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An evidence for surface expression of an immunogenic epitope of sarcoplasmic/endoplasmic reticulum calcium-ATPase2a on antigen-presenting cells from naive mice in the mediation of autoimmune myocarditis

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Abstract

We recently reported identification of sarcoplasmic/endoplasmic reticulum calcium-ATPase2a (SERCA2a) 971–990, which induces atrial myocarditis by generating autoreactive T cells in A/J mice. However, it was unknown how antigen-sensitized T cells could recognize SERCA2a 971–990, since SERCA2a-expression is confined to an intracellular compartment. In this report, we present evidence that antigen-presenting cells (APCs) from lymphoid and non-lymphoid organs in naïve animals present SERCA2a 971–990 and stimulate antigen-specific T cells. Using major histocompatibility complex (MHC) class II dextramers for SERCA2a 971–990, we created a panel of T cell hybridomas and demonstrated that splenocytes from naïve A/J mice stimulated the hybridoma cells without exogenous supplementation of SERCA2a 971–990. We then recapitulated this phenomenon by using SERCA2a 971–990-specific primary T cells, verifying that the T cell responses were MHC-restricted. Furthermore, SERCA2a 971–990-sensitzed T cells exposed to APCs from naïve mice were found to produce the inflammatory cytokines interferon-γ, granulocyte macrophage colony stimulating factor, and interleukin-17A, which are implicated in

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Competing interests statement:

Bharathi Yalaka is employed by Bristol-Myers Squibb. Chandirasegaran Massilamany is employed by CRISPR Therapeutics. All other authors declare no competing interests.

Conflict of interest

The authors declare no financial or commercial conflicts of interest.

the induction of myocarditis. Finally, while T cells exposed to mononuclear cells (MNCs) obtained from heart and liver also responded similarly to splenocytes, endothelial cells (ECs) generated from the corresponding organs displayed opposing effects, in that the proliferative responses were suppressed with the heart ECs, but not with the liver ECs. Taken together, our data suggest that the surface expression of SERCA2a 971–990 by naïve APCs can potentially trigger pathogenic autoreactive T cell responses under conditions of autoimmunity, which may have implications in endothelial dysfunction.

Keywords

autoreactive T cells; self-antigens; myocarditis; autoimmunity; mouse model; APCs; T cells; SERCA2a

1. Introduction

Autoimmune diseases can result from autoreactive T cells or B cells or both, but detection of autoreactive cells in healthy individuals does not mean that clinical manifestations would ensue in their lifetime. It is well established that developing T and B lymphocytes are expected to recognize self-antigens during maturation processes in their generative organs, thymus and bone marrow, respectively, prior to their export to the periphery. Because weakly recognized lymphocytes are allowed to exit the primary organs as a result of positive selection, detection of autoreactive cells in the periphery would not be surprising (1). Yet, most individuals remain healthy, and how such a tolerance can be maintained is a fundamental question in autoimmunity research.

Mechanistically, various theories have been proposed to suggest that self-tolerance can be broken in genetically susceptible individuals under altered environmental conditions, which may include both exogenous (e.g., exposure to microbial infections) and endogenous (e.g., defects in the maturation processes, faulty regulation) factors (1, 2). It is widely believed that the frequencies of antigen-specific lymphocytes are in the range of 1 in 1×10^5 to 1×10^6 cells in healthy individuals (3). Despite such low frequencies, upon exposure to microbial infections, lymphocytes faithfully respond to foreign antigens, which is one of the cardinal features of adaptive immune cells. By contrast, autoreactive lymphocytes, although present in similar frequencies, are not expected to react to self-antigens (4–7), even though they continuously see the antigens. Whether this continuous exposure is a critical requirement for self-reactive lymphocytes to become tolerant in the periphery is not clear. In support of this proposition, however, transgenic expression of self-antigens has been shown to promote tolerance (8–10). Conversely, it has been held that either autoreactive cells recognizing self-antigens undergo apoptosis or such recognition can be suppressed by the mediation of regulatory T cells (1, 11).

Experimentally, use of animal models has enhanced our understanding of how self-tolerance can be broken, leading to the induction of pathogenic responses. We have been engaged in determining the role of antigen-specific T cells in the causation of organ-specific diseases, in particular, heart (12–16). This work led us to identify at least three intracellular proteins – adenine nucleotide translocator (14), branched chain α-ketoacid dehydrogenase kinase (12),

and sarcoplasmic/endoplasmic reticulum calcium-ATPase2a (SERCA2a) (13) − as autoimmune targets in the development of myocarditis/dilated cardiomyopathy in myocarditis-susceptible, A/J mice. SERCA2a is unique in that it contains at least six immunodominant T cell epitopes. One of these, SERCA2a 971–990, can bind two major histocompatibility complex (MHC) class II alleles (IA^k and IE^k) with varied affinities and induce mainly atrial myocarditis by generating antigen-specific T cells that can transfer disease to naïve animals (13). While preferential induction of myocardial lesions in the atria could be correlated with enhanced expression of SERCA2a in the atria rather than the ventricles (13), it was unknown whether SERCA2a peptide can be displayed by the naïve antigen-presenting cells (APCs) to trigger autoreactive T cell responses. To address this question, we generated SERCA2a 971–990-specific T cell hybridomas and demonstrated that hybridoma cells respond to SERCA2a 971–990 when stimulated by splenocytes from naïve mice without exogenous supplementation of peptide. While we confirmed these findings with primary T cells specific to SERCA2a 971–990, we unexpectedly noted that the endothelial cells (ECs) derived from hearts and livers from naïve mice modulated SERCA2a 971–990-reactive T cell responses differentially, which may have implications in our understanding of endothelial dysfunction that might occur in vascular inflammation and coronary heart diseases.

2. Materials and Methods

2.1. Mice

Male A/J mice (6- to 8-wk-old, $H\text{-}2^a$) obtained from the Jackson Laboratory (Bar Harbor, ME) were maintained in accordance with the Institutional Animal Care and Use Committee guidelines, University of Nebraska-Lincoln, Lincoln, NE. Euthanasia was performed using carbon dioxide as recommended by the Panel on Euthanasia, American Veterinary Medical Association.

2.2. Peptide synthesis

SERCA2a 971–990 (Ac-KISLPVILMDETLKFVARNY), bovine ribonuclease (RNase) 43–56 (VNTFVHESLADVQA), and moth cytochrome C (MCC) 82–103 (FAGLKKANERADLIAYLKQATK) (GenScript, Piscataway, NJ) were synthesized by 9-fluorenylmethyloxycarbonyl chemistry. The purity of peptides was more than 90% as evaluated by high-performance liquid chromatography, and their identities were confirmed by mass spectroscopy. Peptides dissolved in ultra-pure water were aliquoted and stored at −20°C until further use.

