CD69 Limits the Severity of Cardiomyopathy After Autoimmune Myocarditis

Aranzazu Cruz-Adalia, PhD; Luis Jesús Jiménez-Borreguero, MD; Marta Ramírez-Huesca, BSc; Isabel Chico-Calero, DVM, PhD; Olga Barreiro, PhD; Erica López-Conesa, DVM, PhD; Manuel Fresno, PhD; Francisco Sánchez-Madrid, PhD; Pilar Martín, PhD

Background — Experimental autoimmune myocarditis (EAM), a mouse model of post–infectious cardiomyopathy, reflects mechanisms of inflammatory cardiomyopathy in humans. EAM is characterized by an infiltration of inflammatory cells into the myocardium that can be followed by myocyte fibrosis, edema, and necrosis, leading to ventricular wall dysfunction and heart failure. Different data indicate that CD69 exerts an important immunoregulatory effect in vivo. However, the possible role of CD69 in autoimmune myocarditis has not been studied.

Methods and Results — We have explored the role of the leukocyte regulatory molecule CD69 in the inflammation that leads to cardiac dysfunction after myocardial injury in EAM. We have found that after induction of EAM, the draining lymph nodes from CD69-deficient mice developed an exacerbated Th17 inflammatory response, resulting in increases in the numbers of infiltrating leukocytes in the myocardium. In the chronic phase of EAM, transthoracic echocardiography revealed a significantly reduced left ventricular fractional shortening and a decreased ejection fraction in CD69-deficient mice, indicative of an impaired cardiac contractility. This condition was accompanied by a greater extent of myocardial fibrosis, an elevated number of sinus pauses on ECG, and an enhanced ratio of heart weight to body weight in CD69−/− mice. Moreover, both bone marrow transplantation and adoptive transfer of Th17 cells isolated from immunized CD69−/− mice with EAM into naive wild-type recipients reproduced the severity of the disease, demonstrating that CD69 exerts its function within the lymphocyte compartment.

Conclusion — Our findings indicate that CD69 negatively regulates heart-specific Th17 responses, cardiac inflammation, and heart failure progression in EAM. (Circulation. 2010;122:1396-1404.)

Key Words: cardiomyopathy ■ echocardiography ■ immune system ■ inflammation ■ myocarditis

Myocarditis and subsequent dilated cardiomyopathy (DCM) are major causes of heart failure in young patients. This condition is characterized by infiltration of inflammatory cells into the myocardium with consequent loss of myocytes and development of fibrosis and necrosis. In a significant fraction of patients, the loss of cardiomyocytes leads to ventricular remodeling, permanent ventricular wall dysfunction, DCM, heart failure, and/or arrhythmias. Myocarditis is induced by a variety of agents, including genetic susceptibility, toxins, viruses, bacteria, and parasites. In addition, myocardial injury can induce an autoimmune response to heart tissue, which has an important role in the pathogenesis of myocarditis and DCM. Experimental autoimmune myocarditis (EAM) is a mouse model of post–infectious myocarditis that can be induced in susceptible mouse strains by immunization with cardiac α-myosin heavy chain (MyHC-α)–derived peptides or by injection of activated MyHC-α–loaded dendritic cells. It has been shown that EAM is a CD4+ T-cell–mediated disease and its development depends critically on the interleukin (IL)-23–STAT4 axis, promoting the expansion of an autoreactive CD4+ T-cell subset characterized by IL-17 production. Mice lacking T-bet, a T-box transcription factor essential for Th1 lineage differentiation, develop a severe form of EAM with increased IL-17 release from heart-infiltrating cells. Accordingly, interferon-γ (IFN-γ) receptor−/− and IFN-γ−/− mice also show an exacerbated and progressive course of disease. These findings further support the notion that IL-17 directly accounts for disease development and point to a negative regulatory role of IFN-γ and Th1 differentiation in the EAM model. Recent data demonstrate that IFN-γ is required for the optimal activation and...
maturation of monocytes and macrophages but that IFN-γ simultaneously limits the expansion of activated T cells by inducing the release of nitric oxide, thus providing protection from exaggerated or even autoggressive T-cell responses.17

