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Sex-Defined T-Cell Responses to Cardiac Self Determine Differential Outcomes of Murine Dilated Cardiomyopathy

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Idiopathic dilated cardiomyopathy (DCM) is a disease of putative autoimmune origin that kills males at a twofold to threefold greater frequency than females. The reasons underlying these differential outcomes may be related to sex-divergent self-recognition. Here we examined sex-specific autoimmune responses to cardiac self and their impact on DCM development. We found that males immunized to the p406-425 peptide derived from mouse cardiac α -myosin heavy chain preferentially develop a predominant Th17 lineage response that provides sustained T-cell memory and a high DCM incidence whereas females preferentially develop a predominant Th1 lineage response that becomes anergized to cardiac self resulting in compensatory protection against DCM. The distinct sex-defined disease phenotypes are interchangeable after *in vivo* manipulation of Th1 (interleukin-2) and Th17 (interleukin-17) cytokines. Our study shows that male and female SWXJ mice differentially respond to cardiac self in ways that lead to distinct autoimmune outcomes and implies that optimized therapy for autoimmunity may require consideration of the qualitatively different ways that males and females engage self. (Am J Pathol 2008, 172:11–21; DOI: 10.2353/ajpath.2008.070324)

Although sex biases are particularly prominent in autoimmune diseases, the skewing of disease incidence does not always favor the same sex.^{1,2} For example, women represent the majority of patients with multiple sclerosis,³ systemic lupus erythematosus, and rheumatoid arthritis, but men die at a twofold to threefold greater frequency than women from idiopathic dilated cardiomyopathy (DCM), a disease believed to have a substantial autoimmune etiological component (Morbidity and Mortality

Chartbook, National Heart, Lung, and Blood Institute, <http://www.nhlbi.nih.gov/resources/docs/cht-book.htm>).^{1,2}

The basis for female-biased incidence in multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis is currently unclear and is particularly confounded by observations that both estrogen and testosterone treatment may be therapeutic.^{3–5} A common explanation for female-biased autoimmune susceptibility contends that it is attributable to a more aggressive proinflammatory Th1-like immune response generated by females in response to self.^{1,2} This view is supported by studies correlating increased frequencies of autoreactive interferon (IFN)- γ -producing T cells and decreased frequencies of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Treg) in females with multiple sclerosis and in female mice with experimental autoimmune encephalomyelitis, a widely used animal model for multiple sclerosis.⁶ However, this view does not apply to myocarditis because male mice develop more severe viral-induced myocarditis^{7,8} and are often used exclusively in studies involving autoimmune-mediated myocarditis.^{9,10}

In this context, we chose to investigate the immunological features surrounding the male-biased incidence seen in human DCM¹¹ using our recently-developed murine model of experimental autoimmune myocarditis (EAM).¹² After adoptive transfer of cardiac α -myosin heavy chain (CAM 406-425) peptide-specific CD4⁺ T cells, SWXJ mice develop EAM with males showing more pronounced complications of myocardial inflammation than females. Male-associated disease is characterized by extensive fibrosis and ventricular dilatation, indicative of cardiac decompensation and DCM.¹³

Here we show that the increased incidence of DCM in male mice is attributable to persistence of autoimmune T-cell memory and that protection from development of DCM

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in female mice is attributable to clonal anergy of their autoreactive T cells. We found that the female cardio-specific repertoire is intrinsically myocarditogenic because *in vivo* potentiation of either interleukin (IL)-17 or IL-2 in females results in the male-like severe DCM phenotype. Likewise, complementary experiments in males involving inhibition of either IL-17 or IL-2 results in anergy and expression of the female-like DCM protective phenotype. We found that programming for anergy in female myocarditogenic T cells occurs during self-priming and is independent of any subsequent sex-specific end-organ modulation of the effector response. Our study shows that males and females differentially engage cardiac self in a manner that leads to qualitatively different autoreactivities and disease outcomes. Our results imply that optimal therapeutic intervention in autoimmune disease may require a sex-based perspective that considers the qualitatively different ways that males and females respond to self.

