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Cardiac Gene Expression Profiling Provides Evidence for Cytokine Pathway as a Molecular Mechanism in Chagas' Disease Cardiomyopathy

Edecio Cunha-Neto,^{*†‡} Victor J. Dzau,[§]
Paul D. Allen,[§] Dimitri Stamatiou,[§]
Luiz Benvenutti,[¶] M. Lourdes Higuchi,[¶]
Natalia S. Koyama,^{||} Joao S. Silva,^{||} Jorge Kalil,^{*†‡}
and Choong-Chin Liew[§]

From the Laboratory of Immunology and the Pathology Service,[¶] Heart Institute (InCor), University of São Paulo, São Paulo, Brazil; the Division of Clinical Immunology and Allergy,[†] University of São Paulo School of Medicine, São Paulo, Brazil; the Department of Immunology,^{||} University of São Paulo School of Medicine—Ribeirão Preto, São Paulo, Brazil; the Institute for Investigation in Immunology/Millennium Institutes,[‡] São Paulo, Brazil; and Brigham and Women's Hospital,[§] Harvard Medical School, Boston, Massachusetts*

Chronic Chagas' disease cardiomyopathy is a leading cause of congestive heart failure in Latin America, affecting more than 3 million people. Chagas' cardiomyopathy is more aggressive than other cardiomyopathies, but little is known of the molecular mechanisms responsible for its severity. We characterized gene expression profiles of human Chagas' cardiomyopathy and dilated cardiomyopathy to identify selective disease pathways and potential therapeutic targets. Both our customized cDNA microarray (Cardiochip) and real-time reverse transcriptase-polymerase chain reaction analysis showed that immune response, lipid metabolism, and mitochondrial oxidative phosphorylation genes were selectively up-regulated in myocardial tissue of the tested Chagas' cardiomyopathy patients. Interferon (IFN)- γ -inducible genes represented 15% of genes specifically up-regulated in Chagas' cardiomyopathy myocardial tissue, indicating the importance of IFN- γ signaling. To assess whether IFN- γ can directly modulate cardiomyocyte gene expression, we exposed fetal murine cardiomyocytes to IFN- γ and the IFN- γ -inducible chemokine monocyte chemoattractant protein-1. Atrial natriuretic factor expression increased 15-fold in response to IFN- γ whereas combined IFN- γ and monocyte chemoattractant protein-1 increased atrial natri-

uretic factor expression 400-fold. Our results suggest IFN- γ and chemokine signaling may directly up-regulate cardiomyocyte expression of genes involved in pathological hypertrophy, which may lead to heart failure. IFN- γ and other cytokine pathways may thus be novel therapeutic targets in Chagas' cardiomyopathy. (Am J Pathol 2005, 167:305–313)

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a significant cause of morbidity and mortality in Central and South America, affecting ~9 million people.¹ Approximately 30% of Chagas' disease patients develop chronic Chagas' disease cardiomyopathy (CCC), a particularly aggressive inflammatory dilated cardiomyopathy that occurs decades after the initial infection. Clinical progression and survival are significantly worse in CCC patients as compared with patients with dilated cardiomyopathy (DCM) of other etiologies.^{2,3} Significantly, myocarditis is found in 93% and 8% of biopsies from CCC and DCM samples, respectively.^{4,5}

CCC heart lesions show histopathological findings consistent with inflammation and a myocardial remodeling process: T cell/macrophage-rich myocarditis, hypertrophy, and fibrosis with heart fiber damage.⁴ Increased local expression of the cytokines interferon-(IFN)- γ and tumor necrosis factor- α ,^{6,7} interleukin (IL)-6 and IL-4,⁶ as well as HLA class I and II molecules, and adhesion molecules were reported.⁸ Autoimmune inflammation may play a role in the myocarditis of CCC.⁹ In murine models, local production of tumor necrosis factor- α , IFN- γ , and IFN- γ -induced chemokines monokine in-

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Address reprint requests to Dr. C.C. Liew, Brigham and Women's Hospital, 77 Louis Pasteur Ave., NRB room 0630K, Boston, MA. E-mail: cliew@rics.bwh.harvard.edu.