Clinical Perspective on p 1404

CD69 is a C-type lectin disulfide–linked homodimer and a member of the natural killer receptor family. Its genes are located in a conserved genomic region known as the natural killer gene cluster on mouse chromosome 6 and human chromosome 12.18–21 In vitro CD69 is expressed early by most leukocytes upon activation and in vivo is detected in chronic inflammatory cell infiltrates.22,23 Several studies with CD69-deficient mice have shown that CD69 has an immunoregulatory role.24 Thus, in the absence of CD69, mice show an enhanced antitumor activity and develop an exacerbated form of collagen-induced arthritis.25 In contrast, the absence of CD69 does not affect neutrophil responses in models of neutrophil-dependent acute inflammatory responses.26 Given that Th17 lymphocytes are the main mediators of collagen-induced arthritis,27 we have hypothesized that CD69 would be a significant negative regulator of EAM, another condition mediated by Th17 cells. Our findings demonstrate that CD69 acts as a brake on Th17-mediated inflammatory responses in myocardial tissue.

Methods

Mice
CD69-deficient mice were generated in the 129-Sv background as described28 and were backcrossed to BALB/c for >12 generations. CD69−/− and control mice used for experiments were 8–12-week-old females and were either littermates or age-matched offspring of these littermates in the BALB/c background. All mice were maintained and used under pathogen-free conditions. All animal procedures were approved by the ethics committee of the Universidad Autónoma de Madrid and were conducted in accordance with institutional guidelines that comply with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.
Circulation
October 5, 2010

Figure 2. Inflammation in the acute phase of EAM is enhanced in the myocardium of CD69<sup>−/−</sup> mice. Mice were immunized with complete Freund adjuvant/MyHC-α peptide, and EAM was analyzed at day 21. A, Quantification of myocardium-infiltrating leukocytes (CD45<sup>+</sup> cells) in WT and CD69<sup>−/−</sup> mice with EAM. Representative flow cytometry histograms are shown; numbers correspond to the percentage of leukocytes. B, Analysis of CD69 leukocyte expression in CD45<sup>+</sup> gated leukocytes isolated from hearts of mice injected with PBS (lined/open) or MyHC-α peptide (gray/solid). CD45 side-scatter plot is representative of 3 experiments. C through F, Representative dot plots showing flow cytometry analysis of CD45<sup>+</sup> gated myocardial leukocytes. Quantification of CD11b<sup>+</sup> Gr1<sup>−/−</sup> neutrophils (C), CD11b<sup>+</sup> F4/80<sup>−/−</sup> macrophages (D), CD4<sup>+</sup> and CD8<sup>+</sup> T cells (E), and CD11b<sup>+</sup> CD11c<sup>−/−</sup> dendritic cells (F). Bar charts show percentage estimates and SD (n=5) of different leukocyte subsets. P values are indicated (2-tailed Student t test).

Induction of EAM
CD69<sup>−/−</sup> and control mice were immunized subcutaneously on days 0 and 7 with 100 μg/0.2 mL MyHC-α peptide (MyHC<sub>614−629</sub>) Ac-RSLKLMATLFSTYASADR-OH emulsified 1:1 in PBS/complete Freund adjuvant (1 mg/mL; H37Ra; Difco Laboratories, Detroit, Mich). The acute phase of EAM was analyzed on day 21; the chronic phase was analyzed between days 58 and 61.

Isolation of Heart-Infiltrating Cells
Mice were anesthetized, the chest cavity was opened, and the root of the aorta was exposed and cannulated with a 23-gauge needle connected to a peristaltic pump. The heart was perfused at a constant flow of 10 mL/min with PBS for 3 minutes and then digested by perfusion with collagenase II (0.5 mg/mL; Sigma-Aldrich) and protease XIV (0.5 mg/mL; Sigma-Aldrich) in PBS at a constant flow of 1.1 mL/min for 7 minutes at 37°C. After the heart was removed and minced, and the cell suspension was sequentially filtered through 70-, 40-, and 15-μm strainers (BD Falcon, Miltenyi Biotec, and Sefar, respectively). After 2 washes with PBS/EDTA/BSA, heart-infiltrating leukocytes were isolated by ficoll-isopaque (density=1.121 g/mL) gradient centrifugation.

Ex Vivo Culture and Analysis of Cytokine Production
Lymph node cells and splenocytes from immunized mice were isolated and restimulated with 10 μg/mL MyHC-α peptide for 20 and 48 hours. Production of inflammatory cytokines was quantified with flow cytomix beads (Bender MedSystems). Intracellular cytokines produced by CD4<sup>+</sup> T cells were measured after exposure of cultures to MyHC-α for 72 hours.