Materials and Methods

Mice and Immunizations

Female SWXJ.Thy-1b/b (H-2^{q,s}) mice expressing the conventional Thy1.2 isoform of CD90 were purchased from the Jackson Laboratory (Bar Harbor, ME). SWXJ.Thy-1a/b mice expressing both Thy1.1 and Thy1.2 were generated by mating male SJL/J.Thy-1a/a (H-2^s) mice (generously provided by Dr. Harley Tse, Wayne State University, Detroit, MI) with female SWR/J.Thy-1b/b (H-2^q) mice. As described previously¹² for active induction of EAM or for generating primed lymph node cells (LNCs), 6- to 8-week-old SWXJ.Thy-1a/b mice were injected subcutaneously in the abdominal flank on day 0 with 200 μ g of CAM 406-425 (KVGNEYVTKGQS-VQQVYYSI) and 400 μ g of *Mycobacteria tuberculosis* H37RA (Difco, Detroit, MI) in 200 μ l of an emulsion of equal volumes of water and IFA (Difco).

Passive Transfer of EAM

LNCs from SWXJ.Thy-1a/b mice were isolated 10 days after immunization with CAM 406-425 and cultured in 24-well flat-bottomed plates (BD Biosciences, San Jose, CA) at 6×10^6 cells/well in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 5% HEPES buffer, 2% L-glutamine, 1% penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY), and 20 μ g/ml of CAM 406-425 in a total volume of 2.0 ml. After 4 days, 2×10^7 washed cells were injected intraperitoneally into naive γ -irradiated (600 rads) SWXJ.Thy-1b/b recipients. Established long-term T-cell lines were derived from peptide-primed Thy1.1-expressing LNCs by alternate cycles of peptide stimulation and the IL-2-supplemented rest on syngeneic γ -irradiated (2000 rads) splenocyte monolayers. After three rounds of stimulation, T-cell lines were transferred at 2×10^7 cells/mouse into γ -irradiated (600 rads) Thy1.1⁻ hosts. In experiments involving sex-mismatched passive transfer of EAM, CD8⁺ T cells were

depleted with three 500- μ g injections of equal amounts of two different CD8 antibodies (clones YTS169 and T IB105; Ligocyte, Bozeman, MT) every other day starting 3 days before transfer and every 4th day thereafter for 5 weeks. CD8⁺ T cells represented $16.7 \pm 3.3\%$ and $0.76 \pm 0.20\%$ of total splenocytes in undepleted and depleted mice, respectively.

Histomorphological Determination of DCM

DCM is traditionally defined by functional criteria involving diminution of cardiac function. In our initial report describing the specific mouse model used in the current study, we showed that cardiac function was severely compromised and that these functional defects involved pathologically altered systolic pressures and ejection fractions as evidenced by pressure recordings from right carotid artery catheterization experiments.¹⁴ We also reported that the histomorphological triad of 1) increased heart weight (mg): body weight (g) ratio (HW:BW), 2) cardiac fibrosis, and 3) ventricular dilatation in mice with autoimmune myocarditis invariably accompanied these observed functional abnormalities and as such were pathognomonic for DCM. In our current study, each heart had to meet this rigid triad of criteria for determination of DCM. Moreover, cardiac histological consistency was ensured because all hearts were arrested during diastole as a result of injection of 2 mol/L KCl infusion during euthanasia.

To prevent postmortem clotting, mice were injected intraperitoneally with 100 IU heparin (Elkins-Sinn, Cherry Hill, NJ) 15 minutes before euthanasia. Removed hearts were perfused with phosphate-buffered saline (PBS) and weighed for determining HW:BW. Hearts were fixed in phosphate-buffered formalin overnight and embedded in paraffin. Multiple serial 5- μ m cross-sections were obtained from the apex of the heart for cross-sectional analysis of left and right ventricles. Sections were stained with either hematoxylin and eosin or Masson's Trichrome and examined by light microscopy for the presence of inflammation and fibrosis. The percentage of heart tissue involved in inflammation was determined as described previously.¹² DCM was determined by the presence of fibrosis, ventricular dilatation involving an increase in the radius of one or both ventricular chambers without a proportionate increase in mural wall thickness, and an increase in HW:BW. All sections were evaluated in a blinded manner.

T-Cell Assays

Thy1.1⁺ T cells were purified >95% from recipient spleens by magnetic bead separation on a MidiMACS cell separator using anti-Thy1.1 microbeads (Miltenyi, Auburn, CA) or by FACS Vantage (BD Biosciences) cell sorting with anti-Thy1.1-FITC (BD Biosciences). Thy1.1⁺ T cells (2×10^5)/well were cultured on a monolayer of 5×10^5 γ -irradiated (2000 rads) sex-matched splenocytes/well in 96-well flat-bottomed microtiter plates (BD Biosciences) in media supplemented as described above in the presence or absence of 100 μ g/ml of CAM 406-425.