duced by IFN- γ (MIG), regulated on activation normal T cell expressed and secreted (RANTES), IFN- γ -inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 α (MIP-1 α) was reported.¹⁰ CCC patients display up-regulated production of IFN- γ in comparison to asymptomatic *T. cruzi*-infected individuals,^{7,11} and up-regulated production of IFN- γ in *T. cruzi*-infected IL-4 $^{-/-}$ mice enhanced late-phase myocarditis,¹² suggesting a pathogenic role for IFN- γ . Together, these observations suggest that chronic myocardial inflammation could contribute to CCC pathogenesis. Accordingly, we hypothesized that the increased aggressiveness of CCC may be due to the activation of specific disease pathways as reflected by a distinct heart gene expression profile. Expression profiling studies were performed in hearts of mice 3 months after *T. cruzi* infection, identifying up-regulation of MHC class I and class II genes, cytokines, chemokines, and receptors, among other genes.^{13,14} However, there has not yet been a comparable study using human patients.

In this study, we used a 10,386-element cDNA microarray, built from cardiovascular cDNA libraries, in combination with real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis to develop the first gene expression profile of CCC using human heart tissue. We compared the gene expression fingerprint of CCC with that of DCM, using nonfailing adult hearts as expression controls, and found that gene expression patterns are markedly different in CCC and DCM, with significant activity of IFN-inducible genes in CCC patients. Gene expression changes of cultured cardiomyocytes in response to IFN- γ and MCP-1 were also studied. These results provide intriguing clues to the molecular mechanisms of this disease and may suggest future targets for specific therapy.

Materials and Methods

Isolation of RNA

RNA samples for the cDNA array analysis came from explanted left ventricular-free wall heart samples from five CCC (serological diagnosis, positive epidemiology) and seven DCM (dilated cardiomyopathy in the absence of ischemic disease, negative epidemiology) end-stage heart failure patients was obtained at transplantation (Table 1). Normal adult heart tissue from the left ventricular-free wall was obtained from four nonfailing donor hearts not used for cardiac transplantation due to size mismatch with available recipients. CCC and DCM patients had been treated with standard heart failure therapy with angiotensin conversion enzyme inhibitors, loop diuretics, and β -blockers; none were receiving amiodarone or milrinone at the time of sample collection. CCC patients were not under anti-trypanosomal treatment. To avoid inclusion of samples undergoing clinicopathological changes secondary to long-standing brain death in the normal donor heart control group, we excluded samples displaying up-regulation of cardiac hypertrophy genes (natriuretic peptides, α -cardiac actin, myosin light

chain-2) greater than twofold in comparison to average expression of each gene in all normal donor heart samples. RNA from endomyocardial biopsy samples from four end-stage CCC patients were also obtained and used for the real-time RT-PCR experiments. Total RNA was isolated with the TRIzol reagent (Life Technologies Inc., Grand Island, NY); we determined RNA quantity by $A_{260/280}$ measurement and assessed RNA integrity by formaldehyde gel electrophoresis. The protocol was approved by the institutional review boards of University of São Paulo School of Medicine, São Paulo, SP, Brazil, and Brigham and Women's Hospital, Boston, MA. Informed consent was obtained from donors. Characteristics of patients and normal donors whose samples were used in this study are described in Table 1.

Construction of a Spotted cDNA Microarray

We spotted 10,368 nonredundant PCR products from a human heart cDNA library (including 96 bacterial clones as negative controls) onto Corning CMT-GAPS amino-silane-coated glass microarray slides (Corning Inc., Corning, NY) using the GMS 417 arrayer (Affymetrix, Santa Clara, CA), and postprocessed the slides according to the manufacturer's manual.¹⁵ The unique clones include 2496 known genes (24.3%), of which 1713 are classified according to function, 3296 matched expressed sequence tags (32.1%), and 4480 novel genes (43.6%), as previously described.¹⁵

