Title
Blood Gene Signatures of Chagas Cardiomyopathy With or Without Ventricular Dysfunction

Journal
The Journal of Infectious Diseases, 215(3)

ISSN
0022-1899

Authors
Ferreira, Ludmila Rodrigues Pinto
Ferreira, Frederico Moraes
Nakaya, Helder Imoto
et al.

Publication Date
2017-02-01

DOI
10.1093/infdis/jiw540

Peer reviewed

The Journal of Infectious Diseases

MAJOR ARTICLE

Blood Gene Signatures of Chagas Cardiomyopathy With or Without Ventricular Dysfunction

Ludmila Rodrigues Pinto Ferreira,[1,3,6,7,a] Frederico Moraes Ferreira,[1,3,6,7,a] Helder Imoto Nakaya,[4,10] Xutao Deng,[11,12] Darlan da Silva Cândido,[1,3,6] Lea Campos de Oliveira,[5] Jean-Noel Billaud,[14] Marion C. Lanteri,[11,13] Vagner Oliveira-Carvalho Rigaud,[1,3,6] Mark Seielstad,[11,13] Jorge Kalil,[1,3,6] Fabio Fernandes,[2] Antonio Luiz P. Ribeiro,[8,9] Ester Cerdeira Sabino,[5,b] and Edecio Cunha-Neto[1,3,6,b]

[1]Laboratory of Immunology and [2]Cardiomyopathy Unit, Heart Institute, and [3]Division of Clinical Immunology and Allergy, School of Medicine, [4]Department of Pathophysiology and Toxicology, School of Pharmaceutical Sciences, and [5]Institute of Tropical Medicine, Department of Infectious Disease, University of São Paulo, [6]Institute for Investigation in Immunology, National Institute of Science and Technology, and [7]Universidade Santo Amaro, São Paulo, and [8]Hospital das Clínicas and [9]Faculdade de Medicina, Universidade Federal de Minas Gerais, Minas Gerais, Brazil; [10]Department of Pathology, Emory University School of Medicine, Atlanta, Georgia; and [11]Blood Systems Research Institute and [12]Department of Laboratory Medicine and [13]Institute for Human Genetics, Department of Laboratory Medicine, University of California, San Francisco, and [14]Department of Bioinformatics, Qiagen, Redwood City, California

Chagas disease, caused by the protozoan parasite *Trypanosoma cruzi*, affects 7 million people in Latin American areas of endemicity. About 30% of infected patients will develop chronic Chagas cardiomyopathy (CCC), an inflammatory cardiomyopathy characterized by hypertrophy, fibrosis, and myocarditis. Further studies are necessary to understand the molecular mechanisms of disease progression. Transcriptome analysis has been increasingly used to identify molecular changes associated with disease outcomes. We thus assessed the whole-blood transcriptome of patients with Chagas disease. Microarray analysis was performed on blood samples from 150 subjects, of whom 30 were uninfected control patients and 120 had Chagas disease (1 group had asymptomatic disease, and 2 groups had CCC with either a preserved or reduced left ventricular ejection fraction [LVEF]). Each Chagas disease group displayed distinct gene expression and functional pathway profiles. The most different expression patterns were between CCC groups with a preserved or reduced LVEF. A more stringent analysis indicated that 27 differentially expressed genes, particularly those related to natural killer (NK)/CD8$^+$ T-cell cytotoxicity, separated the 2 groups. NK/CD8$^+$ T-cell cytotoxicity could play a role in determining Chagas disease progression. Understanding genes associated with disease may lead to improved insight into CCC pathogenesis and the identification of prognostic factors for CCC progression.

Keywords. Chagas disease; whole-blood transcriptome; Cardiomyopathy; NK cells.

Chagas disease is caused by the protozoan *Trypanosoma cruzi* and affects up to 7 million people in Latin American countries where *T. cruzi* is endemic [1]. The parasite is mainly transmitted by hematophagous reduviid bugs, by blood transfusion, and congenitally, and Chagas disease is now a concern in industrialized countries where it is not endemic, owing to immigration [2–4]. The infection presents with an acute phase with high-level parasitemia, which progresses to a chronic low grade infection phase due to strong innate and acquired immunity that controls—but does not completely suppress—blood and tissue parasitism [5–7]. Most patients will develop an indeterminate/asymptomatic form (ASY) showing no signs of cardiac or digestive alterations. Around 30% will have altered electrocardiograph and/or echocardiograph findings that are characteristic of chronic Chagas cardiomyopathy (CCC) or so-called mega-syndromes (ie, megacolon and megaesophagus). These complications are a great cause of morbidity and mortality, resulting a considerable socioeconomic burden [7–9].

CCC is the most frequent and severe clinical consequence of Chagas disease and the major cause of infectious myocarditis in the world [1, 8, 10]. It is an inflammatory cardiomyopathy with electrical heart conduction abnormalities and arrhythmias [6, 11]. One third of patients with CCC further develop left ventricular dysfunction, leading to dilation and refractory heart failure. Patients with CCC have a worse prognosis than patients with other noninflammatory cardiomyopathies [12]. Current anti–*T. cruzi* drugs have shown to be unable to block progression toward the more severe forms of CCC [1]. The mechanisms underlying CCC development are still not fully understood, although inflammatory mechanisms are certainly involved [13]. Detection of *T. cruzi* DNA in peripheral blood is associated with CCC severity [14], suggesting that the parasite load influences disease progression toward CCC with ventricular dysfunction.

Histopathological analysis of hearts from patients with CCC reveals a diffuse T-cell– and macrophage-rich myocarditis, hypertrophy, and fibrosis with scarce tissue parasitism [15–17]. Cytokines and chemokines are also known to play an important role in immune-mediated damage and fibrosis [6].

Received 16 April 2016; editorial decision 31 October 2016; accepted 29 November 2016; published online December 17, 2016.

[a]L. R. P. F. and F. M. F. contributed equally to this work.

[b]E. C. S. and E. C.-N. are co–senior authors and contributed equally to this work.

Correspondence: E. Cunha-Neto, MD, PhD, Laboratory of Immunology, Heart Institute (InCor), Avenida Dr Enéas de Carvalho Aguiar, 44 – Bloco II, 9º Andar, São Paulo, Brazil 05403-900 (edecunha@usp.br).