Enzyme-linked immunosorbent assays (ELISAs) for IFN- γ , IL-2, IL-4, IL-5, and IL-10 were performed on 48-hour culture supernatants using commercially-available capture/detection antibody pairs as previously described.¹² ELISAs were also performed on culture supernatants from T cells removed from heart tissues enzymatically digested by agitation with collagenase type IV (Sigma, St. Louis, MO) in Hanks' balanced salt solution (5 mg/ml) for 30 minutes at 37°C. The released lymphocytes in suspension were then passed through a cell strainer, washed, and cultured at 2×10^5 cells/well in 96-well flat-bottomed microtiter plates (BD Biosciences). Transforming growth factor (TGF)- β 1 was measured according to the manufacturer's specifications (Promega, Madison, WI). Proliferation assays were performed by ^3H -thymidine incorporation at 96 hours of culture as previously described.¹² ELISPOT assays were performed by adding 3×10^5 peptide-activated T cells/well in ELISPOT plates (Polyfiltronics, Rockland, MA) containing wells precoated with IFN- γ , IL-2 (BD Biosciences), or IL-17 (R&D Systems, Minneapolis, MN) capture antibody. At 24 hours, cells were removed, and wells were treated with biotinylated cytokine detection antibodies (BD Biosciences). Spots were visualized using an automated Series-1 immunospot analyzer (Cellular Technology, Cleveland, OH).

In Vivo Manipulation of IL-2 and IL-17

In vivo induction of anergy was performed in male SWXJ mice by injecting 100 μg of CAM 406-425 peptide in PBS three times weekly starting at 2 weeks after adoptive transfer of 2×10^7 Thy1.1⁺ CAM 406-425-specific LNCs. T cells were released from anergy *in vivo* by daily intraperitoneal injection of 3 to 6 $\times 10^4$ IU recombinant human IL-2 (Chiron, Emeryville, CA) throughout a period of 5 weeks in female hosts that had received 2×10^7 Thy1.1⁺ CAM 406-425-specific LNCs.

IL-17 was depleted *in vivo* through daily injection of 100 μg of IL-17-neutralizing antibody for 5 weeks starting at 3 weeks after immunization (R&D Systems). *In vivo* frequencies of IL-17-producing T cells were boosted by culture of CAM 406-425-primed LNCs with 10 ng/ml of IL-23 (R&D Systems) for 4 days before adoptive transfer. The observed increased numbers of IL-17-producing T cells were confirmed by ELISPOT analysis before adoptive transfer.

It is worth noting that our original observations showed sex differences in recall responses to CAM 406-425 8 weeks after adoptive transfer of activated LNCs (Figures 1 and 2). Subsequently, we found that female anergy was observed readily by 5 weeks after adoptive transfer and that the anergy did not undergo any spontaneous reversal, particularly between the 5- to 8-week period after transfer. Thus, we often chose the 5-week postadoptive transfer period for analysis of anergy (Figures 3, 4, and 5). In the *in vivo* manipulation experiments (Figure 6), we reverted to the 8-week postadoptive transfer time point to maximize any observed impact on DCM.

Flow Cytometry Analysis

Cells were analyzed using a FACScan flow cytometer (BD Biosciences) after T-cell culture with or without 100 μg of CAM 406-425 for 72 hours. Fluorescent antibodies against CD3, CD4, CD25, CD40L, CD44, CD62L, CD90.1 (Thy1.1), Annexin V (BD Biosciences), and CTLA-4 (Sero-tec, Raleigh, NC) were obtained commercially. For cell cycle analysis, 2×10^7 10-day rested T-cell lines taken after two stimulation/rest cycles were co-transferred with 100 μg of lipopolysaccharide (serotype 01111:B4; Sigma), retrieved 5 weeks after passive transfer, and examined by flow cytometry analysis after staining with propidium iodide. For determining Treg frequencies 10-day-primed LNCs were activated *in vitro* with 25 $\mu\text{g}/\text{ml}$ of CAM 406-425 for 72 hours. Cells were then stained with CD4 Per-CP and CD25-PE antibodies (BD Biosciences), permeabilized for 4 hours at room temperature, and stained with FoxP3-FITC antibody (eBioscience, San Diego, CA). Treg frequencies were determined by showing CD4⁺FoxP3⁺ double-stained cells in the gated CD25⁺ population.