Synthesis of cDNA Probes and Subsequent Hybridization

We followed procedures previously described for probe generation by RT-PCR and hybridization.¹⁶ Universal Reference RNA (Stratagene, Cedar Creek, TX) was used as a control for each slide. We converted 10 μ g of RNA from each RNA sample to a cDNA probe via reverse transcriptase (RT)-PCR—each RNA sample was oligo-dT primed and then added to a master mix containing nucleotides, the reverse transcription enzyme, corresponding buffers, and Cy3- or Cy5-dUTP fluorescent dye (Amersham Biosciences, Piscataway, NJ). Cy5-labeled probes were always made from myocardial sample RNA, and Cy3-labeled probes were always made from Universal Reference RNA. After the RT-PCR reaction, corresponding pairs of Cy3- and Cy5-labeled probes were mixed and applied to the arrayed slides; after hybridization, slides were scanned using the GMS 418 array scanner (Affymetrix). We considered hybridization experiments from the five distinct CCC patients, as well as the seven distinct DCM patients, as biological replicates.

Image Acquisition and Data Processing

We processed newly-scanned images using ScanAlyze v2.44 (Michael Eisen, Stanford University, Stanford, CA; <http://rana.lbl.gov/eisensoftware.htm>). We used 96 spots representing bacterial clones as a negative control.

Table 1. Characteristics of Patients and Normal Heart Donors Whose Samples Were Used in This Study

Internal no.	Age (years)	Sex	Disease group	Nature	Experiment
CCC patients					
CCC2	47	F	End-stage CCC	Explant (LVFW)	cDNA array
CCC5	53	M	End-stage CCC	Explant (LVFW)	cDNA array
CCC1	49	M	End-stage CCC	Explant (LVFW)	cDNA array, RT-PCR
CCC3	27	M	End-stage CCC	Explant (LVFW)	cDNA array, RT-PCR
CCC4	63	F	End-stage CCC	Explant (LVFW)	cDNA array, RT-PCR
CCC6	52	M	End-stage CCC	Endomyocardial biopsy	RT-PCR
CCC7	51	M	End-stage CCC	Endomyocardial biopsy	RT-PCR
CCC8	49	M	End-stage CCC	Endomyocardial biopsy	RT-PCR
CCC9	50	M	End-stage CCC	Endomyocardial biopsy	RT-PCR
DCM patients					
DCM1	39	F	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
DCM2	23	F	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
DCM3	50	M	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
DCM4	45	F	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
DCM5	38	M	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
DCM6	45	M	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
DCM7	26	F	End-stage DCM	Explant (LVFW)	cDNA array, RT-PCR
Normal samples*					
N1	38	F	Normal heart donor	Explant (LVFW)	cDNA array, RT-PCR
N2	24	M	Normal heart donor	Explant (LVFW)	cDNA array, RT-PCR
N3	16	M	Normal heart donor	Explant (LVFW)	cDNA array, RT-PCR
N4	38	F	Normal heart donor	Explant (LVFW)	cDNA array, RT-PCR

Samples were collected between June 1992 and January 2001. LVFW, left ventricle free wall; explant, surgical sample obtained during heart transplantation; endomyocardial biopsy obtained pre-transplantation. Only explant samples were used for cDNA array. Some explant samples were used for cDNA array and quantitative real-time RT-PCR expression analysis. Heart failure patients received standard therapy with angiotensin conversion enzyme inhibitors, loop diuretics and beta-blockers.

*Normal heart donors were subject to ventilator and vasoactive drugs, and had been under life support for an average of 48 hours (range, 24 to 96 hours).

Spots with net signal intensities that were less than twice the median intensities of the bacterial clones on both channels were eliminated to exclude nonspecific binding.

Bioinformatics

Data normalization and analysis was performed using GeneSpring version 6.0 (Silicon Genetics, Redwood City, CA). Expression levels were first normalized relative to the other genes in the same sample—each measurement was divided by the 50th percentile of all measurements in that sample. In addition, disease samples were normalized to normal control samples; when calculating the mean of the control measurements only those measurements were included that had at least a normalized value of 0.01. After normalization, samples in each disease group (CCC, DCM, normal) were then treated as replicates and averaged. In this way, we obtained a final expression ratio for each gene in CCC and DCM, relative to normal controls. A gene was considered to be significantly up- or down-regulated if the average sample/control expression ratio in a disease group was both 1.5-fold higher or lower than the average expression values in the control samples, and the difference between patient group and control expression values passed statistical significance ($P < 0.05$). We performed hierarchical clustering analysis on the filtered microarray data using GeneSpring version 6.0 (Silicon Genetics), using nonsupervised clustering and correlation by up-regulated expression, with default parameters. We considered the median Cy5/Cy3 intensity ratio for each spot

to be its intensity value for analytical purposes. Genes of known function in the Cardiochip version 6.0 cDNA microarray were classified into functional categories (cell division, cell signaling/communication, cell structure/motility, cell/organism defense, gene/protein expression, metabolism) and related subcategories, as described before.^{17,18}