Adoptive Transfer Experiments
Wild-type (WT) and CD69<sup>−/−</sup> mice were immunized as for EAM induction, and axillary-draining lymph nodes were removed at day 10. CD4<sup>+</sup> T cells were isolated with antibody-coupled paramagnetic beads (Miltenyi). In vitro differentiation toward Th17 was done by culture for 5 days with γ-irradiated (30 Gy) antigen-presenting cells, MyHC-α peptide (50 μg/mL), anti-IFN-γ (4 μg/mL), anti-IL-4 (4 μg/mL), recombinant mouse IL-6 (10 ng/mL), recombinant mouse IL-23 (10 ng/mL), and recombinant human transforming growth factor-β1 (5 ng/mL). Th17-like cells (8×10<sup>6</sup>) were collected, washed, and injected intravenously into sublethally γ-irradiated (6 Gy) WT and CD69<sup>−/−</sup> recipient mice. Animals were monitored by transthoracic echocardiography before and after adoptive transfer to evaluate the evolution of the disease. Mice were killed at day 12 after transfer, and heart infiltrates were analyzed by fluorescence-activated cell sorter.

Chimeric Mice
Ten- to 12-week-old CD69<sup>+/+</sup> and CD69<sup>−/−</sup> female mice were lethally γ-irradiated with 2 doses of 6.5 Gy and transplanted with a mixture of 4×10<sup>6</sup> bone marrow (BM) cells from RAG2<sup>−/−</sup> and CD69<sup>−/−</sup> or RAG2<sup>−/−</sup> and WT mice in a proportion of 3:1 respectively. The reconstituted mice were subsequently analyzed 21 days after immunization with the MyHC-α peptide for their susceptibility to EAM.
Statistical Analysis

Experiments were performed by using a randomized complete block design (4 levels of treatment at 3 different times) or under a complete randomized design. Four or 5 independent replicates for each experimental condition were obtained as indicated. To determine significant differences, data were analyzed by 2-way ANOVA, 1-way ANOVA followed by Bonferroni posthoc multiple-comparisons test, or paired or Student t test. When data did not show normal distribution, they were analyzed by the 2-tailed Mann–Whitney U test. Analysis was performed by with GraphPad Instat version 3.0 software.

Results

Induction of EAM Produces an Enhanced Th17 Inflammatory Response in the Lymphoid Organs of CD69-Deficient Mice

After the induction of EAM as described previously, the immune response in the acute phase was characterized in WT and CD69−/− mice. We detected that the axillary-draining lymph nodes and spleens of CD69−/− EAM mice had significantly higher levels of activated/memory (CD44+CD62LlowCD4+) T cells than WT mice (Figure IA and data not shown). However, no significant differences were detected in the percentages of CD4+, CD8+, or CD25+ T cells or B220+ B lymphocytes (Figure IA through IC in the online-only Data Supplement).

We then assessed whether CD69 affects the cytokine secretion pattern in EAM. We found that IL-17 was the major cytokine produced by lymph node cells from EAM mice and that CD69−/− cell cultures showed significantly higher levels of this cytokine than controls (Figure 1B). Enhanced IL-17 production by CD69−/− lymph node cells was also evident after 20 hours (Figure IIA in the online-only Data Supplement). Furthermore, the inflammatory cytokines IFN-γ and IL-22 were enhanced in the supernatants of CD69−/− axillary-draining lymph node cultures (Figure 1B). Intracellular staining of CD4+ T cells revealed that the proportion of IL-17+ cells was higher in the CD69−/− cultures, whereas the percentage of IFN-γ+ CD4+ cells was similar in WT and CD69-deficient cultures (Figure 1C), indicating that the percentage of Th17 cells is enhanced in CD69−/− axillary-draining lymph nodes during the acute phase of EAM.
Similar results were observed when splenocytes were analyzed (Figure 1D and 1E), and again this difference was evident after 20 hours of culture (Figure IIB in the online-only Data Supplement). These results indicate that CD69−/− mice mount an enhanced inflammatory response in the acute phase of EAM, mediated mainly by Th17 lymphocytes.

**CD69 Deficiency Increases the Inflammatory Immune Response in Heart Myocardium**

Analysis of CD45+ infiltrating cells from the myocardium of WT EAM mice showed a significant increase in CD69+ leukocytes compared with PBS-injected controls (Figure 2A and 2B). The number of CD45+ cells in CD69−/− EAM hearts was 2-fold greater than in immunized WT mice (Figure 2A). Phenotypic characterization of the subsets of heart-infiltrating leukocytes revealed a significant increase in neutrophils (CD11b+Gr-1high) and macrophages (CD11b+F4/80+) in the inflammatory cell infiltrate of CD69−/− EAM mice (Figure 2C and 2D), indicating greater disease severity.29 However, no significant differences were detected in the percentages of CD4+ or CD8+ T lymphocytes or dendritic cells (CD11blowCD11c−; Figure 2E and 2F).