The Journal of Infectious Diseases® 2017;215:387–95

DOI:10.1093/infdis/jiw540

Whole-blood transcriptomic analyses have been used to understand pathogenesis in cardiovascular diseases, such as cardiac allograft rejection and coronary artery disease, and postmyocardial infarction heart failure [18, 19]. Here, we describe for the first time the use of a whole-blood microarray to analyze samples from different clinical/parasitological forms and progression stages of Chagas disease.

METHODS

Samples From Healthy Controls and Patients With Chagas Disease

Samples from patients with Chagas disease were identified by blood bank screening in 1996–2002 (from the cities of São Paulo and Montes Claros in the State of Minas Gerais, Brazil); samples from seronegative donors (ie, controls) were obtained from the same blood banks as previously described [20]. Patients with severe CCC came from the Heart Institute of the University of São Paulo Medical School. Samples are from a subset of the retrospective cohort described previously [21].

The diagnostic criteria for Chagas disease included the detection of antibodies against *T. cruzi* in serological tests. All patients with Chagas disease underwent standard electrocardiography and echocardiography. Left ventricular dimensions and left ventricular ejection fraction (LVEF) were evaluated with a 2-dimensional, M-mode approach, in accordance with the recommendations of the American Society of Echocardiography. Patients with Chagas disease who had no changes in electrocardiographic and echocardiograph findings and no clinical signs of digestive disease (but were not assessed for subclinical digestive disease) are here described as asymptomatic (ASY) subjects. Patients with CCC presented typical conduction abnormalities (ie, right bundle branch block and/or left anterior division hemiblock). Patients with CCC who had a significantly reduced LVEF (ie, ≤40%; average, 30%) are here described as having severe CCC, whereas those with a preserved LVEF (ie, 40%; average, 60%) are here described as having moderate CCC (Table 1). A total of 150 whole-blood samples collected in PAXgene tubes were from 5 different subject groups (30 samples per group). Characteristics of patients and seronegative controls whose samples were used in this study are described in Table 1. This protocol was approved by the Institutional Review Board of the University of the São Paulo School of Medicine and by the Brazilian National Ethics in Research Commission, and written informed consent was obtained from the patients.

Sample Selection and Preparation

Total RNA was isolated from PAXgene whole-blood samples by using the PAXgene Blood RNA Kit (PreAnalytiX) according to the manufacturer's instructions. Quantification of quality assessment of total RNA was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), and all samples had a RNA integrity number of >6. Detection of kinetoplast minicircle *T. cruzi* DNA in 20 mL of blood was performed with a real-time polymerase chain reaction (PCR) assay [14].

Whole-Blood Gene Expression Analysis

Gene expression analysis was performed using the Illumina HumanHT-12 v3 BeadChip. Reverse transcription of 200 ng of total RNA and complementary RNA (cRNA) synthesis were performed using the Illumina TotalPrep-96 RNA Amplification Kit (Ambion, Darmstadt, Germany). Hybridization of 700 ng of each biotinylated cRNA sample with a single array on the BeadChip was performed at 58°C for 16–18 hours.

Data Processing and Statistical Analysis of Gene Expression

BeadChips were scanned using the Illumina Bead Array Reader. The GenomeStudio V 2010.1 Gene Expression Module was used to determine the fluorescence intensities. The uncorrected and nonnormalized raw fluorescence intensities were integrated and derived in the probe profile mode. Subsequently the fluorescence intensities underwent background correction, $\log_2$ transformation, and quantile normalization, using the R/Bioconductor package LUMI (available at: http://www.bioconductor.org/) [22]. Individual arrays were submitted to a batch-correction protocol implemented in the R/Bioconductor package SVA [23]. Sample quality control and outliers detection were assessed using the R/Bioconductor package ARRAYQUALITYMETRICS [24]. Probes were mapped to their unique gene symbols with the LUMIHUMANALL annotation database. To identify genes highly expressed in one or more clinical groups, we used independent 2-class *t* tests ($P < .05$;

Table 1. Clinical Characteristics of Study Subjects, by Study Group

			Result of Testing for *T. cruzi*			LVEF Analysis	
Group	Patients, No.	Age, y	PCR	EIA	EKG	ECG Finding	Value, %
Seronegative controls	30	48 ± 10	Negative	Negative	Normal	Preserved	64 ± 4
ASY *T. cruzi* PCR negative	30	49 ± 9	Negative	Positive	Normal	Preserved	62 ± 4
ASY *T. cruzi* PCR positive	30	47 ± 12	Positive	Positive	Normal	Preserved	62 ± 4
Moderate CCC (with preserved LVEF)	30	50 ± 8	Positive	Positive	Abnormal	Preserved	61 ± 7
Severe CCC (with reduced LVEF)	30	46 ± 10	Positive	Positive	Abnormal	Reduced	30 ± 7

Data are mean ± SD, unless otherwise indicated.

Abbreviations: ASY, asymptomatic; CCC, chronic Chagas cardiomyopathy; ECG, echocardiography; EIA, enzyme-linked immunosorbent assay; EKG, electrocardiogram; LVEF, left ventricular ejection fraction.

fold change, >1.25) [25]. Differentially expressed genes (DEGs) were determined by fitting the expression data to the linear model implemented in the R/Bioconductor package LIMMA [26]. *P* values were submitted to false-discovery rate correction with the Benjamini-Hochberg method and the statistical significance threshold was defined as a *P* value of ≤ .05.

Enrichment Analysis

Probe sets representing the same gene were collapsed by taking the probe set the with highest expression across all samples. Gene expression was subjected *z* normalization (per gene across samples), and the average *z* score of each class was used as the preranked gene lists (1 list per class). The preranked gene lists with 1000 permutations were subjected to gene set enrichment analysis (GSEA) [27, 28] to generate normalized enrichment scores for blood transcription modules (BTMs) from previously generated gene sets [29] (nominal *P* = .01; 1000 permutations).

Network and Pathway Analysis

Ingenuity Pathway Analysis software (IPA; Qiagen, Redwood City, California) maintains a graphical database of networks of interacting genes (Ingenuity Knowledge Base). The lists of differentially expressed genes (DEGs) in patients with moderate CCC as compared to those with severe CCC were uploaded to IPA and analyzed on the basis of the content available during December 2015. The significance of the association between each list and the pathway was measured by the Fisher exact test. The biological relationship between 2 molecules is represented as an edge (line).