2.3. Immunization procedures, and generation of primary T cell cultures

On days 0 and 7, animals were immunized subcutaneously in the inguinal and sternum regions with emulsions of SERCA2a 971–990 (50 μg/animal) prepared in complete Freund's adjuvant containing *Mycobacterium tuberculosis* H37RA extract (5 mg/ml; Difco Laboratories, Detroit, MI, USA) (17, 18). At termination on day 21 post-immunization, single cell suspensions were prepared from the draining lymph nodes. Cells were stimulated with SERCA2a 971–990 (20 μg/ml) at a density of 5×10^6 cells/ml for 2 days in growth medium (RPMI medium supplemented with 10% fetal bovine serum [FBS], 1 mM sodium

pyruvate, 4 mM L-glutamine, 1x each of non-essential amino acids and vitamin mixture, and 100 U/ml penicillin-streptomycin [Lonza, Walkersville, MD, USA])(18), and growth medium containing interleukin (IL)-2 was then added. Cells were then stimulated 3 to 4 times with SERCA2a 971–990 as above, using syngeneic APCs.

2.4. Creation of MHC class II/IA^k or IE^k dextramers and dextramer staining

We recently reported the generation of MHC dextramers, more sensitive reagents than tetramers, and used them to detect antigen-specific T cells in a variety of experimental systems (12, 13, 15, 19–22). We also had demonstrated their utility in generating T cell hybridomas for the central nervous system antigen proteolipid protein (PLP) 139–151 (23). We adopted a similar approach to create SERCA2a 971–990-specific T cell hybridomas. Essentially, A/J mice express two MHC class II alleles: IA^k and IE^k (15). We generated two sets of MHC class II dextramers, with one set each for IA^k (SERCA2a 971–990/RNase 43–56) and IE^k (SERCA2a 971–990/MCC 82–103), where RNase 43–56 and MCC 82–103 were used as controls for IA^k and IE^k molecules, respectively (13). The sequences for all the above were covalently tethered to the β chains of the corresponding MHC class II alleles. In brief, α and β constructs of IA^k and IE^k molecules were expressed in baculovirus in Sf9 insect cells. After affinity-column purifications using anti-IA^k and anti-IE^k antibodies, soluble IA^k/SERCA2a 971–990 and RNase 43–56, as well as IE^k/SERCA2a 971–990 and MCC 82–103 monomers, were biotinylated, and dextramers were derived using streptavidin (SA)/fluorophore-conjugated dextran molecules as we have described previously (21). For dextramer staining, cells were stained with IA^k- (SERCA2a 971–990 and RNase 43–56) or IE^k-(SERCA2a 971–990 and MCC 82–103) dextramers, followed by anti-CD4 FITC (Fluorescein isothiocyanate) and 7-aminoactinomycin-D (7-AAD). After washing, cells were acquired by flow cytometry, and the percent dextramer$^+$ cells were analyzed using FlowJo software (Tree Star, Ashland, OR, USA) (21, 24, 25).

2.5. Derivation of T cell hybridomas specific to SERCA2a 971–990

By using MHC class II dextramers as screening and sorting tools, we generated a panel of 92 T cell hybridoma clones as we have described previously (23), and we selected one clone (80-P8–8-E2), designated hereafter, clone 80 for further characterization. While, antigen-specificity of this clone was determined based on dextramer staining as described above (23), expression of various markers was analyzed by standard flow cytometry.

2.6. Intracellular cytokine staining

T cell hybridomas were briefly stimulated for 4 to 5 hours with an activation cocktail containing phorbol 12-myristate 13-acetate (PMA), ionomycin, and Brefeldin A (Biolegend). After staining with anti-CD4 FITC and 7-AAD, cells were fixed and permeabilized, followed by staining with cytokine antibodies as recommended by the manufacturer (Biolegend). The following cytokine antibodies used were (clone numbers indicated in parentheses): IL-2 (JES6); interferon (IFN)-γ (XMG1.2) (Th1 subset); IL-4 (11B11); IL-5 (TRFK5); IL-13 (eBio13A) (Th2 subset); IL-17A (eBio17B7); IL-17F (eBio18F10); IL-22 (IH8PWSR) (Th17 subset); IL-6 (MP5-20F3); tumor necrosis factor (TNF)-α (MP6-X722); and granulocyte macrophage colony stimulating factor (GM-CSF) (MP1-22E9) (other inflammatory cytokines). In experiments involving T cells sensitized

with SERCA2a 971–990, T cells were cultured with splenocytes from naïve mice in the presence or absence of SERCA2a 971–990 for two days where monensin (1x) was added in the last 12 hours at each of the four time points namely, 6, 12, 24 and 36 hours. After washing and staining with anti-CD4-FITC and 7-AAD, cells were fixed and permebilized and stained with three cocktails of cytokine antibodies namely, IFN-γ-allophycocyanin (APC) and IL-2-phycoerythrin (PE), IL-17A-APC and GM-CSF-PE and their corresponding controls (rat IgG1-APC and rat IgG2a/IgG2b-PE). After acquiring the cells by flow cytometry, percentages of cytokine-producing, viable (7-AAD$^-$)cells within the CD4 subset were analyzed using FlowJo software. In all the treatment groups, while, APC-conjugated, IFN-γ, IL-17A and isotype controls are shown in the Y axis, the PE-conjugated, IL-2, GM-CSF and isotype controls are shown in the X axis in the flow cytometric plots. To determine the frequencies of IFN-γ, IL-17A and their controls, percentage values of the upper left and upper right quadrants were added in their corresponding flow cytometric plots. Similarly, percentage values in the upper right and lower right quadrants were added to obtain the frequencies of cells producing IL-2, GM-CSF and their controls, in their respective plots.

2.7. Proliferative responses of SERCA2a 971–990-specific T cell hybridoma and Primary T cells to splenocytes from naïve mice

To determine whether APCs spontaneously stimulate SERCA2a 971–990-specific T cells, we used both hybridoma cells (clone 80) and primary T cells. For hybridomas, cells were cultured for 24 hours (2.5×10^4 cells/ml) with splenocytes obtained from naïve A/J mice at different ratios (1:1 to 1:20) in the presence or absence of SERCA2a 971–990 or control (MCC 82–103) (50 μg/ml). After pulsing with ^{3}[H] thymidine for 16 hours, proliferative responses were measured as cpm. Similarly, primary T cells were also cultured with splenocytes at a ratio of 1:1 with or without peptides for 2 days, and proliferative responses were measured 16 hours after pulsing with ^{3}[H] thymidine (26–28). In MHC blocking experiments, Fc-blocker-treated splenocytes from naïve mice were cultured with or without T cells supplemented with anti-IA^k (10–2.16, clone TIB 93, 20 μg/ml) and anti-IE^k (M5/114.15.2, clone TIB 120, 100 μg/ml; American Tissue Culture Collection, Manassas, VA, USA) or Rat IgG (control; 100 μg/ml, MP Biomedicals, Santa Ana, CA, USA). The proliferative responses were measured as above (29, 30).