Cardiac inflammation was further characterized by hematoxylin and eosin staining of ventricular sections in the acute phase of EAM. These studies revealed larger areas occupied by heart-infiltrating cells in the myocardium of CD69−/− mice (Figure 3A). Quantitative histoscore analysis showed higher numbers of moderately to severely inflamed areas in the hearts of CD69−/− EAM mice compared with WT (Figure 3B). Accordingly, immunofluorescence analysis of myocardial sections in the acute phase of the disease revealed enhanced levels of platelet endothelial cell adhesion molecule-1/CD31 and CD68+ macrophages and Gr-1+ neutrophils in the hearts of CD69−/− EAM mice (Figure 3C and 3D).

CD69−/− mice developed a more exacerbated form of EAM than WT mice, with a significantly higher increase in the ratio of heart weight to body weight (HW/BW; Figure 4A). Accordingly, hearts from CD69−/− EAM mice were larger than those from WT animals (Figure 4A, upper and lower). After the onset of inflammation, leukocytes and myocytes become apoptotic, and there was evidence of necrosis of cardiac muscle resulting in cardiac fibrosis.32,33 Masson trichrome staining in sections from the chronic phase of EAM scored as previously described34 showed a significantly greater extent of myocardial fibrosis in CD69-deficient hearts compared with WT in the chronic phase of EAM (Figure 4B and 4C).

**CD69−/− Mice Exhibit Severe Ventricular Dysfunction**

A pilot electrophysiological study with implantable telemetry revealed a 3-fold increase in the number of sinus pauses in EAM CD69−/− mice compared with WT (Figure 5A and data not shown). We then assessed cardiac function in the chronic phase of EAM in WT and CD69−/− mice by transthoracic echocardiography. M-mode echocardiography of left ventricular function revealed a reduction in cardiac contractility in immunized CD69−/− mice (Figure 5B). Both left ventricular fractional shortening and ejection fraction (EF) in CD69-deficient EAM mice were significantly diminished compared with immunized WT mice (Figure 5C). Analysis of B-mode EF distinguished groups of CD69−/− mice with extensive myocarditis from the control group but could not discriminate the mild extension of myocarditis of WT EAM mice from the nonimmunized control group (Figure 5D). However, visual scoring detected significant differences between all groups (see the Methods section in the online-only Data Supplement). In comparison, B-mode EF measured by segmentation of the left ventricular endocardial edge in several planes has
the advantage of yielding objective data on global function (Mov­ies I through IV in the online-only Data Supplement). Using this global disease severity score, we found that the absence of CD69 alters cardiac function and impairs heart contractility in EAM (Figure 5E).

Adoptive Transfer of Th17 Cells From CD69→−/− EAM Mice Reproduces Exaggerated Inflammation in Nonimmunized WT Mice

We transferred exogenously differentiated Th17 cells from WT or CD69→−/− EAM mice into nonimmunized WT and CD69→−/− recipient mice (Figure 6A). Th17-polarized cell populations from WT and knockout mice contained similar proportions of IL-17+ and IFN-γ+ cells, and there were no differences in their expression levels of activation markers (Figure III in the online-only Data Supplement).

Trans­thoracic echocardiography revealed a significant decrease in heart contractility in most of the mice tested on day 12 (Figure IV in the online-only Data Supplement). Inter­restingly, we detected CD69-expressing cells within CD69→−/− mouse hearts, indicating that Th17 cells transferred from WT donors are able to migrate to the myocardium (Figure 6B). Moreover, the hearts of mice injected with CD69→−/− Th17 cells contained twice as many infiltrating neutrophils as the hearts of mice injected with WT Th17 cells (Figure 6C). These data demonstrate that CD69→−/− Th17 cells can induce an increase in heart infiltration independently of the genotype of the recipient.

To ascertain the role of CD69 expression on the lymphocyte compartment, we performed chimera experiments. Mice reconstituted with a mixture of BM from RAG2→−/− and CD69→−/− BM cells had a higher HW/BW ratio (Figure 6D) and larger number of infiltrating neutrophils (Figure 6E) than mice reconstituted with RAG2→−/− plus WT BM cells. Moreover, the increase in the disease parameters observed after reconstitution with RAG2→−/− plus CD69→−/− BM cells was independent of the genotype of the recipient mice. Therefore, these data unequivocally demonstrate that CD69 exerts its function within the lymphocyte compartment.