Analysis of Messenger RNA Expression by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

Real-time qRT-PCR analysis of selected genes was performed using TaqMan Gene expression assays according to manufacturer's instructions. 18S ribosomal RNA was used for normalization. The reactions were done in the QuantStudio12K PCR System (Applied Biosystems). All reagents were provided by Thermo Fisher Scientific. Reactions were performed in triplicate and cycle threshold (Ct) values were averaged for the replicates; negative controls were included. The relative expression was calculated for each gene in each group as individual data points by the $2^{-\Delta\Delta Ct}$ method, as previously described [30]. Severe CCC was used as the calibrator in the $2^{-\Delta\Delta Ct}$ method for comparison between CCC groups; therefore, the relative expression in each sample was calculated with respect to the average expression value for patients with severe CCC (value set as 1). Statistical analysis was performed with the Mann-Whitney rank sum test and the Fisher exact test.

RESULTS

Different Groups of Patients With Chagas Disease Display Distinct Gene Expression Profiles

We collected whole-blood samples from 150 patients (n = 30 per group) in 4 different groups based on clinical/parasitological characteristics of Chagas disease and *T. cruzi* PCR status (Table 1), as well as from seronegative controls. The blood transcriptome of these patients was assessed by microarray analysis. All possible comparisons between any 2 groups (10 comparisons in total) were performed to identify genes highly expressed in one of the groups (Figure 1). For each comparison, genes were considered upregulated in one group compared to another if the *P* value was <.05 and the fold change was >1.25. Of the 4 possible comparisons for a given group, genes were considered highly expressed in the group if they were upregulated in at least 2 comparisons. Figure 1 presents 3 sets of genes, comprising 16, 6, and 9 genes, that were highly expressed in control, ASY PCR-negative, and ASY PCR-positive groups, respectively. In addition, 2 other sets of genes, comprising 53 and 196 genes, were highly expressed in the moderate and severe CCC groups, respectively. The expression profiles of these genes in the moderate and severe groups were remarkably different. Figure 1B lists some genes that were upregulated in each group. Of note, several genes with high expression in the moderate CCC group and low expression in the severe CCC group were related to natural killer (NK)/cytotoxic CD8⁺ T cells.

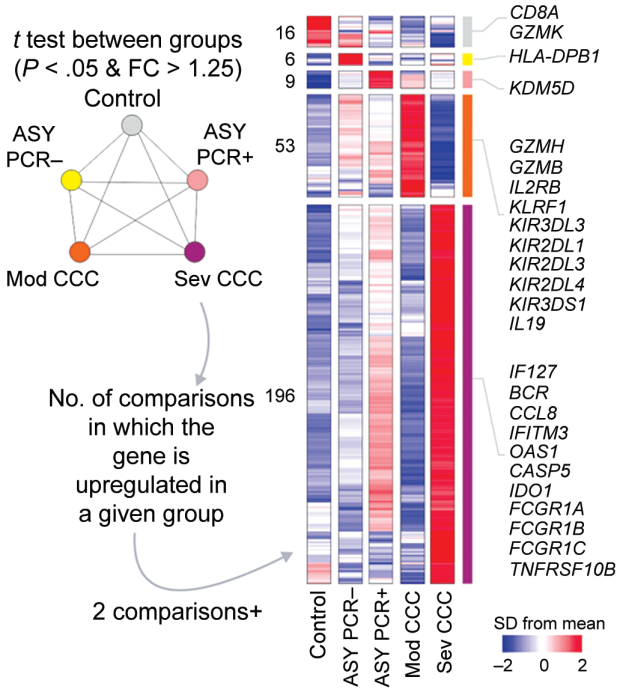

Figure 1. Whole-blood microarray analysis of patients with Chagas disease. Whole-blood transcriptome for 150 patients (30 per group) assigned to 4 different clinical/parasitological groups based on Chagas disease and *Trypanosoma cruzi* polymerase chain reaction (PCR) status, as well as seronegative controls, was assessed by microarray analysis. All possible comparisons between any 2 groups (10 comparisons in total) were performed to identify genes highly expressed in one of the groups. Genes were considered highly expressed in the group if they were upregulated in at least 2 comparisons ($P < .05$; fold change, >1.25) in the control group, the asymptomatic (ASY), PCR-negative group, and the ASY PCR-positive group. Abbreviations: CCC, chronic Chagas cardiomyopathy; FC, fold change; Mod, moderate; Sev, severe; –, negative; +, positive.

GSEA Discloses Functional Pathways Modulated in Each Chagas Disease Group

To describe the functional content of the transcriptional profiles in each subject group, we performed GSEA on the lists of genes ranked by the average *z*-normalized expression across all subjects in a group (Figure 2). We have used as gene sets the BTMs previously developed [29]. The BTMs include a collection of 346 gene sets that, taken together, describe various aspects of the functioning of the immune system in blood circulating cells. BTMs with increased activity in the peripheral blood of patients with severe CCC included functions related to the inflammatory response and innate immunity. BTMs with reduced activity in patients with severe CCC included NK-cell, B-cell, and T-cell activation; phosphatidylinositol signaling; and chaperonin-mediated protein folding. BTMs with increased activity in patients with moderate CCC included NK-cell and phosphatidylinositol signaling, while monocyte-related functions were negatively modulated in this group. BTMs with increased activity in the ASY *T. cruzi* PCR–positive group included polo-like kinase 1 (PLK1) signaling and the mitotic cell cycle in stimulated CD4$^+$ T cells. Both of these functions were negatively modulated in the ASY *T. cruzi* PCR–negative group, along with BTMs related to inflammation and innate immunity. As shown in Figure 2, among the BTMs found to be active in the ASY *T. cruzi* PCR–negative group were NK-cell signaling, chaperonin-mediated protein folding, and clustering of major histocompatibility complex–Toll-like receptor 7 (TLR7)–TLR8. The control group showed enrichment of functions related to B-cell activation, T-cell activation, and chaperonin-mediated protein folding. PLK1 signaling events, the NK-cell surface signature, and complement activation were negatively modulated in the control group.