2.8. Determination of SERCA2a 971–990-reactive T cell responses to mononuclear cells (MNCs) generated from heart and liver

Groups of 5 to 6 naïve A/J mice were euthanized, and animals were immediately perfused with 10 ml of ice-cold 1x phosphate-buffered saline (PBS) prior to harvesting of hearts and livers (12). Briefly, hearts were cut into two halves and the pooled tissues were minced and transferred to gentleMACS C Tube (Miltenyi Biotech, San Diego, CA, USA) containing 4.7 ml of Hank's Balanced Salt Solution supplemented with Collagenase II (600 U/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA) and DNase I (60 U/ml; AppliChem GmbH, Darmstadt, Germany). After mixing by MACS Dissociator, samples were incubated for 30 minutes at 37°C using the MACSmix Tube Rotator (Miltenyi Biotec); the tissue suspensions were mixed using the MACS Dissociator. The homogenate was centrifuged and cell suspensions were filtered using a 70-μm cell strainer; after washing, red blood cells were lysed and the debris removed using Debris Removal Solution as

recommended (Miltenyi Biotech). Finally, cell pellets were resuspended in growth medium, and cells were treated with Fc blocker (Biolegend), followed by staining with 7-AAD and anti-CD45. After washing, $CD45^+$ cells within the live subset ($7\text{-}AAD^-$) were sorted by Aria flow cytometry (FACS Aria, BD Biosciences, San Jose, CA, USA). To obtain single cell suspensions from livers, tissues were minced and centrifuged. The homogenate was digested in Hank's Balanced Salt Solution containing Collagenase II (Worthington Biochemical Corporation) and DNase I (AppliChem GmbH) at 37°C for 30 minutes, followed by centrifugation to remove hepatocytes. Cell pellets were washed with 1x PBS, and cell suspensions were then subjected to Percoll density gradient centrifugation. The interphase representing the MNCs was harvested, and, after washing and treating with red blood cell lysis buffer, samples were centrifuged to obtain the MNC fraction. Cells were then treated with Fc blocker, and after staining with CD45 and 7-AAD, $CD45^+$ cells were sorted by flow cytometry as above. Finally, cells derived from both hearts and livers were evaluated for CD11b, CD11c, CD19, and IE^k, as well as for T cell responses, using SERCA2a 971–990-specific primary T cells by ^{3}[H] thymidine-incorporation assay as described above.

2.9. Isolation of primary ECs from hearts and livers and evaluation of T cell responses to the ECs

ECs from hearts and livers of groups of 5 to 6 naïve A/J mice were isolated using the previously published method with some modifications (31–33). In brief, animals were euthanized using 30% isoflurane, and ECs were isolated by positive selection using immunomagnetic beads specific to PECAM-1 (CD31) (34–37). Essentially, the CD31-bound magnetic beads were freshly prepareda day prior to isolating the cells by mixing sheep anti-rat IgG Dynabeads (Thermo Fisher Scientific) and anti-CD31 (Clone: MEC13.3, Biolegend) in a final volume of 500 μl after a series of washing steps. Finally, the CD31-conjugated beads were washed 4 times and resuspended in a volume of 200 μl for further use. To isolate ECs, heart and liver tissues were minced with scissors to make small pieces (1 to 2 mm^3) and digested with Collagenase II and Dispase (1mg/ml each in DMEM/F12; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 37°C for 40 minutes. The digested tissues were washed with DMEM/F12 medium containing 10% FBS; suspensions were filtered using 70-μm and 40-μm meshes and centrifuged at 500 g for 10 minutes at 4°C. Cell suspensions were then incubated with the Dynabeads coated with PECAM-1 (CD31) antibody. The bead-bound cells were collected using BD IMag (BD Biosciences) and washed 3 times. Cells that bound to the magnetic beads were re-suspended and cultured in 0.1% gelatin-coated tissue culture plates in EC growth medium (Cell Biologics). After growing for 5 to 7 days, cells were harvested at >80% confluence with trypsin-EDTA and used for experiments; purity of ECs was ascertained by anti-CD31 staining. For proliferation experiments, ECs and SERCA2a 971–990-sensitized T cells were cultured at a ratio of 1:1 individually or together with or without SERCA2a 971–990 or MCC 82–103 for 2 days, and proliferative responses were measured by pulsing the cells with ^{3}[H] thymidine 16 hours later.

2.10. IFN-γ treatment of ECs

The $CD31^+$ heart and liver ECs plated in gelatin-coated culture flasks (T25 cm^2) were grown to ~80% confluency. On day 7 post-culturing, cells were harvested by trypsinization (0.05%) and treated with or without Mouse IFN-γ (500 ng/ml) (Biolegend) (38). After 3 days, cells

were stained with antibodies for IA^k, IE^k, CD80, CD86, and CD40 molecules and 7-AAD. Cells were acquired by flow cytometry, and percentages of cells positive for each marker were analyzed in the live ($7\text{-}AAD^-$) subset using FlowJo software.

2.11. Statistics

We used Wilcoxon rank-sum followed by Bonferonni multiple test correction to determine differences in T cell responses. Observations for the T cell responses were normalized prior to statistical testing per repeat experiment based on the average sum of input hybridoma or primary T cells and APCs or ECs. $p \leq 0.05$ was considered significant.

3. Results and Discussion

We recently reported that SERCA2a contains multiple antigenic determinants, and one of these epitopes, SERCA2a 971–990, was found to induce atrial myocarditis in A/J mice by generating both T cell and antibody responses (13). However, it was unknown as to how SERCA2a can be recognized by autoreactive T cells. Although our efforts to localize the expression of SERCA2a in cardiac myocytes provided leads to predict that its expression may occur close to the plasma membrane (13), supporting evidence was lacking. Thus, we made an effort to derive T cell hybridomas for SERCA2a 971–990 with the expectation that T cells can respond to antigens if they are displayed by APCs in naïve animals. From a panel of 92 hybridoma clones, we chose clone 80 that was positive for CD3, CD4, TCR vβ10b, CD45, CD5, CD62L, CD44, CD28, PD-1, PD-L1, CD2, LFA-2 and CCR4. We determined antigen specificity of this clone by staining with IE^k and IA^k dextramers for SERCA2a 971–990, expecting that the IE^k-specific clone 80 hybridoma cells should bind only to IE^k, but not IA^k dextramers or controls (MCC 82–103 or RNase 43–56), and it was the case (Figure 1A, top panel). We also verified the cytokine-producing ability of hybridoma cells to be in the order of TNF-α, IL-2, IL-4, and IL-6 (Figure 1B, bottom panel). Similar profiles have been described for other antigens (23).