Discussion

In this study, we show that CD69→−/− mice develop a severe form of autoimmune myocarditis that progresses to DCM and heart failure. This pathogenic effect is exerted mainly through an increased Th17-mediated inflammatory response. Moreover, adoptive transfer of Th17 cells from immunized CD69→−/− mice and chimera experiments with RAG2→−/− and CD69→−/− BM cells reproduce severe myocardial inflammation in WT recipients, mimicking the phenotype observed in CD69→−/− mice immunized with MyHC-α peptide. Thus, our data indicate that CD69 is an impor­tant negative regulator of the pathogenesis and tissue damage observed in EAM. It is
very feasible that a similar phenomenon occurs in humans with myocarditis and subsequent heart failure.

The involvement of Th17 lymphocytes in the pathogenesis of EAM has previously been documented.12,13 Hence, mice lacking T-bet, a T-box transcription factor essential for Th1 lineage differentiation, develop severe autoimmune myocarditis with an enhanced IL-17 production by heart-infiltrating lymphocytes.12 Moreover, IFN-γ−/− deficient mice also develop a severe form of EAM that leads to DCM and heart failure.15,16,35 In contrast, IL-12 receptor α1/β1, STAT-4/−/− and STAT-4/−/− mice do not develop myocarditis,11 indicating that IL-12 might be required for the development of the disease, although the p40 subunit of this receptor is shared with IL-23 and IL-12p40−/− deficient mice are resistant to EAM.13 Thus, all these data suggest that IL-23, rather than IL-12, is a determining factor in the development of myocarditis. IL-23 is also involved in the production of IL-17 and in the survival of Th17 cells.36 Nevertheless, recent data indicate that IL-15/−/− mice develop severe myocarditis with low levels of IL-17 in the heart. In this setting, the severity of the disease might be a consequence of a compensatory enhancement in Th1 responses, resulting in high levels of IFN-γ, which, in turn, increase the population of classic macrophages and the levels of proinflammatory cytokines involved in the enhancement of the disease.37 Thus, these data indicate that cytokines from both Th1 and Th17 cells are essential to the pathogenesis of EAM.38

Remarkably, our data demonstrate that Th17 cells transferred from CD69−/− mice into WT mice induced a stronger immune cell infiltrate in the myocardium, supporting the notion that CD69 acts as a regulator of heart inflammation and function. This is in accordance with the higher infiltration by neutrophils (CD11b+ and Gr-1+) in the myocardium 12 days after Th17 cell transfer. D, CD69 WT and knockout (KO) mice were lethally irradiated and reconstituted with a mix of BM cells from RAG2−−/− plus CD69−/− or RAG2−−/− plus CD69+/+ mice. HW/BW ratios of individual chimeric mice after the induction of EAM are shown (dots); horizontal bars represent means. Representative myocardial cross sections are shown below the chart. E, Analysis of infiltrating neutrophils in the hearts of chimeric mice after reconstitution and 21 days after EAM induction. Data correspond to the arithmetic mean and SD (n=6), and P values are indicated (1-way ANOVA and Bonferroni multiple-comparisons test). Ax-dLNs indicates axillary-draining lymph nodes.

Figure 6. Adoptively transferred CD69−/− Th17 cells can induce severe myocarditis in WT mice. A, WT and CD69−/− Th17 cells were generated by sensitizing mice to MyHC-α peptide followed by in vitro derivation (see Methods) and injected into either WT or CD69−/− recipient mice. B, Flow cytometry analysis of the expression of CD69 on CD45+ and CD4+ cells infiltrating the myocardium 12 days after transfer. C, Analysis of inflammation in recipient hearts. Bars represent the proportion of infiltrating neutrophils (CD11b+ and Gr-1+) in the myocardium 12 days after Th17 cell transfer. D, CD69 WT and knockout (KO) mice were lethally irradiated and reconstituted with a mix of BM cells from RAG2−−/− plus CD69−/− or RAG2−−/− plus CD69+/+ mice. HW/BW ratios of individual chimeric mice after the induction of EAM are shown (dots); horizontal bars represent means. Representative myocardial cross sections are shown below the chart. E, Analysis of infiltrating neutrophils in the hearts of chimeric mice after reconstitution and 21 days after EAM induction. Data correspond to the arithmetic mean and SD (n=6), and P values are indicated (1-way ANOVA and Bonferroni multiple-comparisons test). Ax-dLNs indicates axillary-draining lymph nodes.
characterized by chronic ventricular dilatation with normal or reduced left ventricular wall thickness and deficient contraction.\(^1\)\(^,\)\(^4\)\(^,\)\(^1\) \(^4\) During the late stage of myocarditis, inflammation is replaced by reparative cardiac fibrosis and cardiac remodeling, resulting in cardiac dilatation and loss of contractile function of the heart.\(^3\)\(^,\)\(^4\)\(^,\)\(^2\) Consistent with this, our data show increased myocardial tissue fibrosis in the hearts of CD69\(^{-/-}\) mice compared with WT counterparts. Accordingly, hearts of CD69\(^{-/-}\) mice are enlarged compared with WT hearts and the HW/BW ratio of CD69\(^{-/-}\) mice is significantly increased.