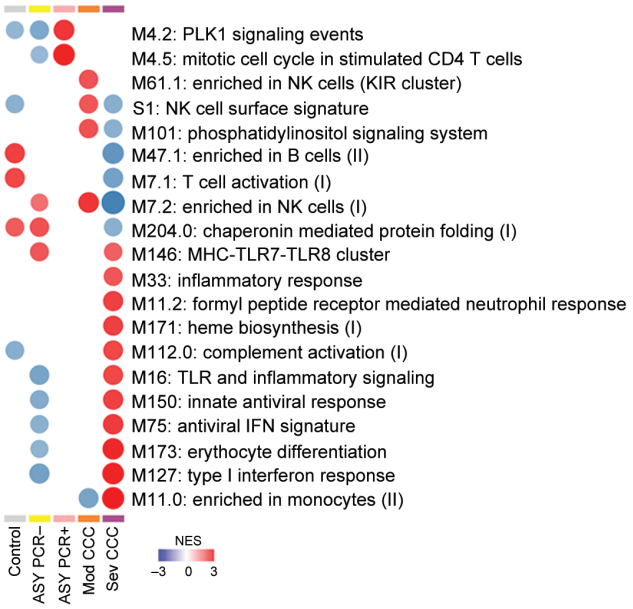

Figure 2. Findings of gene set enrichment analysis. Corrplot analysis of gene expression results shows blood transcription modules and functional pathways that have increased (red) or reduced activated (blue) in the peripheral blood of each subject group. Abbreviations: CCC, chronic Chagas cardiomyopathy; IFN, interferon; MHC, major histocompatibility complex; Mod, moderate; NES, normalized enrichment score; NK, natural killer; Sev, severe; TLR, Toll-like receptor; –, negative; +, positive.

An NK/CD8$^+$ T-Cell Cytotoxicity Gene Signature Is Present in Patients With Moderate CCC

We then performed a more stringent analysis to determine the DEGs, considering an adjusted *P* value of .05 and a fold change of 1.5. Expression profiles of 150 samples distributed in 5 groups (30 per group; Table 1) were assessed. The analysis revealed 27 DEGs between the moderate versus severe CCC groups (Table 2). We have also found 11 DEGs when patients with severe CCC were compared to seronegative controls, 7 DEGs when the ASY *T. cruzi* PCR–negative group was compared to patients with severe CCC, 6 DEGs when the ASY *T. cruzi* PCR–positive group was compared to the control group, 2 DEGs when the ASY *T. cruzi* PCR–positive group was compared to patients with severe CCC, and 1 DEG when patients with moderate CCC were compared to the control group Supplementary Tables 1–5. The other 4 remaining comparisons did not present any DEGs. Once again, the highest number of DEGs was found in the contrast between the moderate and severe CCC groups. Interestingly, 8 of 13 DEGs upregulated in the moderate CCC group as compared to the severe CCC group belonged to the NK/CD8$^+$ T-cell cytotoxicity pathways, including GZMB (which encodes granzyme B); PRF1; GLNY (granulysin); KIR3DS1, KIR2DL4, and KIR2DL1/KIR2DL3 (killer immunoglobulin-like receptors); and KLRC2/NKGD2 and KLRF1/NKp80 (killer cell lectin-like receptors).

Network and Pathway Analysis

The list containing the 27 DEGs (Table 2) capable of segregating moderate from severe CCC with their respective fold changes and *P* values was uploaded in IPA. The most significant network was termed "cell death and survival, hematological disease, immunological disease" ($P = 10^{-39}$; Figure 3). In this network, we could find multiple genes associated with NK/CD8$^+$ cytotoxic T-cell function, such as GZMB, PRF1, GLNY, KLRC2, KIR2DL4, and KIR2DL1/KIR2DL3.

Validation of Microarray Results by Real-Time qRT-PCR

To assess the expression of each gene belonging to the NK/CD8$^+$ T-cell cytotoxic profile in each individual in the 2 cardiomyopathy groups, we performed real-time qRT-PCR assays using specific primers for each of the 8 genes belonging to the NK/CD8$^+$ T-cell cytotoxicity pathways. For KIR2DL4, we had undetectable expression in most samples and thus limited the analysis of qPCR results to the remaining 7 genes. The validation of microarray results is shown in Figure 4. The number of upregulated genes for each sample is depicted in Supplementary

Table 2. Differentially Expressed Genes in the Comparison Between Blood Samples From Patients With Moderate and Those With Severe Chronic Chagas Cardiomyopathy

Symbol	Entrez Gene Name	Fold Change	*P*adjusted
ADGRG1	Adhesion G protein–coupled receptor G1	1.615	4.93E−03
ALAS2	5′-aminolevulinate synthase 2	−1.563	4.48E−02
BCL2L1	BCL2-like 1	−1.562	2.50E−02
CES3	Carboxylesterase 3	−1.590	9.79E−03
FCGR1B	Fc fragment of immunoglobulin G, high-affinity Ib, receptor (CD64)	−1.661	5.23E−03
FCGR1C	Fc fragment of immunoglobulin G, high-affinity Ic, receptor (CD64), pseudogene	−1.541	6.72E−03
FGFBP2	Fibroblast growth factor binding protein 2	1.504	2.19E−02
GK5	Glycerol kinase 5 (putative)	1.557	6.71E−04
GNLY	Granulysin	1.504	2.90E−02
GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte–associated serine esterase 1)	1.688	1.38E−03
ITLN1	Intelectin 1 (galactofuranose binding)	−1.581	4.02E−02
KIR2DL1/ KIR2DL3	Killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail, 3	2.002	1.51E−03
KIR2DL4	Killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic tail, 4	1.845	1.22E−03
KIR3DS1	Killer cell immunoglobulin-like receptor, three domains, short cytoplasmic tail, 1	2.236	4.18E−03
KLC3	Kinesin light chain 3	−1.681	6.16E−03
KLRC2	Killer cell lectin-like receptor subfamily C, member 2	1.671	2.58E−02
KLRF1	Killer cell lectin-like receptor subfamily F, member 1	1.690	1.09E−03
KRT1	Keratin 1, type II	−1.775	4.87E−02
MIR181A2HG	MIR181A2 host gene	1.669	5.97E−04
MYBL1	v-myb avian myeloblastosis viral oncogene homolog-like 1	1.504	1.34E−03
POLR2J4	Polymerase (RNA) II (DNA directed) polypeptide J4, pseudogene	2.234	2.74E−03
PPDPF	Pancreatic progenitor cell differentiation and proliferation factor	−1.560	3.18E−03
PRF1	Perforin 1 (pore-forming protein)	1.574	1.34E−03
SMIM24	Small integral membrane protein 24	−1.536	2.57E−02
ST6GALNAC4	ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 4	−1.514	9.18E−03
TGFBR3	Transforming growth factor β receptor III	1.529	1.94E−03
TMOD1	Tropomodulin 1	−1.544	2.47E−02

The analysis revealed 27 differentially expressed genes between the 2 groups (fold change, 1.5; cutoff adjusted $P < .05$).