3.1. Splenocytes from naïve A/J mice trigger antigen-specific T cell responses spontaneously

We took advantage of SERCA2a 971–990-specific T cell hybridomas to test a hypothesis that APCs from naïve animals display MHC/SERCA2a complexes that can trigger antigen-specific T cell responses. First, we cultured the clone 80 cells with splenocytes generated from naïve A/J mice in the presence or absence of SERCA2a 971–990 or MCC 82–103 (control) (39–41). The clone 80 cells responded spontaneously to splenocytes at ratios ranging from 1:1 to 1:20 with ratios up to 1:10 yielded significant differences between treatments (Figure 2). Responses were antigen specific, since SERCA2a 971–990, but not control (MCC 82–103), stimulated clone 80 cells. Of note, proliferative responses obtained by co-culturing splenocytes with clone 80 cells alone, and with control (MCC 82–103) were also comparable. The data suggest that naïve APCs express SERCA2a 971–990 and trigger T cell responses.

To investigate whether splenocytes from naïve animals can stimulate primary T cells, we generated T cell cultures from A/J mice immunized with SERCA2a 971–990. We evaluated

T cell responses to splenocytes pulsed with or without SERCA2a 971–990 or MCC 82–103 (control) at a 1:1 ratio (Figure 3A). We noted that the T cells co-cultured with splenocytes alone responded significantly, and expectedly, SERCA2a 971–990, further increased the response, whereas responses obtained with T cells cultured with MCC 82–103 were comparable with those obtained with splenocytes alone (Figure 3A). Further, we verified the T cell responses to be MHC-restricted, as anti-IA^k and IE^k antibodies significantly blunted the proliferation of T cells co-cultured with APCs alone (Figure 3B, left panel) or with SERCA2a 971–990, but not with control (Figure 3B, right panel). The data suggest that the APCs from naïve mice can induce proliferation of SERCA2a 971–990-sensitized T cells through MHC class II pathway, thereby potentially modulating their functional responses.

3.2. SERCA2a 971–990-sensitized T cells exposed to splenocytes from naïve mice produce inflammatory cytokines implicated in the development of autoimmune myocarditis

We had previously shown that the SERCA2a 971–990-senstized T cells generated from immunized animals produced mainly Th1 and Th17 cytokines that can transfer disease to naïve animals (13). We reasoned that the SERCA2a 971–990-sensitized T cells exposed to APCs from naïve mice produce cytokines similar to those produced in response to SERCA2a 971–990 antigen-specifically. By intracellular cytokine staining, we analyzed the frequencies of cytokine-producing T cells in co-culture experiments, where we cultured SERCA2a 971–990-sensitized T cells with splenocytes (APCs) from naïve animals in the absence or presence of SERCA2a 971–990 or MCC 82–103 (control). Cytokine analysis was performed at four time points (18, 24, 36 and 48 hours) using cocktails containing IFN-γ and IL-2, IL-17A and GM-CSF and their corresponding controls as described in the Methods (Section, 2.6). As shown in Figure 4, T cells with no exposure to APCs, contain a low frequency of cytokine producing cells except IFN-γ, if any at 18 hours (6.3%). In contrast, cytokine-producing cells were absent in the cultures containing APCs-alone. However, upon exposure to APCs, SERCA2a 971–990-sensitized T cells were found to produce the inflammatory cytokines implicated in the induction of various autoimmune diseases including myocarditis (13, 17, 55–57). These include mainly, IFN-γ (63.5%) and GM-CSF (6.0%%), and, to a lesser extent, IL-17A (1.6%%) and IL-2 (1.2%) with their peaks reaching at 24 hours. As expected, cytokine responses were also upregulated and reached peaks at 18 or 24 hours in the presence of SERCA2a 971–990 (IFN-γ, 69.3.4%; GM-CSF, 20.5%; IL-17A, 9.1%; and IL-2, 4.2%) (Figure 4). Likewise, cytokine responses in cultures exposed to control (MCC 82–103) were also comparable to the T cells co-cultured with APCs alone during the same period (IFN- γ, 58%; GM-CSF, 5.0%; IL-17A, 1.6%; and IL-2, 1.0%). These data indicate that the SERCA2a 971–990-sensitized T cells responded to the antigen displayed by the APCs and produce inflammatory cytokines that may have a pathogenic role in the development of autoimmune myocarditis.

3.3. Heart and liver APCs and ECs differentially modulate SERCA2a 971–990-sensitized T cell responses

First, we asked whether APCs derived from non-lymphoid organs can also present SERCA2a 971–990 by isolating MNCs from the hearts and livers of naïve A/J mice. We obtained MNCs from groups of A/J mice, and, after staining with anti-CD45 and 7-AAD,

we sorted $CD45^+CD3^-$ viable ($7\text{-}AAD^-$) cells by flow cytometry. We noted that the $CD45^+$ cells contained cells in the order of B cells ($CD19^+$), followed by macrophages ($CD11b^+$) and dendritic cells ($CD11c^+$) and the MNCs also expressed the MHC class II molecule IE^k (Supplementary Figure 1A). Next, we cultured both heart and liver MNCs with SERCA2a 971–990-reactive T cells as described above. The data revealed enhanced proliferative responses when T cells were cultured with either MNCs alone or with peptides (SERCA2a 971–990 or MCC 82–103). Responses obtained with MCC 82–103 were comparable to those for cells cultured with MNCs alone (Supplementary Figure 1B), and these patterns were very similar to those noted with splenocytes described above. Collectively, the data suggest that APCs present in both lymphoid and non-lymphoid organs can display SERCA2a 971–990 and stimulate antigen-primed T cells. Of note, SERCA exists in multiple isoforms (42–44). While, SERCA2a is preferentially expressed in cardiomyocytes, vascular smooth muscle cells and ECs, SERCA2b can be expressed in APCs like macrophages (45) that also has an equivalent epitope of SERCA2a 971–990. Thus, it is possible that epitope from multiple isoforms can potentially stimulate SERCA2a 971–990-senstized T cells. It is also possible that the APCs in the target organs like heart can acquire self-antigens from other SERCA2a-expressing cells through phagocytosis of dead cells or autophagy that migrate to the spleens (46, 47).

Next, since SERCA2a is known to be expressed in non-professional APCs, we tested a hypothesis that these cells are not expected to stimulate T cells. To this end, we purified $CD31^+$ ECs by magnetic separation from hearts of naïve A/J mice as previously described (31–33). After verifying the purity of ECs to be more than 90% (Figure 5A), we used them in co-culture experiments using SERCA2a 971–990-reactive T cells in a proliferation assay. The data revealed that the T cell responses in cultures involving heart ECs and T cells were blunted (Figure 5B, left panel). However, upon supplementation with SERCA2a 971–990, the T cell responses were further blunted, but not in the cultures exposed to MCC 82–103 (control) (Figure 5B, left panel). We then tested whether liver ECs follow a pattern similar to heart ECs by sorting the ECs from liver (Figure 5A, right panel). In co-culture experiments, ECs stimulated the SERCA2a 971–990-primed T cells (Figure 5B, right panel), and the patterns of T cell responses were similar to those obtained with splenocytes as described above. The finding that the basal proliferative responses obtained with heart ECs were high (Figure 5B), but reduced upon exposure to T cells, suggests that the heart ECs might have been suppressed by T cells leading to their death. But why, then, exposure to SERCA2a 971–990, but not MCC 82–103 led to reduced proliferative responses? Similarly, why such a pattern was lacking with the liver ECs? One possibility is that the ECs in various organs can behave differently, and their phenotypes can differ in the expression of MHC and co-stimulatory molecules (31, 48).