Biostatistical Analysis

The two-sided Fisher's exact test was used to determine differences in DCM incidence between male and female mice. The Student's *t*-test was used to analyze differences between all other experimental groups.

Results

Males with EAM Develop a High Incidence of DCM Compared to Females

We induced EAM in both male and female SWXJ mice by adoptive transfer of 2×10^7 activated sex-matched CAM 406-425-primed LNCs. Eight weeks after cell transfer, female hearts selectively underwent hypertrophy of their ventricular walls consistent with physiological compensation (Figure 1A, arrows). In contrast, male hearts preferentially showed a decompensated phenotype with ventricular chamber dilatation and thinning of the mural wall (Figure 1B). The difference in DCM incidence between males (13 of 22) and females (2 of 22) was significant ($P = 0.015$). Moreover, female EAM hearts (Figure 1C, middle) showed significantly ($P = 0.03$) less fibrotic replacement ($4.0\% \pm 1.7$ SD) than male EAM hearts ($13.0\% \pm 5.4$ SD; Figure 1C, right). Thus, compared to females, males with EAM developed a high incidence of DCM defined as heart size enlargement, parenchymal fibrosis, and ventricular dilatation.

Males but Not Females with EAM Show Sustained Memory to Cardiac Self

Insight into the underlying mechanism for enhanced EAM and more frequent DCM in males emerged after comparison of T-cell recall responses to CAM 406-425

Figure 1. Males with EAM develop a high incidence of DCM compared to females. We induced EAM in SWXJ mice by transferring activated sex-matched CAM 406-425-primed LNCs. Eight weeks later we examined heart sections stained with H&E. **A:** EAM female hearts typically show thickening of the mural wall (arrows) consistent with compensatory hypertrophy (DCM, 2 of 22). **B:** EAM male hearts typically show ventricular dilatation (asterisks) with thinning of the mural wall consistent with decompensation and DCM (DCM, 13 of 22). The difference in DCM incidence between males and females is significant ($P = 0.015$). **C:** Representative Masson's Trichrome-stained section from normal untreated control mouse shows no fibrotic changes (left). In contrast, female hosts (middle) showed significantly less ($P = 0.03$) fibrotic replacement of inflammation, comprising $4.0\% \pm 1.7$ SD of female heart tissue compared to $13\% \pm 5.4$ SD of male hearts.

by male- and female-primed LNCs both before (Figure 2, top) and 8 weeks after (Figure 2, bottom) adoptive transfer. Memory responses were determined by lymphocyte proliferation (Figure 2, left), cytokine production (Figure 2, middle), and ELISPOT frequencies of IFN- γ -producing effector T cells (Figure 2, right). Despite substantially greater proliferative and cytokine responses before adoptive transfer, female T-cell responses were qualitatively attenuated when compared to analogous male T-cell responses 8 weeks after adoptive transfer. Thus, males showed sustained memory responsiveness to CAM 406-425 whereas females did not. Because females showed a greater number of effector cytokine-secreting T cells and a greater production of Th1-associated cytokines before transfer, we concluded that the inversion of sex-defined response patterns after transfer of autoreactive T cells was not because of a selectively smaller T-cell precursor pool reactive to CAM 406-425 in females nor to a defect in Th1 differentiation occurring pretransfer. Rather, the failure of T-cell memory in females appeared to be attributable to a posttransfer tolerogenic process.