Table 6. We found that patients with moderate CCC displayed a mean (±standard error of the mean) of 4.63 ± 0.39 upregulated NK/CD8$^+$ T-cell cytotoxic genes, while patients with severe CCC displayed 2.42 ± 0.39 upregulated genes ($P = .0011$). Supplementary Figure 1A shows that the frequency of samples displaying upregulated expression of combinations of genes (≥5, ≥4, ≥3, and ≥2) in the NK/CD8$^+$ T-cell cytotoxic signature was significantly higher in the moderate CC group than in the severe CCC group. The best discrimination was found to be the upregulated expression of ≥3 genes belonging to the NK/CD8$^+$ T-cell cytotoxic signature (frequencies, 80% vs 33.33% in the moderate and severe CCC groups, respectively; $P = .0005$). Supplementary Figure 1*B* shows that several genes were more frequently upregulated in samples from patients with moderate CCC, compared with those from patients with severe CCC samples, especially KIR3DS1 (86.6% vs 29.6%, respectively; $P < .0001$). Together, results indicate that many more samples in the moderate CCC group than in the severe CCC group displayed upregulated expression of several genes in the NK/CD8$^+$ T-cell cytotoxic signature.

DISCUSSION

Whole-blood transcriptome analyses of Chagas disease clinical groups and *T. cruzi*–seronegative control subjects revealed specific signatures of each clinical/parasitological group, with expression profiles between CCC groups being the most distinct. GSEA of immune-related functions also generated similar findings, where the severe CCC group displayed the highest number of modulated BTMs. A more stringent analysis revealed a low number of DEGs, probably due to the high interindividual variability often found in chronic diseases. Yet, the largest list of DEGs came from the comparison between the moderate CCC group (comprising patients with a preserved LVEF) and the severe CCC group (comprising patients with a reduced LVEF). Several groups have reported differential immune pathways in patients with Chagas disease belonging to different clinical groups. Patients with Chagas cardiomyopathy displayed a skewed proinflammatory, Th1-type cytokine profile as compared to patients with the ASY form [31–33]. Increased levels of plasma TNF-α, IL-6, and IL-10 were detected in patients with CCC who had a reduced LVEF as compared to those who had a preserved LVEF [34, 35]. GSEA established further differences between clinical/parasitological groups. Increased inflammation and innate immunity BTMs in the severe CCC group is in line with the increased production of proinflammatory cytokines in patients with CCC who had a reduced LVEF [34] and may be related to the association of *T. cruzi* parasitism with severe CCC [14]. Conversely, the lower activity of inflammatory and innate immunity BTMs in the ASY *T. cruzi* PCR–negative group may be related to the reduced parasitism displayed by this group. The finding that BTMs related to T-cell activation showed a reduced activity in the severe CCC group is in line with recent studies showing that T cells from the peripheral blood of patients with CCC displayed reduced activation when stimulated with antigen [36, 37]. Incubation of T cells with *T. cruzi* trypomastigotes reduced the expression of CD3ζ, proliferation indexes, and IL-2

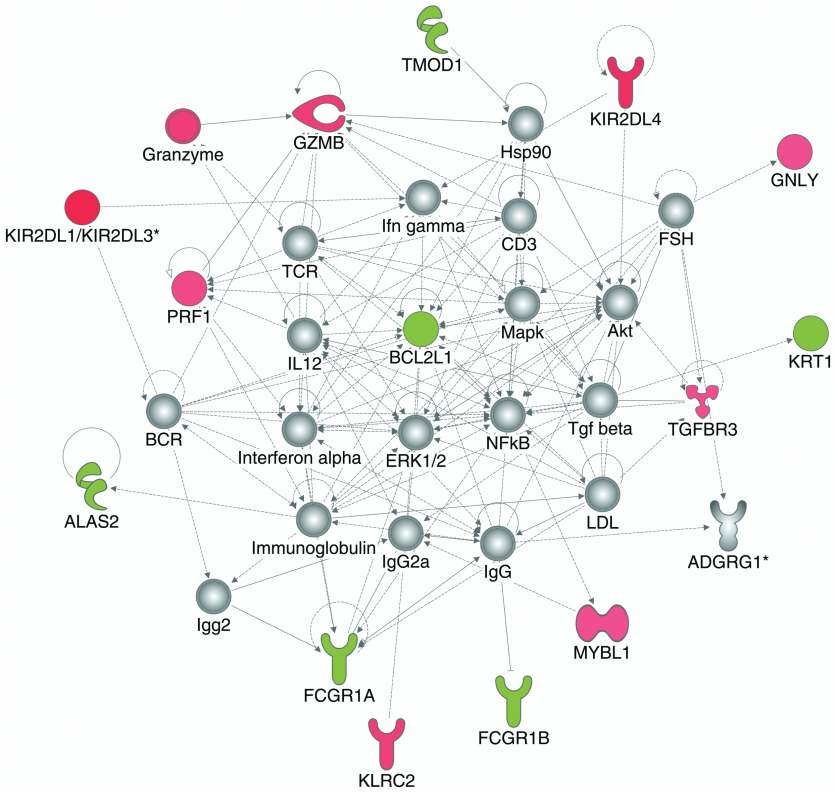

Figure 3. Findings of network and pathway analysis. Ingenuity Pathway Analysis was performed with the list containing the 27 differentially expressed genes capable of segregating patients with moderate chronic Chagas cardiomyopathy (CCC) from those with severe CCC on the basis of their respective fold changes and *P* values. The most significant network generated was related to cell death and survival, hematological disease, immunological disease ($P = 10^{-39}$). Genes upregulated in moderate as compared to severe CCC are depicted in red, while downregulated genes are depicted in green.

production [37, 38], possibly indicating a direct effect of parasitism on the T-cell activation status.