To address the above questions, we examined the expression of MHC class II (IA^k and IE^k), MHC class I ($H\text{-}2D^d$, K^d and L^d), and co-stimulatory (CD80, CD86, and CD40) molecules in both heart and liver ECs by flow cytometry. These analyses revealed that heart ECs lacked the expression of both IA^k and IE^k, CD86, and CD40 and showed only low levels of CD80 (4%) (Figure 6, top left panel). Similar analysis of liver ECs showed the expression of IE^k, but not IA^k, whereas other molecules (CD80, CD86 and CD40) were detected in the range of ~60 to 90% (Figure 6, top right panel). Heart and liver ECs, however, expressed all three

MHC class I molecules as expected (data not shown). We then exposed the heart and liver ECs to IFN-γ, a cytokine known to promote the expression of MHC class II and co-stimulatory molecules, to determine its effects on ECs (49–55). Expectedly, heart ECs treated with IFN-γ revealed upregulation of both MHC class II molecules (IA^k, 49% and IE^k, 21%), as well as CD80 (97%) and CD40 (77%), but expression of CD86 remained unaltered (Figure 6, bottom left panel). Similarly, expression of the MHC class II molecule IA^k in liver ECs, which was lacking prior to exposure to IFN-γ, could now be detected with IFN-γ treatment (Figure 6, bottom right panel). Overall, the data suggest that lack of expression of MHC class II molecules in heart ECs correlates with the absence of proliferative responses of T cells, since MHC class II molecules are needed for antigen presentation. Conversely, since liver ECs express at least one of the two MHC class II molecules (IE^k), they are able to present SERCA2a 971–990 to the responding T cells. This may be the reason the proliferative responses obtained in cultures containing liver ECs and T cells merely tended to be greater (Figure 5B, right panel). While these explanations can be logically construed, it was difficult to explain why the proliferative responses of ECs were blunted in the heart. One possibility is that the SERCA2a 971–990 may contain antigenic determinants for CD8 T cells, and if so, they may potentially induce EC death. Identification of CD8 T cell determinants, if any may provide new insights as to their significance.

In summary, by creating T cell hybridomas for SERCA2a 971–990, we addressed a hypothesis that naïve APCs can spontaneously induce T cell responses antigen specifically. This hypothesis was tested using APCs generated from both lymphoid and non-lymphoid organs, and demonstrate that the responding T cells can produce pro-inflammatory cytokines. Of note, presentation of MHC/peptide complexes has been previously described for other cardiac antigens like cardiac myosin heavy chain-α (27), but the pathologic significance was not tested. Likewise, constitutive presentation of such complexes has also been reported for self-antigens of the central nervous system such as myelin basic protein (MBP) 1–44, MBP 83–102, PLP 139–151 and PLP 178–191 (58). Furthermore, we had demonstrated that A/J mice although contain SERCA2a 971–990-reactive T cells in their naïve periphery (13), they remain healthy (13). It is currently unknown whether enhanced production of inflammatory cytokines (in particular, IL-17A) that occurs in the immunized animals is a missing factor (13). Alternatively, antigen-specific T cells in the naïve animals may have a capacity to induce disease, but they could be potentially suppressed by regulatory T cells. We have not addressed these possibilities. While our data have provided an explanation as to how SERCA2a 971–990-reactive T cells can induce myocarditis (13), the data also revealed new insights into the possibility that SERCA2a 971–990-reactive T cells can potentially induce EC death. This may be an important mechanism because EC dysfunction has been proposed as an important predisposing factor for induction of vascular inflammation and coronary heart diseases like atherosclerosis (59–62).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

RA, BK, CM, XS and JR: Conceived and designed the experiments; RA, BK, SA, NL, and SJ: performed the experiments; JJR, analyzed the data; and RA, XS and JR: wrote the paper.

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List of abbreviations:

SERCA2a	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase2a
MHC	major histocompatibility complex
APCs	antigen presenting cells
ECs	endothelial cells
RNase	bovine ribonuclease
MCC	moth cytochrome C
FBS	fetal bovine serum
^{3}[H]	tritiated-thymidine
cpm	counts per minute
IL	interleukin
SA	streptavidin
7-AAD	7-aminoactinomycin D
RT	room temperature
TCR	T cell receptor
PBS	phosphate-buffered saline
MNCs	mononuclear cells
IFN	interferon
TNF	tumor necrosis factor
GM-CSF	granulocyte macrophage colony stimulating factor
SR	sarcoplasmic reticulum
Th	T helper
PI	propidium iodide
PD-1	programmed cell death protein 1

PD-L1	programmed death-ligand 1
PMA	phorbol 12-myristate 13-acetate
PLP	proteolipid protein
MBP	mylein basic protein
DNase1	deoxyribonuclease I
PECAM	platelet endothelial cell adhesion molecule
Sf	*Spodoptera frugiperda*
DMEM	dulbecco's modified eagle medium
HBSS	hank's balanced salt solution
CCR	chemokine receptor
LFA	lymphocyte function associated antigen
FITC	fluorescein isothiocyanate
APC	allophycocyanin
PE	phycoerythrin

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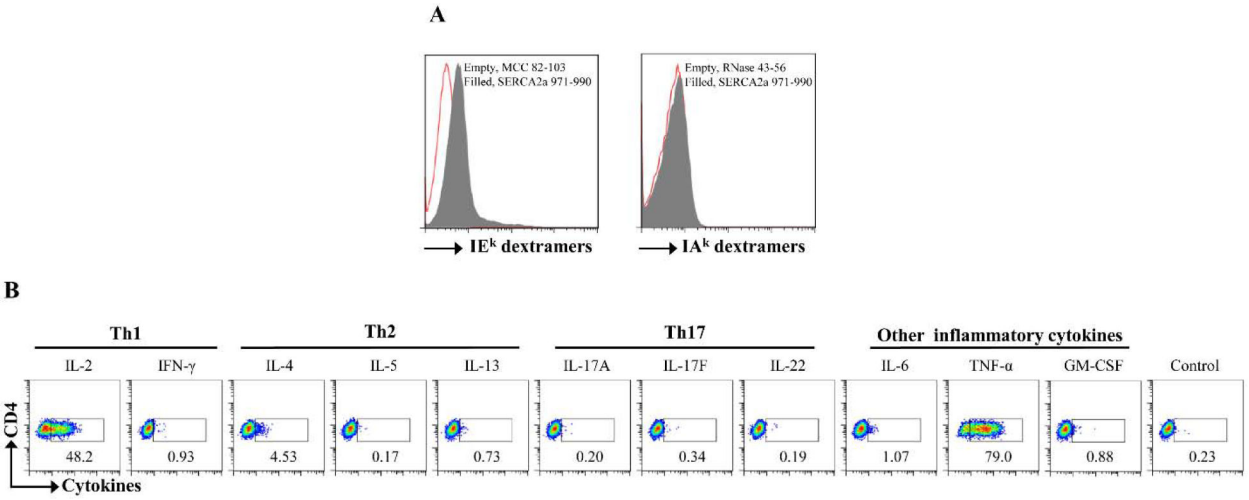

Figure 1. Characterization of hybridoma cells.