Analysis of mouse ECGs has shown episodes of heart block that appear to be strain dependent.\(^4\)\(^,\) Our electrophysiological study with implantable telemetry\(^4\) revealed a higher number of sinus pauses in CD69\(^{-/-}\) mice than in WT animals, indicating that CD69 deficiency alters cardiac function. This finding is supported by our analysis of cardiac function by echocardiography, which identified a severe defect in left ventricular contractility in the chronic phase of EAM in CD69-deficient mice compared with the mild defect observed in WT mice. Together, our findings demonstrate an important role for CD69 in the control of the severity of myocarditis and subsequent DCM. Additional evidence that CD69 regulates inflammation through the control of Th17 immune responses is provided by the fact that EAM-induced Th17 cells from CD69\(^{-/-}\) mice adoptively transferred into nonimmunized WT mice reproduce the severity of the inflammation within the myocardium.

Conclusions

Our study supports an unexpected role for the leukocyte antigen CD69 as a brake on the progression and severity of autoimmune myocarditis and the development of DCM. In addition, our study paves the way to investigations into whether defects in CD69 expression or function influence the development of DCM in humans. These findings increase our knowledge of the development of myocarditis, providing a cellular and molecular basis for the development of novel specific therapies.

Acknowledgments

We thank R. González-Amaro and S. Bartlett for comments and critical reading of the manuscript, Pedro López-Romero for assistance with the statistical analysis of the data, and Miguel González for his excellent technical support.

Sources of Funding

This work was supported by grant SAF2008-02719 from the Spanish Ministry of Science and Innovation to Dr Martín and grants SAF2008-02635 from the Spanish Ministry of Science and Innovation, INSINET 01592006 from Comunidad de Madrid, and RECAVA (RD06/0014-0030) from the Instituto de Salud Carlos III to Dr Sánchez-Madrid. Dr Martín is an investigator from Spanish Ministry of Science and Innovation “Ramón y Cajal Program” (Ryc-2006). The Centro Nacional de Investigaciones Cardiovasculares Carlos III is supported by the Ministry of Science and Innovation and the Pro Centro Nacional de Investigaciones Cardiovasculares Carlos III Foundation.

Disclosures

None.

References

15. Eriksson U, Kurrer MO, Sebild W, Brombacher F, Kopf M. Dual role of the IL-12/IFN-gamma axis in the development of autoimmune myo-

Cruz-Adalia et al

CD69 Regulates Inflammation and Heart Failure 1403


**CLINICAL PERSPECTIVE**

The mechanisms involved in the immune activation observed during the progression of human chronic heart failure and dilated cardiomyopathy have not been fully elucidated. However, a number of recent studies suggest that, besides genetic susceptibility and infections, chronic immune-mediated inflammation after acute myocarditis may lead to dilated cardiomyopathy. This report investigates the role of the leukocyte activation antigen CD69 in the modulation of the inflammatory response in a mouse model of autoimmune myocarditis. Our data demonstrate that CD69, through the regulation of Th17 effector responses, limits myocardial inflammation and subsequent heart failure. It is very feasible that a similar phenomenon occurs in humans with myocarditis and subsequent dilated cardiomyopathy. These findings reveal the involvement of a novel molecular actor in the immunopathogenesis of myocarditis, which could be a potential therapeutic target.
CD69 Limits the Severity of Cardiomyopathy After Autoimmune Myocarditis

Aranzazu Cruz-Adalia, Luis Jesús Jiménez-Borreguero, Marta Ramírez-Huesca, Isabel Chico-Calero, Olga Barreiro, Érica López-Conesa, Manuel Fresno, Francisco Sánchez-Madrid and Pilar Martín