A more stringent computational analysis of the DEGs upregulated in patients with moderate CCC revealed an enrichment of genes related to NK cells, cytotoxicity, and cell death pathways. Likewise, GSEA revealed that the NK cell BTM was more active in ASY *T. cruzi* PCR–negative and moderate CCC groups than in the severe CCC group. This was reinforced by IPA of the high-stringency moderate versus severe CCC DEGs, which identified the cell death and survival, hematological disease, immunological disease network as highly significant. The finding that 8 of 13 DEGs upregulated in the moderate CCC group as compared to the severe CCC group belong to the NK/$CD8^+$ T-cell cytotoxicity pathways provided a clue for pathogenesis. The validation of the microarray findings with qPCR indicated that those findings were biologically relevant. Furthermore, analysis of qPCR results indicated that the upregulated expression of genes from the cytotoxic signature may be a general feature of blood samples from patients with moderate CCC, even when taken individually. NK cells have been reported to interact with *T. cruzi*–infected fibroblasts [39] and to be associated with acute *T. cruzi* infection control [40]. An increased frequency of NK and NKT cells and a decreased frequency of NKT cells have been observed in ASY and severe CCC cases, respectively [41, 42]. Furthermore, cytotoxicity toward the NK target cell line K562 has been reported to be low in peripheral blood mononuclear cells from symptomatic patients with CCC [43]. Significantly, the cytolytic pathway is shared between NK and cytolytic $CD8^+$ T cells, especially the cytolytic effector molecules perforin, granzyme B, and granulysin. The genes encoding these molecules, all of which were upregulated DEGs in blood samples from patients with moderate CCC, play a key role in the killing of intracellular *T. cruzi* in murine and cellular infection models [44]. The other genes (KIR2DL1, KIR2DL1, KLR2/NKGC2, and KLRF1/NKp80) displayed high expression in NK cells, but some have been reported to be expressed by a smaller number of $CD8^+$ T cells [45]. $CD8^+$ T cells are involved in the control of *T. cruzi* infection in murine models of acute infection [46]. Thus, we cannot exclude that the increased expression of genes belonging to the cytotoxic signature observed here could be due to circulating $CD8^+$ T cells. Accordingly, the NK cell BTM was found to be active in the ASY *T. cruzi* PCR–negative group, which shows more complete control of parasitism. The presence of *T. cruzi* DNA in blood was significantly associated with known markers of disease progression, suggesting parasite load and persistence influence evolution from moderate-to-severe CCC [14]. Concordantly, a higher level of parasitism in the acute phase is

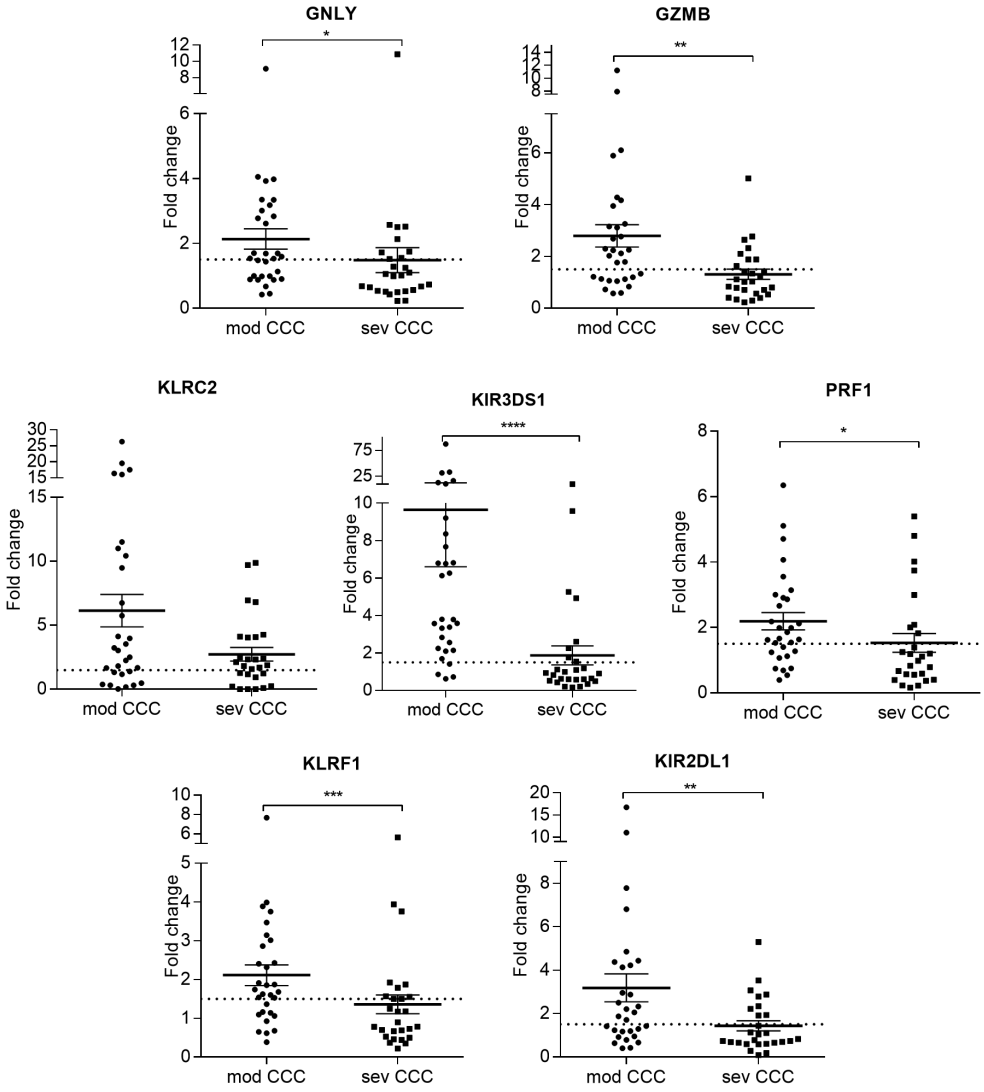

Figure 4. Validation of microarray results by real-time quantitative reverse transcription polymerase chain reaction analysis (qRT-PCR). Real-time qRT-PCR assays for 7 genes belonging to the natural killer (NK)/CD8+ T-cell cytotoxicity pathways: GZMB (which encodes granzyme B), PRF1 (perforin 1), GLNY (granulysin), KIR2DL1/KIR2DL3 (killer immunoglobulin-like receptor genes), and KLRC2 and KLRF1 (killer cell lectin-like receptor genes). The fold change was calculated as the mean ± SD for each group as individual data points, by the $2^{-\Delta\Delta Ct}$ method. *$P < .05$, **$P < .01$, and ***$P < .0001$ by the Mann–Whitney rank sum test.