(A) Antigen-specificity. Clone 80 hybridoma cells were stained with two sets of dextramers (IE^k/SERCA2a 971–990 and MCC 82–103; and IA^k/SERCA2a 971–990 and RNase 43–56) for 2 hours, followed by anti-CD3, anti-CD4, and 7-AAD. After washing, cells were acquired by flow cytometry and the $dext^+$ cells were determined in the live ($7\text{-}AAD^-$) $CD3^+CD4^+$ subset. **(B) Cytokine analysis.** Hybridoma cells were stimulated with a cocktail of PMA/Ionomycin/GolgiStop for 5 hours. After washing, cells were fixed and permeabilized to stain with the indicated cytokine antibodies and their corresponding controls. After acquiring by flow cytometry, percentages of $cytokine^+CD4^+$ T cells were determined. Representative data from 3 individual experiments are shown.

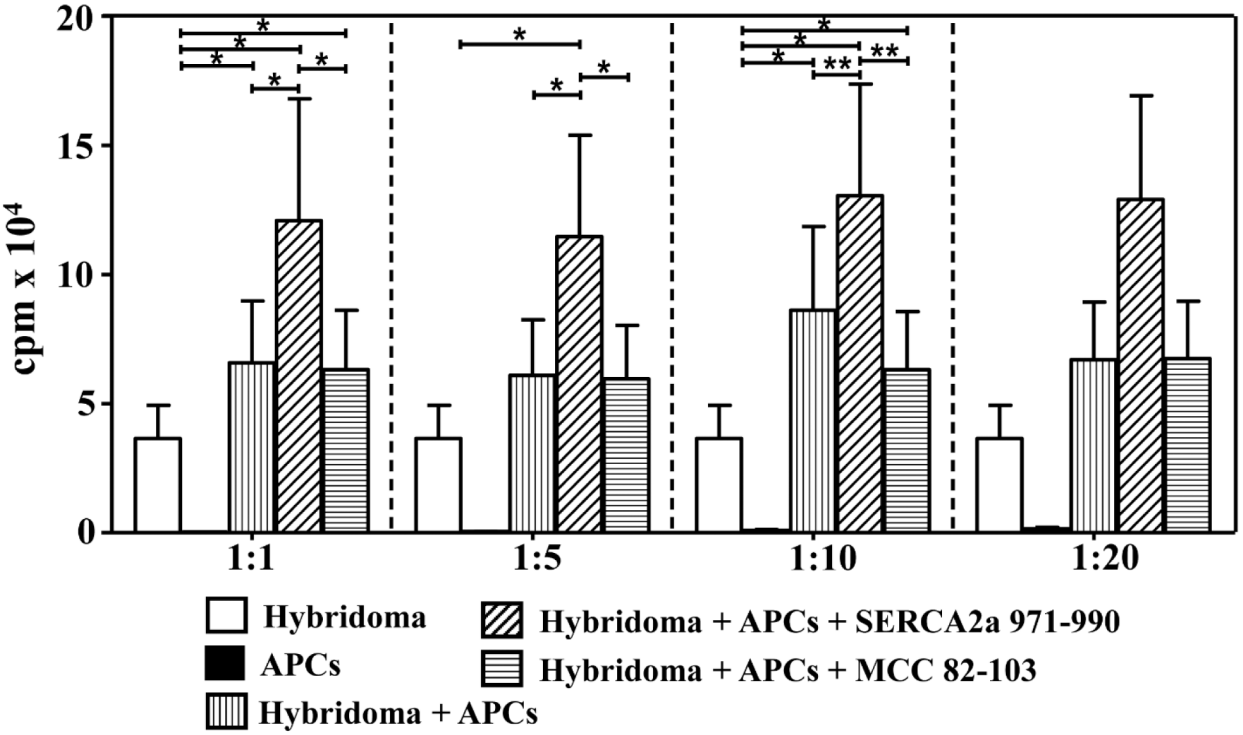

Figure 2. Proliferative responses of SERCA2a 971–990-specific hybridoma cells cultured with or without splenocytes from naïve mice and antigens.

Clone 80 cells were cultured with splenocytes from A/J mice with or without SERCA2a 971–990 (specific) or MCC 82–103 (control) at different ratios for 24 hours. After pulsing the cells with ^{3}[H] thymidine for 16 hours, proliferative responses were measured. Mean ± SEM values are shown (n=3). *p≤0.001, **p=0.023.