Real-Time Reverse Transcriptase(RT)-PCR Analysis

We designed forward and reverse primers for quantitative real-time RT-PCR to confirm expression patterns or to analyze genes not present in the array: IL-7 receptor, IFN- γ receptor, atrial natriuretic factor (ANF), human leukocyte antigen (HLA)-DR, chemokines stromal cell-derived factor-1 (SDF-1), RANTES, MCP-1, IP-10, MIG, and chemokine receptors (CCR1, CCR2, CXCR3, CXCR4, CCR5) using the Primer3 internet site (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi>). Primers were 19 to 21 bases long, had a melting point of 59 to 61°C, generating PCR products of 100 to 150 bp. Using RNA samples from CCC, DCM, and donor control heart used in cDNA array analysis as well as additional endomyocardial biopsy samples of CCC samples, we performed 50- μ l real-time RT-PCR reactions in 96-well plates, using SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA), in the Perkin-Elmer ABI Prism 7700 sequence detection system. (15 seconds at 94°C and 1 minute at 60°C) as described.¹⁵ Normalization and fold change were calculated with the $\Delta\Delta$ Ct method with

Figure 1. Cluster analysis of gene expression (Cardiochip version 6.0 microarray data) in the CCC, DCM, and N left ventricular-free wall heart tissue mRNA samples. Data reveals CCC- and DCM-specific global gene expression profiles, with disease-specific and shared clusters of up- or down-regulated genes. Each individual column stands for the gene expression pattern of an individual heart mRNA sample belonging to CCC, DCM, or N groups; green dendrograms on the **top** of the figure represent degrees of similarity of gene expression among samples. Each **horizontal line** (y-axis) corresponds to the expression of a single gene across all samples. Gene expression clusters (green dendrograms to the **left** of the figure) represent relatedness of expression patterns among the 10,368 genes across several samples and patient groups. Expression values for each gene in each sample in relation to average control values normal tissue samples are expressed in a color scale (**right** of figure): yellow, unchanged; orange-red, up-regulated; blue, down-regulated. Nonsupervised hierarchical clustering performed with Genespring version 6.0, using correlation by up-regulated expression with default parameters, of microarray results for five CCC, seven DCM, and four normal heart tissue samples. Samples from each disease group are marked to facilitate their identification, with red, green, and white, standing for samples for CCC, DCM, and normal donor heart, respectively.

GAPDH as the reference mRNA species according to ABI Prism 7700 manufacturer's instructions. Statistical significance was defined by $P < 0.05$ using two-tailed Student's *t*-test. Real-time RT-PCR was also used to investigate the effect of cytokines in cardiomyocyte gene expression. Heart cell cultures from hearts of 20-day-old embryonic mice were prepared as described,¹⁹ incubated with IFN- γ (100 U/ml; R&D Diagnostics, Minneapolis, MN) and/or MCP-1 (10 ng/ml; Peprotech, Rocky Hill, NJ) for 8 hours before RNA extraction; primers for murine ANF were designed as described above.

Results

Cluster Analysis

When we subjected gene expression data obtained on the Cardiochip microarray to hierarchical cluster analysis (Figure 1), all of the CCC, and most of the DCM and normal heart samples (Figure 1, individual columns; green dendrograms on the top) clustered together with other members of their groups, with a single DCM patient

Figure 2. Venn diagram, CCC-specific, DCM-specific, and shared classified, nonredundant genes of known function in end-stage heart failure samples, as assessed by cDNA microarrays. Selected genes and gene groups (in bold-face) are listed in **boxes** below each area. **A:** Genes up-regulated ≥ 1.5 -fold, $P < 0.05$. **B:** Genes down-regulated ≥ 1.5 -fold, $P < 0.05$.

clustering with a normal heart sample. This showed the consistency of disease-specific gene expression. Gene expression clusters (Figure 1, lines; green dendrograms to the left) specific to CCC, as the up-regulated cluster on the top, or the down-regulated cluster toward the bottom of the figure, along with gene clusters shared between CCC and DCM or DCM-specific, were clearly demonstrated.