Circulation. 2010;122:1396-1404; originally published online September 20, 2010; doi: 10.1161/CIRCULATIONAHA.110.952820

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/122/14/1396

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2010/09/16/CIRCULATIONAHA.110.952820.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

CD69 limits the severity of cardiomyopathy after autoimmune myocarditis

Short Title; CD69 regulates inflammation and heart failure

Aranzazu Cruz-Adalia, PhD\textsuperscript{1}; Luis Jesús Jiménez-Borreguero, MD\textsuperscript{2,3}; Marta Ramírez-Huesca, BSc\textsuperscript{1}; Isabel Chico-Calero, DVM, PhD\textsuperscript{5}; Olga Barreiro, PhD\textsuperscript{1}; Erica López-Conesa, DVM, PhD\textsuperscript{6}; Manuel Fresno, PhD\textsuperscript{5}; Francisco Sánchez-Madrid, PhD\textsuperscript{1,4} and Pilar Martín, PhD\textsuperscript{1}

1 Dept. Vascular Biology and Inflammation. Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid 28029, Spain.
2 Dept. Atherothrombosis and Cardiovascular Imaging. Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid 28029, Spain.
3 Servicio de Cardiología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid 28006, Spain.
4 Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid, Madrid 28006, Spain.
5 Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Cantoblanco, Madrid 28049, Spain.
6 Animal Facility Unit. Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid 28029, Spain.

Correspondence to Pilar Martín, Department of Vascular Biology and Inflammation. Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC). Melchor Fernández Almagro, E-28029 Madrid, Spain. E-mail pmartinf@cnic.es
Phone: 34-91-4531200, Fax: 34-91-4531265
Supplemental Methods

**FACS analysis.** Cell suspensions were stained with fluorochrome-conjugated mouse-specific antibodies against CD3, CD4, CD8α, CD45.2, CD11c, CD11b, CD69, CD25, B220, Gr-1, IFN-gamma and IL-17. All antibodies were purchased from BD Biosciences. Before intracellular staining, cells were re-stimulated for 4 h with 50ng/ml PMA and 500ng/ml ionomycin in the presence of 1ug/ml brefeldin. Samples were analyzed in a FACSCalibur flow cytometer. For analysis of heart-infiltrating cells, data were acquired until at least 100 000 events were collected from a live gate using forward/side scatter plots. Surface markers of interest were analyzed by gating CD45⁺ leukocytes in the CD45⁺/side scatter plots.

**Histopathology and immunofluorescence analysis.** Hearts were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were cut and stained with Haematoxilin-eosin to determine the level of inflammation and with Masson’s thichrome to detect collagen deposition. Histological analysis was performed on a Nikon Eclipse 90i microscope. H&E stained sections were scored for inflammation as follows: 0, no inflammatory infiltrates; 1, small foci of inflammatory cells; 2, larger foci of >100 inflammatory cells; 3, 10-30% of a cross section occupied by inflammatory cells; 4, >30% of a cross section occupied. Myocardial fibrosis was evaluated by histopathological microscopy of the percent area of myocardium with fibrosis as described by Blyszczuk P, et al.. For immunofluorescence, hearts were embeded in “Tissue-Tek” tissue-freezing medium (O.C.T.™, Sakura) and cryosections were
immunolabeled for CD31, CD68 and Gr-1. Nuclei were stained with Hoescht. Glass coverslips were mounted in Prolong and examined with a Leica TCS SP5 confocal system.

**Echocardiography.** Mice were anaesthetized by inhalation of isoflurane/oxygen (1.25%/98.75%) and examined by a 30MHz transthoracic echocardiography probe. Images were obtained with Vevo 770 (VisualSonics, Toronto, Canada) from immunized CD69 knockout and WT mice during the chronic phase of EAM. Short-axis and long axis, B-Mode and 2D M-Mode views were obtained as described previously. From these images, left ventricle (LV) function was estimated by three validated methods as the shortening fraction (SF) and ejection fraction (EFm) obtained from the M mode, quantitative ejection fraction measured from the B mode (EFb), and visual assessment from the B mode by an expert in echocardiography in a blind fashion. For SF measurements a long or short axis view of the heart was selected to obtain an M mode registration in a line perpendicular to the LV septum and posterior wall at the level of the mitral chordae tendineae. Diastolic and systolic manual segmentation of the LV endocardial edge in four chambers views of the heart was used to quantify EFb by the formula;
Ventricular volumens;

\[
\frac{4\pi}{3} \times \frac{\text{diameter}}{2} \times \left( \frac{\text{area}}{\pi \left( \frac{\text{diameter}}{2} \right)^2} \right)^2
\]