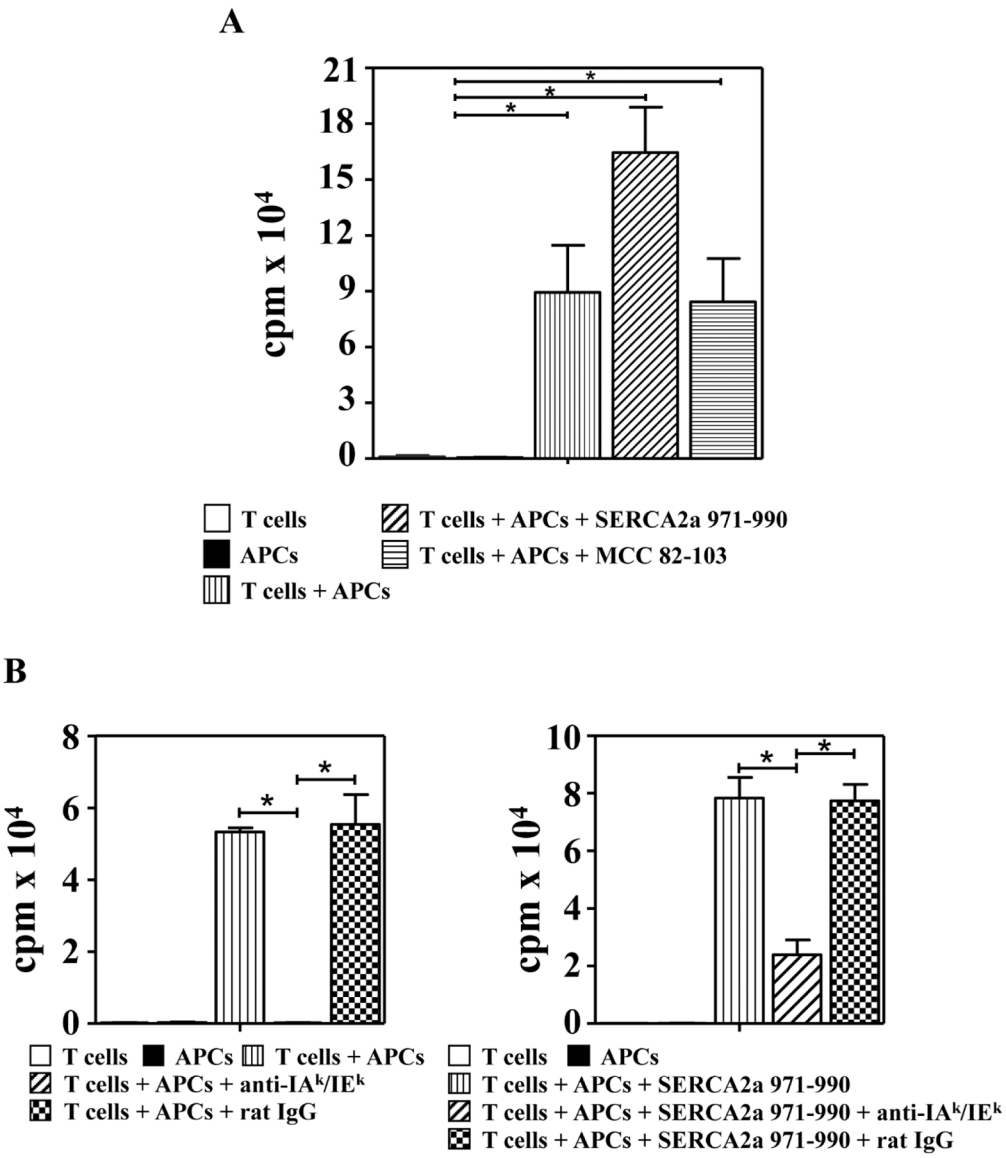

Figure 3. Determination of proliferative responses of SERCA2a 971–990-sensitized T cells to APCs loaded with or without peptides in the presence or absence of MHC antibodies.

(A) Responses to APCs loaded with or without peptides. SERCA2a 971–990-sensitizedT cells were generated from A/J mice immunized with SERCA2a 971–990, and cells were cultured with SERCA2a 971–990 or MCC 82–103 in the presence or absence of splenocytes generated from A/J mice. Forty-eight hours later, cells were pulsed with ^{3}[H] thymidine, and after 16 hours, proliferative responses were measured. **(B) Responses to APCs loaded with or without peptides in the presence or absence of MHC antibodies.** Splenocytes from naïve mice were treated with Fc blocker and cultured with or without T cells in the absence (left panel) or presence of SERCA2a 971–990 (right panel) and anti-IA^{k} and anti-IE^{k} antibodies or rat IgG (control). Proliferative responses were measured as above after pulsing with ^{3}[H] thymidine. Mean ± SEM values are shown (n=3). *p≤0.001,

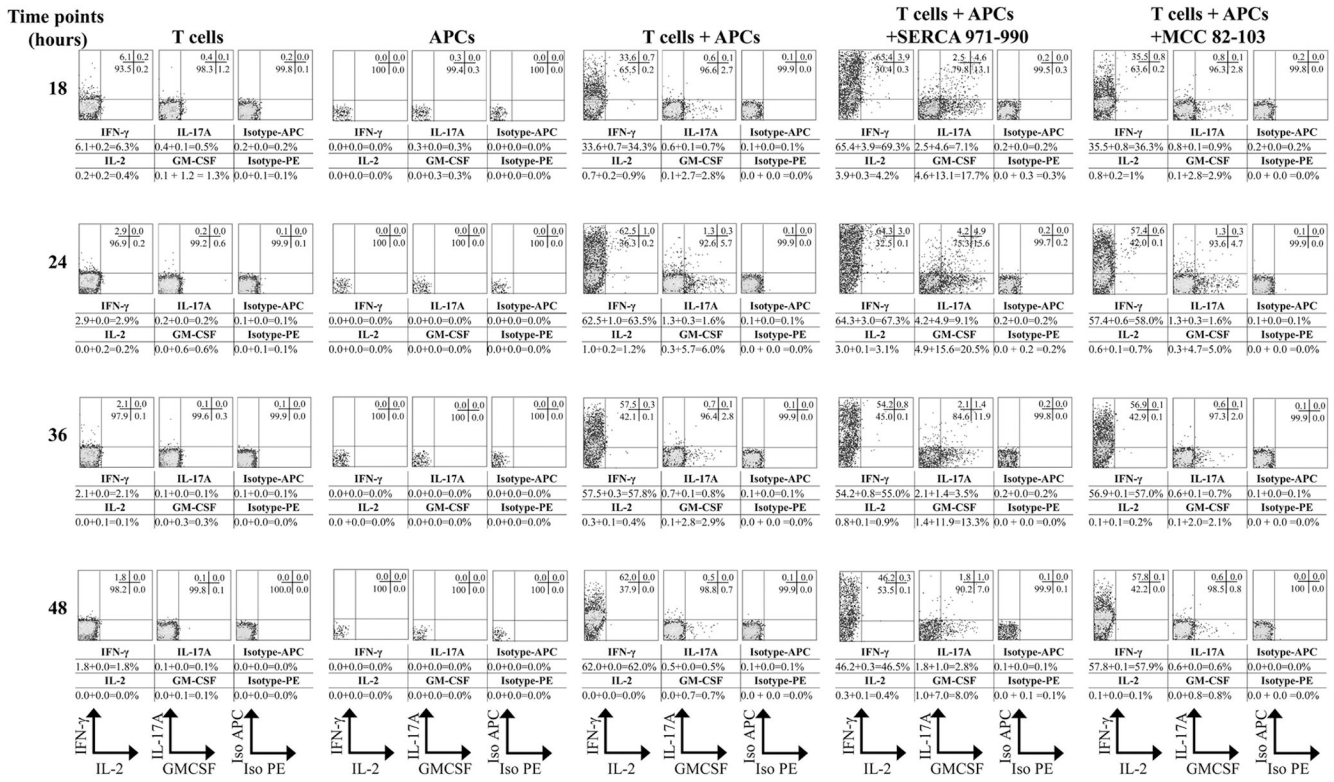

Figure 4. Cytokine responses in SERCA2a 971–990-sensitized T cells exposed to splenocytes from naïve animals.

