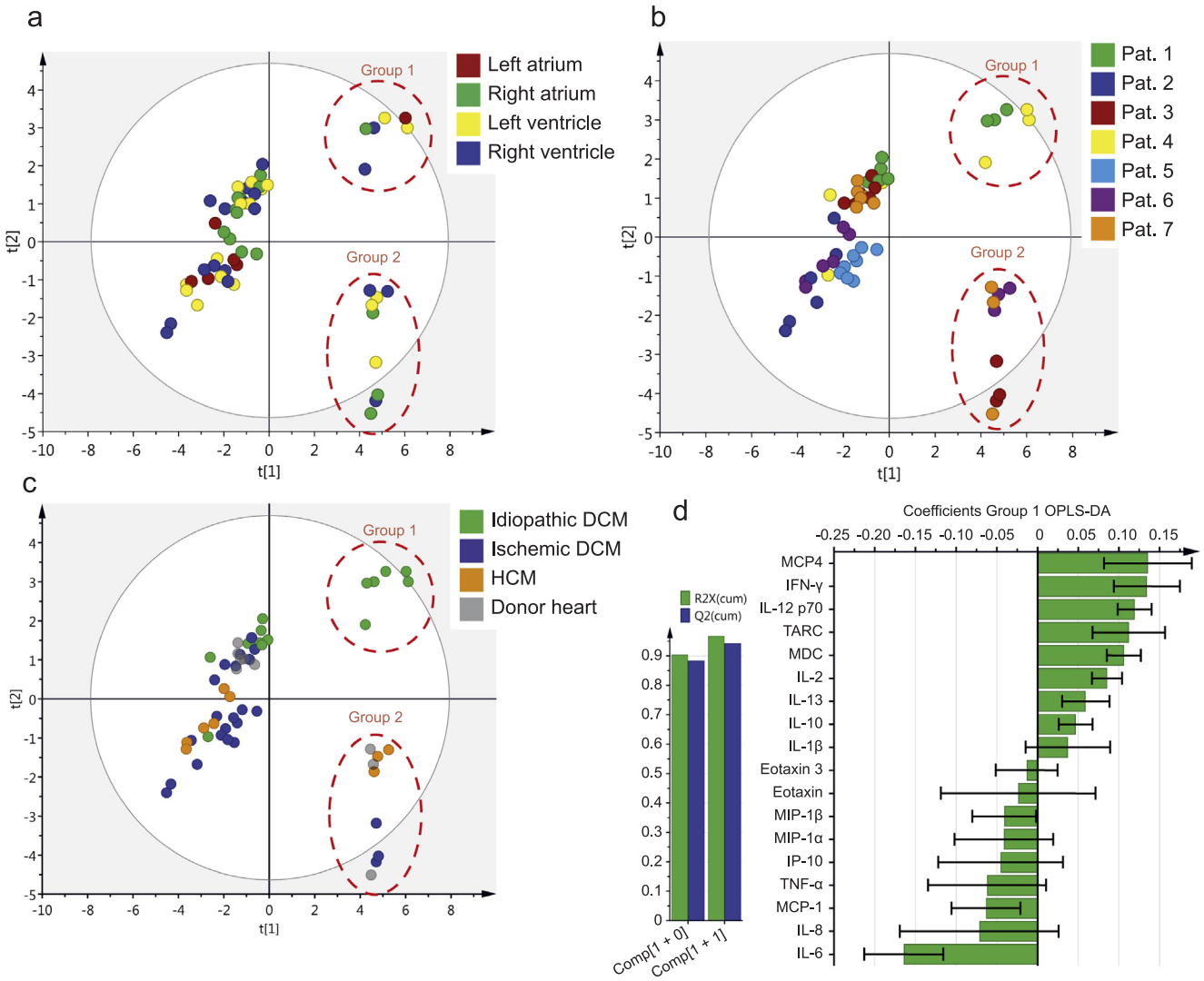


**Fig. 3.** Principal Component Analysis (PCA) score plots of the analysed cytokines/chemokines (first two components) with different colour codes to elucidate possible reasons for the observed clustering of LPS-treated samples. (a) Colour codes according to the compartment of the heart. (b) Colour codes according to patient. (c) Colour codes according to disease background. (d) Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) model of group 1 and group 2 samples to determine which cytokines/chemokines that contributed significantly to the separation of the two groups. Cytokines/chemokines with coefficients $> 0$ were more highly expressed in group 1, whereas cytokines/chemokines with coefficients $< 0$ were more highly expressed in group 2.

similar cytokine profile upon hypoxic and pro-inflammatory stimuli, compared to fibroblasts from hearts suffering from HF.

Some limitations of the present study should also be acknowledged. Due to the severe limitation in the number of cardiac transplantation patients and donors who were suitable for enrolment in the study, the sample size is small. To verify the results from the study, especially regarding the differences between the cytokine profiles of idiopathic DCM patients and other patients, further studies will be necessary. Nevertheless, it is worth emphasizing that the tissue material in this study is unique, since human heart tissue for isolating cardiac cells is generally very hard to obtain, particularly from all four chambers of the same heart.

In conclusion, this study shows that cytokine and chemokine levels are markedly increased in inflammatory-activated human cardiac fibroblasts by LPS, whereas hypoxic stimulation did not result in any clear cytokine/chemokine response. Fibroblasts from all compartments of the heart displayed a similar response. In the inflammatory-activated fibroblasts, two subgroups of patients were noted that need to be investigated further. The pronounced effects on inflammatory markers in cardiac fibroblasts suggest that these cells play an important role in inflammation associated with the ethology of cardiac diseases.

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## Author contributions

Conceived and designed the experiments: JS, MS AL, and LMH. Contributed reagents/materials and analytic tools: JS, AJ, AL, LMH. Performed the experiments: AL, MS, MJ, JS, LMH. Analysed the data: JS, MS, VRS, AL, LMH. All authors contributed to the writing and editing of this manuscript.

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## Conflict of interest

None.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.09.021.

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