associated with more-aggressive heart disease in the chronic phase of experimental *T. cruzi* infection [47, 48]. The differential expression patterns between moderate and severe CCC may suggest that the peripheral blood expression profile has a bearing on progression to severe cardiomyopathy. However, it is possible that this differential expression profile may be a consequence of pathophysiological alterations of heart failure itself. We have compared the list of DEGs in the severe versus moderate CCC contrasts with postmyocardial infarction heart failure [19]. The comparison revealed a single common downregulated DEG (TGFBR3). This suggests that the DEGs in our study are primary to CCC and not secondary to heart failure. However, one of the main limitations of our study is that we did not follow up patients in the cohort. Our study was therefore unable to assess the possible predictive value of these markers for progression to the more severe form of CCC. Another caveat of the present analysis is that transcriptome measured in peripheral blood will reflect both the change of transcriptional programs within individual cells, as well as the change in the representation of cell populations [49]. So, it is difficult to ascertain which of the possibilities might be taking place. In addition, we observed a low number of DEGs. This is not unexpected in a chronic disease, given the heterogeneity often observed among human samples, which leads to signals that are rather weak. Considering these limitations, the present study shows that Chagas disease clinical/parasitological groups and *T. cruzi*–seronegative control samples displayed a distinct whole-blood gene signature in which the gene expression profiles in samples from the moderate CCC (preserved LVEF) and severe CCC (reduced LVEF) groups were the most distinct. Taken together, our data suggest that cytotoxic peripheral NK or CD8+

T cells may play a role in the control of *T. cruzi* parasitism, which might be important for Chagas disease–associated heart disease progression and severity, although this still has to be functionally validated. Understanding genes and pathways associated with the disease may lead to improved insight into CCC pathogenesis and the identification of prognostic factors for CCC progression.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgment. We thank Dr Christophe Chevillard (Aix-Marseille Université, France) for sharing reagents.

Financial support. This work was supported by the National Institute of Allergy and Infectious Diseases (grant P50AI098461), the National Heart, Lung, and Blood Institute (grant HHSN268200417175C), the São Paulo (Brazil) Research Foundation (FAPESP) (grant 2013/50302–3 to E. C.-N.), and the Brazilian National Council for Scientific and Technological Development (CNPq) (research productivity awards to H. I. N., A. L. R., E. C.-N., E. S., and J. K.).