Differential Gene Expression Profiles in CCC, DCM, and Normal Heart Tissue

Using a 1.5-fold and $P < 0.05$ cutoff, 582 and 364 genes were significantly up-regulated in CCC and DCM, respectively, including 126 genes that were up-regulated in both conditions. Similarly, 465 genes were significantly down-regulated in CCC and 32 in DCM. We classified differentially expressed genes of known function into subcategories as mentioned in Material and Methods (Figures 2 and 3; and in Supplemental Material at <http://ajp.amjpathol.org>).

Among genes selectively up-regulated in CCC, we observed that the participation of cell defense and metabolism categories was substantially higher than the frequency of representation that categories in the gene array (Figure 2A and Figure 3A). Furthermore, 15 of 16 (94%) cell defense genes up-regulated specifically in CCC are immune response genes, whereas the propor-

Figure 3. A: The percentage of known genes in each functional category that are up-regulated exclusively in myocardial samples from CCC, DCM, or shared by both diseases. **B:** Percentage of down-regulated known genes in each functional category in CCC. Representation of genes in each category among up-regulated genes in a patient group was calculated as follows: number of up-regulated genes in the category/total number of known up-regulated genes in that patient group. The same was performed for each patient sample, for the shared gene list, and for the down-regulated CCC gene list.

tion of immune response genes among cell defense genes in the gene array is 49%. Among these genes, we found the MHC class I/class II molecules, IFN- γ receptor, IL-7 receptor, T-cell antigen receptor, immunoglobulin chains, cytotoxic granule nucleolysin, and IFN-inducible genes (Supplemental Material, <http://ajp.amjpathol.org>). Nineteen of the up-regulated metabolism genes were related to energy metabolism, including seven proteins of the oxidative phosphorylation and proton transfer chains, and eight enzymes involved in lipid metabolism. Up-regulated structural genes comprise sarcomere-building contractile and cytoskeletal proteins, and collagens I and III. Signaling genes already associated to heart failure,^{20,21} including angiotensin II receptor 2, G protein β polypeptide 2, several low-molecular weight GTPases such as rhoB, RAB1, RalB, along with IKK- α and mitogen-activated kinase MKK4, protein kinase H11, and its induced anti-apoptotic gene Akt-1, were also up-regulated. We also found factors involved in protein/RNA synthesis, such as hypoxia-inducible factor 1 and TATA-box-binding protein-associated factor, to be up-regulated specifically in CCC samples.

In contrast, the proportion of immune response genes among genes up-regulated in DCM was similar to their

representation in the Cardiochip. Most genes selectively up-regulated in DCM belonged to the gene/protein expression category (13 genes, of which 8 encode ribosomal proteins); translation factors are also up-regulated. Of the 11 up-regulated metabolism genes, 5 belong to the glycolysis/sugar metabolism pathway, and none to the lipid metabolism pathway, consistent with known changes of energy metabolism in DCM.²² Several unique signaling elements were up-regulated: ryanodine receptor, phospholamban, ras-related proteins, G protein pathway suppressor 1, cAMP-dependent protein kinase, as described before for dilated cardiomyopathy.^{15,16,20} Structural genes include mainly cytoskeletal proteins and skeletal muscle tropomyosin. Thirty-one classified, known genes were up-regulated in both CCC and DCM (Figure 2). Structural genes, including cardiac myosin heavy chain, cardiac myosin light chain-2, several α -actin isoforms, smooth muscle myosin, actin-binding proteins, and collagen α XIII, comprised 35% of shared up-regulated genes, followed by protein/gene expression, metabolism (glycolysis/TCA cycle and carbohydrate energy metabolism), and signaling, including natriuretic peptide genes (ANF and brain natriuretic peptide precursor, ion channels, tyrosine kinase ZAP-70). Most of these genes comprise the fetal gene expression program characteristic of the hypertrophic response, and the expression of structural genes is associated to the downstream structural phase of the ventricular remodeling process, common to end-state dilated cardiomyopathy of several etiologies.^{16,23,24} Among down-regulated known genes (39 in CCC and 7 in DCM), only three are shared by both diseases, among them mitochondrial 16S rRNA (Figure 2, and in Supplemental Material at <http://ajp.amjpathol.org>). Most genes selectively down-regulated in CCC belong to the gene/protein expression, metabolism, and signaling categories (Figure 3B), including SERCA-2 Ca^{++} ATPase, G α q protein, Mek1, glutathione S-transferase M3, DNA ligase I, transferrin receptor, CREB2, cysteine proteinase inhibitor cystatin B. The finding that structural genes were basically not down-modulated is in line with the significant number of sarcomeric and cytoskeletal genes whose transcription is up-regulated (Supplemental Material, <http://ajp.amjpathol.org>).