Abrogated Memory in EAM Females Is Not Attributable to Clonal Deletion or Suppression

We investigated the possibility that failure of T-cell recall responses may be attributable to apoptotic deletion of cardio-specific T cells chronically exposed to high levels of cognate antigen.^{15,16} Peptide-activated male or female Thy1.1⁺-primed LNCs or Thy1.1⁺ peptide-specific long-term T-cell lines were transferred

Figure 2. Males but not females with EAM show sustained memory to cardiac self. Before transfer, CAM 406-425-primed LNCs from females show significantly enhanced peptide-induced proliferation (top left, $P = 0.001$ at 10μ g/ml and $P = 0.01$ at 100μ g/ml) and production of the Th1 cytokines (top middle) IFN- γ ($P = 0.03$) and IL-2 ($P = 0.04$) when compared to males. Eight weeks after transfer, this response pattern inverts with male T cells showing significantly enhanced peptide-induced proliferation (bottom left, $P = 0.003$ at 10μ g/ml and $P = 0.002$ at 100μ g/ml) and production of proinflammatory cytokines (bottom middle) IFN- γ ($P = 0.01$) and IL-2 ($P = 0.0001$) when compared to females. This inversion involves a relative decline throughout time in frequencies of peptide-specific IFN- γ -producing female T cells, which were substantially higher than males before transfer (top right, $n = 11$) but markedly lower than males 8 weeks after transfer (bottom right, $n = 3$). All error bars indicate $\pm$ SD. Mean background cpm $\pm$ SD before transfer were 1409 ± 678 (males) and 1139 ± 321 (females) and were not significantly different ($P = 0.72$). Mean background cpm $\pm$ SD after transfer were 6643 ± 791 (males) and 5909 ± 225 (females) and were not significantly different ($P = 0.12$). IFN- γ and IL-2 levels were always elevated in unstimulated cultures as a result of *in vivo* exposure to antigen. However, these responses were always less than 25% of those observed in peptide-activated cultures. Equivalent low levels of IL-4, IL-5, and IL-10 were measured in unstimulated and peptide-activated cultures.

into sex-matched Thy1.1⁺ hosts, and total recovered cells were assessed throughout time. Although we observed a decrease in the recovery of donor Thy1.1⁺ T cells throughout time, there was no detectable difference between the percentage of recovered male or female Thy1.1⁺ T cells after transferring 10-day-primed LNCs (Figure 3A, top) or peptide-specific Thy1.1⁺ T-cell lines (Figure 3A, bottom).

The equivalent survival profiles of male and female autoreactive T cells were paralleled both by a gradual decline in female Thy1.1⁺ cardiac-specific T-cell responsiveness and potentiated autoreactivity of male Thy1.1⁺ T cells. One week after transfer, female T cells showed greater proliferative responsiveness than male T cells (Figure 3B). However, by 4 to 5 weeks, this response pattern became inverted with male T cells showing potentiated autoreactivity both in terms of proliferation (Figure 3B) and production of IFN- γ and IL-2 (Figure 3C). Because these divergent male and female memory T-cell responses were not associated with a correspondingly discrepant percentage of recovered Thy1.1⁺ T cells, we concluded that the failure of female T cells to sustain

autoimmune memory was not attributable to any female-biased apoptotic deletion.

Thy1.1⁺ T cells recovered from both male and female hosts consistently expressed the CD4⁺CD44⁺CD62L⁺ phenotype of memory T cells (data not shown). Female T cells additionally showed a CD4⁺CD25⁺ surface phenotype, prompting investigation as to whether they had acquired regulatory/suppressor activity.¹⁷ Female Thy1.1⁺ T cells were transferred into Thy1.1⁺ recipients, and T cells from donor (Figure 3D, gate R3) and host (Figure 3D, gate R2) as well as non-T-cell populations (Figure 3D, gate R1) were sorted and cultured at varying cell ratios with established CAM 406-425-specific T-cell lines. Donor-derived Thy1.1⁺CD4⁺CD25⁺ T cells, host-derived Thy1.1⁺ T cells, and host-derived non-T cells were unable to suppress responder cell proliferation at any cell ratio tested. However, naturally occurring CD4⁺CD25⁺ regulatory T cells purified from normal mouse spleens were able to inhibit proliferative responses of the CAM 406-425-specific T-cell lines. Moreover, when stimulated with anti-CD3, retrieved female Thy1.1⁺ T cells did not produce substantial amounts of IL-10 or TGF- β (Figure 3E, top) and did not express CTLA-4 (Figure 3E, bottom) in a manner comparable to naturally occurring CD4⁺CD25⁺ regulatory T cells.¹⁷

To determine whether female-specific suppression of autoreactive CD4⁺ T cells had occurred *in vivo*, we transferred female or male CAM 406-425-specific CD4⁺ Thy1.1⁺ T-cell lines into sex-matched and sex-mismatched recipients and recovered Thy1.1⁺ T cells 5 weeks after transfer. To avoid host-directed responsiveness to male-specific antigens,