SERCA2a 971–990-sensitized T cells were cultured with splenocytes from naïve mice supplemented with or without SERCA2a 971–990 (specific) or MCC 82–103 (control) for two days where monensin was added during the last 12 hours in each of the four different time points (18, 24, 36 and 48 hours). At each point, cells were harvested and stained with anti-CD4, 7AAD. After fixation and permeabilization steps, cells were stained with three cocktails of antibodies, each representing IFN-γ-APC and IL-2-PE, IL-17A-APC and GM-CSF-PE and their corresponding isotype controls (rat IgG1-APC and rat IgG2a/IgG2b-PE). Cells were acquired by flow cytometry to analyze the percentages of cytokine-producing CD4 T cells within the live (7-AAD^{-}) subset, where cells producing IFN-γ, IL-17A and their corresponding controls (all APC-conjugated) are shown in the Y axis and the cells producing IL-2, GM-CSF and their controls (all PE-conjugated) are shown in the X axis in the respective plots. Frequencies of cytokine producing cells are shown in the tables below each plot by adding the percentage values of the upper left and upper right quadrants for APC-conjugated cytokine antibodies (IFN-γ, IL-17A and their controls). Similarly, percentage values of the upper right and lower right quadrants were added for PE-conjugated cytokine antibodies (IL-2, GM-CSF and their controls). Representative data sets from 3 individual experiments are shown.

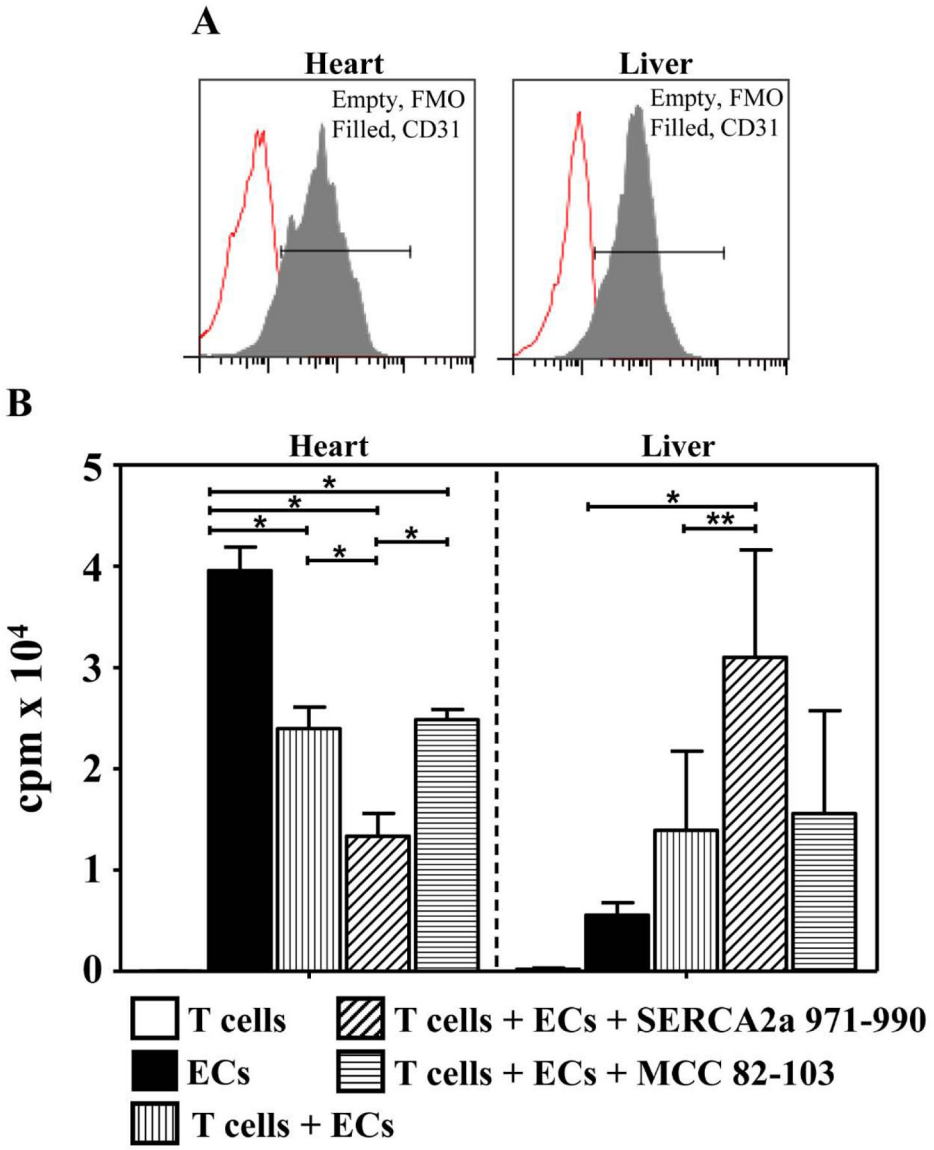

Figure 5. Determination of proliferative responses of SERCA2a 971–990-sensitized T cells cultured with or without ECs.

(A) CD31 staining. Heparin was administered to groups of naive A/J mice, and after 10 minutes, animals were euthanized to collect hearts and livers. Organs were washed with ice-cold DPBS, and tissue homogenates were used to purify ECs as described in the Methods section. Cells were plated in culture dishes pre-coated with gelatin, and after 5 to 7 days they were examined for CD31 expression by flow cytometry. **(B) Proliferative response.** SERCA2a 971–990-sensitized T cells were cultured for 2 days with heart and liver ECs supplemented with or without SERCA2a 971–990 (specific) or MCC 82–103 (control). Cells were pulsed with ^{3}[H] thymidine, and 16 hours later proliferative responses were measured. Mean ± SEM values representing 3 individual experiments with 5 to 6 animals in each are shown. *p≤0.001, **p=0.023.

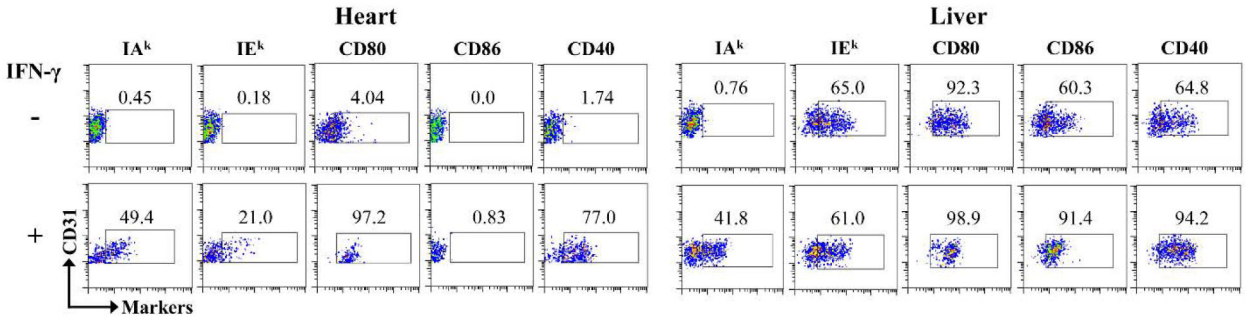

Figure 6. Effect of IFN-γ on ECs.

Naïve A/J mice were used to prepare ECs from hearts and livers as described in the Methods section, and cells were treated with or without IFN-γ for 3 days. After trypsinization, cells were washed and stained with the indicated markers and 7-AAD. Cells were acquired by flow cytometry to analyze the expression of indicated markers with the live (7-AAD^-) $CD31^+$ subset.