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Morillo CA, Marin-Neto JA, Avezum A, et al; BENEFIT investigators. Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. N Engl J Med **2015**; 373:1295–306.
2. Coura JR. Special issue on Chagas disease. Mem Inst Oswaldo Cruz **2015**; 110:275–6.
3. Gascon J, Vilasanjuan R, Lucas A. The need for global collaboration to tackle hidden public health crisis of Chagas disease. Expert Rev Anti Infect Ther **2014**; 12:393–5.
4. Kalil-Filho R. Globalization of Chagas disease burden and new treatment perspectives. J Am Coll Cardiol **2015**; 66:1190–2.
5. Cunha-Neto E, Bilate AM, Hyland KV, Fonseca SG, Kalil J, Engman DM. Induction of cardiac autoimmunity in Chagas heart disease: a case for molecular mimicry. Autoimmunity **2006**; 39:41–54.
6. Cunha-Neto E, Chevillard C. Chagas disease cardiomyopathy: immunopathology and genetics. Mediators Inflamm **2014**; 2014:683230.
7. Marin-Neto JA, Rassi A Jr. Update on Chagas heart disease on the first centenary of its discovery. Rev Esp Cardiol **2009**; 62:1211–6.
8. Rassi A, Jr., Rassi A, Marin-Neto JA. Chagas disease. Lancet **2010**; 375:1388–402.
9. Rassi Jr A, Rassi A, Marin-Neto JA. Chagas heart disease: pathophysiologic mechanisms, prognostic factors and risk stratification. Mem Inst Oswaldo Cruz **2009**; 104 Suppl 1:152–8.
10. Hidron A, Vogenthaler N, Santos-Preciado JI, Rodriguez-Morales AJ, Franco-Paredes C, Rassi A Jr. Cardiac involvement with parasitic infections. Clin Microbiol Rev **2010**; 23:324–49.
11. Nunes MC, Dones W, Morillo CA, Encina JJ, Ribeiro AL; Council on Chagas Disease of the Interamerican Society of Cardiology. Chagas disease: an overview of clinical and epidemiological aspects. J Am Coll Cardiol **2013**; 62:767–76.
12. Silva CP, Del Carlo CH, Oliveira Junior MT, et al. Why do patients with chagasic cardiomyopathy have worse outcomes than those with non-chagasic cardiomyopathy? Arq Bras Cardiol **2008**; 91:358–62.
13. Marin-Neto JA, Cunha-Neto E, Maciel BC, Simões MV. Pathogenesis of chronic Chagas heart disease. Circulation **2007**; 115:1109–23.
14. Sabino EC, Ribeiro AL, Lee TH, et al.; Chagas Study Group of the NHLBI Retrovirus Epidemiology Donor Study-II, International Component. Detection of Trypanosoma cruzi DNA in blood by PCR is associated with Chagas cardiomyopathy and disease severity. Eur J Heart Fail **2015**; 17:416–23.
15. Higuchi ML, De Morais CF, Pereira Barreto AC, et al. The role of active myocarditis in the development of heart failure in chronic Chagas' disease: a study based on endomyocardial biopsies. Clin Cardiol **1987**; 10:665–70.
16. Higuchi ML. [Chagas disease. Importance of the parasite in the pathogenesis of the cardiac chronic disease]. Arq Bras Cardiol **1995**; 64:251–4.
17. Reis DD, Jones EM, Tostes S Jr, et al. Characterization of inflammatory infiltrates in chronic chagasic myocardial lesions: presence of tumor necrosis factor-alpha+ cells and dominance of granzyme A+, CD8+ lymphocytes. Am J Trop Med Hyg **1993**; 48:637–44.
18. Pedrotty DM, Morley MP, Cappola TP. Transcriptomic biomarkers of cardiovascular disease. Prog Cardiovasc Dis **2012**; 55:64–9.
19. Maciejak A, Kiliszek M, Michalak M, et al. Gene expression profiling reveals potential prognostic biomarkers associated with the progression of heart failure. Genome Med **2015**; 7:26.
20. Sabino EC, Ribeiro AL, Salemi VM, et al.; National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study-II (REDS-II), International Component. Ten-year incidence of Chagas cardiomyopathy among asymptomatic Trypanosoma cruzi-seropositive former blood donors. Circulation **2013**; 127:1105–15.
21. Keating SM, Deng X, Fernandes F, et al.; NHLBI Retrovirus Epidemiology Donor Study-II (REDS-II), International Component. Inflammatory and cardiac biomarkers are differentially expressed in clinical stages of Chagas disease. Int J Cardiol **2015**; 199:451–9.
22. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. Bioinformatics **2008**; 24:1547–8.
23. Song JS, Johnson WE, Zhu X, et al. Model-based analysis of two-color arrays (MA2C). Genome Biol **2007**; 8:R178.
24. Kauffmann A, Rayner TF, Parkinson H, et al. Importing ArrayExpress datasets into R/Bioconductor. Bioinformatics **2009**; 25:2092–4.
25. Nakaya HI, Wrammert J, Lee EK, et al. Systems biology of vaccination for seasonal influenza in humans. Nat Immunol **2011**; 12:786–95.
26. Ritchie ME, Phipson B, Wu D, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res **2015**; 43:e47.
27. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A **2005**; 102:15545–50.
28. Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP. GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics **2007**; 23:3251–3.
29. Li S, Rouphael N, Duraisingham S, et al. Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. Nat Immunol **2014**; 15:195–204.
30. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc **2008**; 3:1101–8.
31. Abel LC, Rizzo LV, Ianni B, et al. Chronic Chagas' disease cardiomyopathy patients display an increased IFN-gamma response to *Trypanosoma cruzi* infection. J Autoimmun **2001**; 17:99–107.
32. Gomes JA, Bahia-Oliveira LM, Rocha MO, Martins-Filho OA, Gazzinelli G, Correa-Oliveira R. Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response. Infect Immun **2003**; 71:1185–93.
33. Araujo FF, Gomes JA, Rocha MO, et al. Potential role of CD4+CD25HIGH regulatory T cells in morbidity in Chagas disease. Front Biosci **2007**; 12:2797–806.
34. Ferreira RC, Ianni BM, Abel LC, et al. Increased plasma levels of tumor necrosis factor-alpha in asymptomatic/"indeterminate" and Chagas disease cardiomyopathy patients. Mem Inst Oswaldo Cruz **2003**; 98:407–11.
35. Talvani A, Rocha MO, Cogan J, et al. Brain natriuretic peptide and left ventricular dysfunction in chagasic cardiomyopathy. Mem Inst Oswaldo Cruz **2004**; 99:645–9.
36. Longhi SA, Atienza A, Perez Prados G, et al. Cytokine production but lack of proliferation in peripheral blood mononuclear cells from chronic Chagas' disease cardiomyopathy patients in response to *T. cruzi* ribosomal P proteins. PLoS Negl Trop Dis **2014**; 8:e2906.
37. Giraldo NA, Bolaños NI, Cuellar A, et al. T lymphocytes from chagasic patients are activated but lack proliferative capacity and down-regulate CD28 and CD3ζ. PLoS Negl Trop Dis **2013**; 7:e2038.
38. Abel LC, Ferreira LR, Cunha Navarro I, et al. Induction of IL-12 production in human peripheral monocytes by *Trypanosoma cruzi* Is mediated by glycosylphosphatidylinositol-anchored mucin-like glycoproteins and potentiated by IFN- γ and CD40-CD40L interactions. Mediators Inflamm **2014**; 2014:345659.
39. Lieke T, Steeg C, Graefe SE, Fleischer B, Jacobs T. Interaction of natural killer cells with *Trypanosoma cruzi*-infected fibroblasts. Clin Exp Immunol **2006**; 145:357–64.
40. Cardillo F, Voltarelli JC, Reed SG, Silva JS. Regulation of *Trypanosoma cruzi* infection in mice by gamma interferon and interleukin 10: role of NK cells. Infect Immun **1996**; 64:128–34.

41. Vitelli-Avelar DM, Sathler-Avelar R, Dias JC, et al. Chagasic patients with indeterminate clinical form of the disease have high frequencies of circulating CD3+CD16-CD56+ natural killer T cells and CD4+CD25High regulatory T lymphocytes. Scand J Immunol **2005**; 62:297–308.
42. Vitelli-Avelar DM, Sathler-Avelar R, Massara RL, et al. Are increased frequency of macrophage-like and natural killer (NK) cells, together with high levels of NKT and CD4+CD25high T cells balancing activated CD8+ T cells, the key to control Chagas' disease morbidity? Clin Exp Immunol **2006**; 145:81–92.
43. Brodskyn CI, Barral A, Bulhões MA, Souto T, Machado WC, Barral-Netto M. Cytotoxicity in patients with different clinical forms of Chagas' disease. Clin Exp Immunol **1996**; 105:450–5.
44. Dotiwala F, Mulik S, Polidoro RB, et al. Killer lymphocytes use granulysin, perforin and granzymes to kill intracellular parasites. Nat Med **2016**; 22:210–6.
45. Björkström NK, Béziat V, Cichocki F, et al. CD8 T cells express randomly selected KIRs with distinct specificities compared with NK cells. Blood **2012**; 120:3455–65.
46. Padilla AM, Bustamante JM, Tarleton RL. CD8+ T cells in *Trypanosoma cruzi* infection. Curr Opin Immunol **2009**; 21:385–90.
47. Bilate AM, Salemi VM, Ramires FJ, et al. The Syrian hamster as a model for the dilated cardiomyopathy of Chagas' disease: a quantitative echocardiographical and histopathological analysis. Microbes Infect **2003**; 5:1116–24.
48. Marinho CR, D'Império Lima MR, Grisotto MG, Alvarez JM. Influence of acute-phase parasite load on pathology, parasitism, and activation of the immune system at the late chronic phase of Chagas' disease. Infect Immun **1999**; 67:308–18.
49. Li S, Nakaya HI, Kazmin DA, Oh JZ, Pulendran B. Systems biological approaches to measure and understand vaccine immunity in humans. Semin Immunol **2013**; 25:209–18.