Figure 3. Abrogated memory in EAM females is not attributable to clonal deletion or suppression. We induced EAM by transfer of CAM 406-425-specific Thy1.1⁺ LNCs or by transfer of long-term established Thy1.1⁺ T cell lines into Thy1.1⁺ hosts. **A:** Deletion rates of transferred male and female T cells are similar because there are no discernible differences in the percentage of male and female donor Thy1.1⁺ T cells retrieved from spleens at each time point after transfer of CAM 406-425-primed LNCs (**top**) or after transfer of long-term peptide-specific T-cell lines (**bottom**). **B:** In recall responses to 100 μ g/ml of peptide, female splenocytes show a decline in proliferation throughout time that is clearly evident 4 to 5 weeks after transfer, whereas male Thy1.1⁺ T cells maintain recall responsiveness at all times after transfer. At 1 week after transfer, females show significantly enhanced peptide-induced proliferation compared to males ($P = 0.01$), whereas at 5 weeks after transfer, males show significantly enhanced proliferation compared to females ($P = 0.04$). **C:** The inverted proliferation response pattern is accompanied by an inversion of proinflammatory cytokine production with females showing substantially decreased T-cell production of IFN- γ by the 2nd week after transfer (**top**) and decreased production of IL-2 by the first week (**bottom**). In contrast, males show an overall gradual increase in T-cell production of IFN- γ (**top**) and IL-2 (**bottom**) throughout the 5-week period. **D:** Retrieved donor-derived female CD3⁺Thy1.1⁺ T cells, host-derived CD3⁺Thy1.1⁺ T cells, or host-derived CD3⁺Thy1.1⁺ non-T cells are unable to suppress proliferation of established CAM 406-425-specific T-cell lines, whereas CD4⁺CD25⁺ Treg cells from normal untreated mice are suppressive. **E:** Anti-CD3-activated female T cells do not produce IL-10 or TGF- β (**top solid**) and do not up-regulate CTLA-4 (**bottom shaded**) compared to anti-CD3-activated CD4⁺CD25⁺ regulatory T cells derived from normal untreated mice, which produce IL-10 and TGF- β (**top hatched**) and express CTLA-4 (**bottom dotted line**). **F:** Adoptive transfer of CAM 406-425-specific female Thy1.1⁺ T cells into either female (**left**) or male (**middle**) recipients results in the female EAM phenotype characterized by protection from development of DCM, compensatory hypertrophy (**arrows**), and hyporesponsiveness to CAM 406-425 (**right**). **G:** In contrast, transfer of male T cells into either female (**left**) or male (**middle**) recipients results in the male EAM phenotype characterized by DCM with ventricular dilatation (**asterisks**) and maintenance of autoimmune memory to CAM 406-425 (**right**). Sex-mismatched recipients underwent depletion of CD8⁺ T cells to prevent responses to male-specific autoantigens on donor T cells. All error bars indicate $\pm$ SD.

Figure 4. Autoimmune memory failure in females is attributable to T-cell anergy. CAM 406-425-specific Thy1.1⁺ donor T cells from primed LNCs or from established T-cell lines were retrieved from recipient spleens of EAM mice 5 weeks after transfer. **A:** Recovered female T cells are hypoproliferative in response to CAM 406-425 but recover responsiveness after pretreatment with IL-2 (**left**). Thy1.1⁺ T cells retrieved from males respond to CAM 406-425 but become unresponsive after IL-2 pretreatment (**right**). For both males and females, $n = 5$. When compared to peptide-activated female T cells, CAM 406-425 activated male T cells show threefold greater frequencies of cells in G₂ and S phases of the cell cycle (**B**), produce fourfold more IL-2 (**C**, **left**; $P = 0.001$), and up-regulate CD25 (**right bottom shaded**). Activated female T cells down-modulate CD25 in the presence of CAM 406-425 (**right top shaded**). Solid black lines indicate CD25 expression in nonactivated cells. Dotted black lines show isotype controls. **D:** CAM 406-425-stimulated female T cells produce IFN- γ (**left solid**, $P = 0.07$) and up-regulate CD40L (**right shaded**) in a manner similar to male T cells (**left hatched** and **right solid line**, respectively). **E:** After IL-2 pretreatment, male T cells become apoptotic with (**top shaded**) or without (**top solid line**) addition of peptide whereas female T cells show minimal apoptosis with (**bottom shaded**) or without (**bottom solid line**) peptide activation. All data are representative of experiments repeated two to four times. All error bars indicate $\pm$ SD.