Given the prominent local production of IFN- γ in CCC heart lesions,^{7,11} and because IFN-induced genes were up-regulated in CCC heart lesions, we identified 78 genes present in the Cardiochip cDNA microarray that were reported to be IFN- γ -inducible.^{25–28} Fifteen percent of the genes selectively up-regulated in CCC are IFN- γ -inducible (Figure 2, Table 2). Significantly, along with inflammatory response genes likely to be expressed by infiltrating inflammatory cells (eg, immunoglobulin, T-cell receptor genes, cytokine receptors), we observed several genes not known to be expressed by inflammatory cells (cardiovascular 27-kd hsp, angiotensin II receptor 2, fatty acid-binding protein 5). Of interest, the SERCA Ca^{++} -ATPase, involved in cardiac calcium metabolism and down-modulated in CCC hearts, is also repressible by IFN- γ . On the other hand, only two IFN- γ inducible genes, polyubiquitin UbC and IFN- γ -inducible mda-9

Table 2. Expression of IFN- γ -Inducible Genes in CCC and DCM Hearts

Fold	Common name	Accession no.
A: IFN- γ -inducible genes up-regulated in CCC		
Lymphoid/inflammatory genes		
6.1	Immunoglobulin κ light chain	D90158
2.1	Cytotoxic T cell granule nucleolysin TIAR	M96954
1.6	MHC class II HLA-DR α 11- β chain	M21966
Lymphoid and nonlymphoid genes		
2.7	Interferon stimulated ISG-54K gene	M14660
2.3	Interferon-inducible protein MX1	M30817
2.1	Major histocompatibility complex, class I (HLA-B)	M95531
2.0	Tropomyosin TM30 (nm), cytoskeletal	X04588
1.6	Interferon- γ receptor	J03143
1.6	Interferon consensus sequence DNA-binding protein	M91196
1.6	Interferon- γ inducible mda-9 protein*	AF006636
Non-lymphoid genes		
2.3	Fatty acid binding protein 5 (psoriasis-associated)	M94856
1.9	Angiotensin receptor 2 (AGTR2)	L34579
1.6	Cardiovascular 27 kd heat shock protein	R73707
B: IFN- γ down modulated genes, down-regulated in CCC		
3.1	SERCA-2 calcium-ATPase (hk2), cardiac	M23278
C: IFN- γ inducible genes up-regulated in DCM		
1.5	Polyubiquitin UbC	AB009010
1.9	Interferon- γ inducible mda-9 protein*	AF006636

*Up-regulated in both CCC and DCM.

protein, were up-regulated in DCM; mda-9 is also up-regulated in CCC.