Ejection fraction from B Mode (EFb):

\[
\frac{\text{Endocardial volume (diastole)} - \text{Endocardial volume (systole)}}{\text{Endocardial volume (diastole)}} \times 100
\]

Visual evaluations of the regional and global LV wall motion were made in real time in B mode studies in long axis views and along the short axis views from the base to the apex of the left ventricle and were recorded for a second visual evaluation. LV regional and global function was scored as follows: normal (1), regional hipokinesia in more than one segment but not in all (2), regional akinesis in more than one segment (3), and global dysfunction in all segments (4). This method evaluates regional wall motion abnormalities globally and in greater detail compared to M mode and B mode. Regional kinetic abnormalities in mice with myocarditis in segments other than the LV septum and the postero-lateral wall are outside the region where FS and EFm are measured.

**Telemetric ECG recordings.** Telemetric surface ECG recordings, analogous to Holter monitoring in humans, were obtained from mice fitted with implanted transmitters. Mice were administered with analgesics and were anesthetized with isofluorane. A wireless radiofrequency transmitter (TA10ETA-F20; DataSciences International, St. Paul, MN) was aseptically implanted into the abdominal cavity, with subcutaneous leads placed in the conventional lead II position. Animals were individually housed, with each cage placed on top of a computer-linked receiver to detect and record biopotential signals for
display and later analysis. Analgesic drugs were administered in drinking water for a week post-surgery. All recordings were performed on conscious, free-moving animals under the same environmental conditions. The analog output option for the telemetry system was used to produce a high-level, single-channel, analog electrocardiogram. Surface ECG digital recordings were stored on a computer and subsequently analyzed with DSI-custom software.
Supplemental Figures and Figures Legends

Supplemental Figure 1. Normal subsets of lymphocytes after MyHC-α peptide immunization. Ax-dLNs from immunized mice were isolated and re-stimulated with 10μg/ml MyHC-α peptide. Cell suspensions were collected 21 days later and stained with fluorochrome-conjugated mouse-specific antibodies against CD4, CD8α A, CD25 B and B220 C. Bar charts show means +/- SD of 3 independent experiments performed with 5 mice per condition.
Supplemental Figure 2. Cytokine response in Ax-dLNs and spleen in WT and CD69−/− mice after MyHC-α immunization. Analysis of cytokine profiles of ax-dLNs (A) and spleens (B) 21 h post inoculation and re-stimulated in vitro with MyHC-α peptide for 20 h. Production of proinflammatory cytokines was assessed by Flow Cytomix cytokine array and analyzed by FACS after 21 hours. Bar charts show means +/- SD of 3 independent experiments performed with 5 mice per condition; *P<0.05; (Student’s t-test).
Supplemental Figure 3. Activation status of Th17 from WT and KO mice. Analysis of in vitro differentiated Th17 cells from WT and CD69 KO mice. (A) Quantification of IFNγ+ and IL-17+ cells by intracellular staining and flow cytometry. (B) Histograms represent the levels of different activation markers for Th17 WT and KO cells. (C) Dot plots show the percentage of naïve (CD44CD62L+) and memory (CD44+CD62L-) T cells.
Supplemental Figure 4. Evaluation of the progression of the disease by transthoracic echocardiography. Mice were monitored by transthoracic echocardiography before (day 1) and after (day 12) adoptive transfer to evaluate the evolution of the disease.
Supplemental Video Legend

Four representative videos of LV short axis views performed by transthoracic echocardiography. Visual evaluations of the regional and global LV wall motion were made in real time in B mode studies and were recorded for visual evaluation.

Video 1 and Video 2 show LV short axis view from WT and CD69−/− control mice respectively. It is appreciated normal ventricular contractility in both mice. The WT control mouse revealed 70% of EFb and 51% of SF and the CD69−/− control mouse 61% of EFb and 37% of SF.

Video 3 shows LV short axis view from immunized WT mouse during the chronic phase of EAM. EFb and SF obtained from this mouse were 54% and 38% respectively.

Video 4 illustrates LV short axis view corresponds to an immunized CD69−/− mouse during the chronic phase of EAM. It is appreciated hypokinesia in the inferior interventricular septum as indication of localized myocarditis. EFb and SF calculated from this mouse correspond to 44% and 27% respectively.