To determine the expression of IFN- γ -inducible chemokines and chemokine receptors (important mediators of the inflammatory response absent from the cDNA array) we conducted real-time RT-PCR experiments, using seven CCC, four DCM, and three normal heart RNA samples. The expression levels of chemokine receptors CCR2, CXCR3, and the IFN- γ -inducible chemokines MCP-1, IP-10, and MIG were selectively up-regulated among CCC samples; the fact that only CCR5, SDF1, IP-10 reached statistical significance probably indicated microheterogeneity in inflammatory foci in the different samples (Figure 4A). We also used real-time RT-PCR to validate the microarray results of four genes up-regulated in CCC. All four genes (ANF, IL-7 receptor, HLA-DR, and IFN- γ receptor) were significantly up-regulated; IL-7 receptor and HLA-DR were selectively up-regulated among CCC samples (Figure 4B). The fold changes in RT-PCR were usually greater than those shown in microarray experiments for the same genes, as observed before by ourselves¹⁶ and others.^{29,30} Rajeevan and colleagues³⁰ observed fold values at SYBR real-time PCR more than 10 times larger than those observed at the microarray experiment, and argued that this could be due to cross-hybridization in microarrays and a more specific detection in the PCR reaction.

To assess whether IFN- γ and the IFN- γ -inducible chemokine MCP-1, also expressed in CCC heart lesions (Figure 3A) and involved in cardiomyopathy^{31,32} can directly modulate expression of pathogenetically relevant genes in cardiomyocytes, we exposed fetal murine cardiomyocytes to IFN- γ and/or MCP-1, using ANF expression as an index of fetal/hypertrophic gene expression. Expression of ANF increased 15-fold in response to IFN- γ , and the combination of IFN- γ and MCP-1 in-

creased ANF expression more than 400-fold compared to control values (Figure 4C).

Discussion

In this study of myocardial tissues from end-stage heart failure, CCC heart tissue displays a global gene expression profile notably distinct from that of DCM heart tissue, as observed from the clustering of samples according to disease type, the identification of disease-specific gene clusters (Figures 1 and 2), concordant with previous observations.^{15,16,24} We observed that the gene expression profiles of CCC and DCM showed some predictable similarities but differed dramatically in key areas. The hypertrophy/heart failure pattern (ie, up-regulation of expression of fetal contractile proteins and natriuretic peptides; sarcomere-building genes; myoglobin and tricarboxylic acid enzymes),^{20,33,34} is evident in both CCC and DCM; consistent with a hypertrophic and ventricular remodeling response to an intense ongoing stimulus. Together with the clustering analysis, the finding of an increased number of differentially expressed genes in CCC as compared to DCM myocardium may indicate that the gene expression profiles of CCC and DCM are qualitatively and quantitatively different. The finding of a significantly lower number of down-regulated than up-regulated genes in cDNA microarray analysis of idiopathic dilated cardiomyopathy has been previously observed by our own group and others.^{16,24} The fact that those reports used distinct data normalization and analysis (Lowess R on pooled samples,¹⁶ and Affymetrix GeneChip software²⁴) suggests that the lower number of down-regulated genes in DCM observed in our study has a true biological basis, rather than being an artifact.

Figure 4. Real-time RT-PCR analysis of selected genes in CCC or DCM myocardium. **A:** Chemokine/chemokine receptor genes. **B:** Genes identified by microarray analysis. **C:** Analysis of ANF expression in fetal cardiomyocytes after 8 hours of stimulus with MCP-1, IFN- γ and their combination. M, culture medium. Cardiomyocyte culture performed as described.¹⁷ In **A** and **B**, results are expressed as average fold change from normal heart (column) and SD (error bar). Asterisks, $P < 0.05$ versus normal. Human (**A**, **B**) or murine (**C**) GAPDH was used as the reference mRNA species, and GAPDH Ct values did not change significantly between disease categories or *in vitro* stimulus.

The observed overrepresentation of up-regulated genes in the cell defense category in CCC in comparison to DCM, or the proportion of defense genes in the gene array (Figure 2 and in Supplemental Material at <http://ajp.amjpathol.org>), is consistent with myocarditis, present in each of the tested CCC samples. These results are in line with the expression profiling experiments performed with *T. cruzi*-infected mice.^{13,14} The considerable proportion of IFN- γ -inducible genes found to be up-regulated specifically in CCC heart samples (Table 2) indicates significant signaling by IFN- γ , consistent with its prominent local production.^{6,7} Several such genes are not known to be expressed by inflammatory cells, suggesting the possibility that IFN- γ could directly modulate gene expression in cardiac cells. The finding that the SERCA-2 cardiac Ca⁺⁺-ATPase gene, involved in calcium metab-

olism and heart failure and down-modulated in CCC heart tissue (Table 2), was found to be repressible by IFN- γ ,²⁷ further supports the idea that this cytokine may directly modulate cardiomyocyte genes involved in heart failure and the hypertrophic phenotype. IFN- γ -inducible chemokines also had increased expression in CCC when compared to DCM or normal heart samples using real-time RT-PCR (Figure 4B), providing further confirmation of IFN- γ signaling. Although previous studies have suggested that MCP-1 and other chemokines can contribute to heart disease,^{31,32} and the proinflammatory cytokines tumor necrosis factor- α ³⁵ and IL-1 β ³³ can induce cardiomyocyte hypertrophy, we have demonstrated that IFN- γ , alone or in combination with MCP-1, can by itself induce increased expression of ANF in fetal cardiomyocytes (Figure 4C). Our study provides direct evidence for the first time that IFN- γ , a key T-cell-derived cytokine, can modulate cardiomyocyte gene expression. The induction of the natriuretic peptides is a feature of hypertrophy in all mammalian species and is a prognostic indicator of clinical severity,³⁴ and the demonstration that ANF is inducible by IFN- γ identifies the T-cell-dependent cytokine as a possible stimulus for cardiomyocyte hypertrophy. If this property of ANF inducibility by IFN- γ is indeed shared with the metabolically distinct adult cardiomyocytes, this could have an even more direct bearing on pathogenesis. The fact that CCC patients display an increased frequency of IFN- γ -producing T cells in peripheral blood^{7,11} may also contribute to the increased signaling in the heart.

We also noted that genes involved in lipid metabolism, oxidative phosphorylation, and the proton transfer chain are selectively up-regulated in CCC but not in DCM tissue, suggesting that the energy metabolism in CCC is distinct from the reported DCM pattern²² (Figures 2 and 3, and in Supplemental Material at <http://ajp.amjpathol.org>). Our group has recently shown that protein levels of creatine kinase, a key enzyme in the generation of cytoplasmic ATP, are reduced in myocardial tissue of CCC but not DCM patients.³⁶ The increase in oxidative phosphorylation and lipid metabolism observed in CCC samples (Figures 2 and 3, and in Supplemental Material at <http://ajp.amjpathol.org>) may be a compensatory mechanism for cytoplasmic ATP depletion, as observed in mice genetically deficient of creatine kinase.³⁷ Interestingly, IFN- γ treatment of cardiomyocytes reduced expression of creatine kinase,²⁷ inhibited oxidative metabolism,³⁸ and reduced ATP levels,³⁹ suggesting a possible link between IFN- γ signaling and energy metabolism changes observed in CCC. Moreover, expression profiling in hearts of mice infected by *T. cruzi* also showed diminished myocardial energetic metabolism and altered oxidative phosphorylation.^{13,14} Significantly, mice infected for 100 days showed morphological alterations in mitochondria and diminished expression of genes from the oxidative phosphorylation pathway, with a detectable reduction of OXPHOS-mediated mitochondrial ATP production.⁴⁰

In conclusion, our results indicate that gene expression patterns are markedly different between the CCC and DCM samples tested, suggesting the differential in-

involvement of disease pathways in their pathogenesis. Our data suggests, for the first time, that locally produced T-cell-dependent IFN- γ , MCP-1, and possibly other inflammatory cytokines may directly up-regulate the myocardial expression of ANF, a hypertrophy-related gene, in addition to inflammatory effector-mediated myocardial cell death. We postulate that this cytokine-induced gene modulation of myocardial gene expression may lead to the increased ventricular remodeling, morbidity, and mortality of CCC patients as compared to other cardiomyopathies. Although our results were obtained with myocardial samples from a small number of CCC patients, they shared a representative phenotype (significant myocarditis, hypertrophy, fibrosis) that is common to other end-stage CCC patients. It is likely that the unique properties of the myocardium from CCC patients sharing these features may be due to their dramatically altered energy metabolism. Our study may also have a bearing on other situations of T-cell-dependent myocardial inflammation, such as allograft rejection or viral myocarditis. Further investigation, including the testing of larger CCC patient sets to confirm the present findings, may suggest therapeutic and diagnostic approaches based on molecular rather than clinical portraits of cardiomyopathy.

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