CD8⁺ T cells were depleted in sex-mismatched recipients by repeated injection with CD8 antibody. Female (Figure 3F, left) or male (Figure 3F, middle) hosts that received female-derived Thy1.1⁺ T-cell lines failed to develop DCM (none of four and none of four, respectively), and the retrieved female Thy1.1⁺ T cells recovered from both female and male recipients showed hypoproliferative responses to CAM 406-425 (Figure 3F, right). Hypoproliferative responses to the cognate CAM 406-425 were also elicited by female Thy1.1⁺ T cells adoptively transferred into neonatally thymectomized Thy1.1⁻ female hosts deficient in production of endogenous Treg populations (data not shown). In contrast, female hosts (Figure 3G, left) or male hosts (Figure 3G, middle) receiving male-derived Thy1.1⁺ T-cell lines developed a relatively high incidence of DCM (three of four and two of four, respectively), and the retrieved male Thy1.1⁺ T cells from both male and female hosts showed persistent recall responses to CAM 406-425 (Figure 3G, right). Thus, autoreactive female T cells exhibited no inherent regulatory/suppressor function and showed no indication of being selectively suppressed in the female host milieu.

Autoimmune Memory Failure in Females Is Attributable to T-Cell Anergy

We next determined whether the memory failure in females was attributable to anergy. Five weeks after transfer, CAM 406-425-specific Thy1.1⁺ donor T cells were retrieved from recipient spleens and cultured *in vitro* with either 100 μ g/ml of peptide alone or with 100 ng/ml of IL-2 for 7 days before addition of peptide. IL-2-mediated recovery from anergy was determined by ³H-thymidine incorporation in response to 100 μ g/ml of CAM 406-425. We found that female T cells, whether derived from transfer of primed LNCs or established T-cell lines, were hypoproliferative in response to direct challenge with CAM 406-425 but recovered their responsiveness after *in vitro* pretreatment with IL-2 (Figure 4A, left). In contrast, recovered Thy1.1⁺ male T cells showed recall responsiveness to CAM 406-425 without IL-2 pretreatment and became hypoproliferative only after pretreatment with IL-2 (Figure 4A, right). Thus, male and female autoreactive T cells show opposite responsiveness to IL-2 with male T cells

becoming hypoproliferative and female T cells showing classic IL-2-mediated recovery from anergy.¹⁸

The anergy observed in females was confirmed using propidium iodide cell cycle analysis of retrieved Thy1.1⁺ T cells. Unstimulated Thy1.1⁺ T cells from both male and female hosts resided almost entirely within the G₁ phase of the cell cycle (Figure 4B, top). However, after *in vitro* activation with 100 μ g/ml of CAM 406-425, male Thy1.1⁺ T cells entered G₂ and S phases of the cell cycle at a threefold greater frequency than analogously treated female T cells (27% versus 9%, respectively; Figure 4B, bottom). G₁ arrest of peptide-activated female Thy1.1⁺ T cells was accompanied by approximately a fourfold lower production of IL-2 when compared to male Thy1.1⁺ T cells (Figure 4C, left) and down-modulation of the high-affinity CD25 IL-2 receptor (Figure 4C, right). Consistent with the features of clonal anergy and not adaptive tolerance,¹⁹ peptide-activated female T cells retained the ability to produce IFN- γ (Figure 4D, left) and up-regulate CD40L (Figure 4D, right) in a manner similar to male T cells. The ablation of proliferation in IL-2 pretreated male T cells was found to be attributable to a relative increase in the frequency of apoptotic male T cells as measured by Annexin V staining (Figure 4E). Our data indicate that whereas male T cells maintain long-term memory to cardiac self, female T cells become clonally anergized.

Anergy and Not Sex-Specific End-Organ Differences Protects Female Hosts from DCM

Several studies have shown uncoupling of effector cytokine production and effector T-cell function. For example, viral-specific CD8⁺ helpless T cells primed in the absence of CD4⁺ T cells may undergo several division cycles and up-regulate intracellular stores of IFN- γ without acquiring the ability to mount protective anti-viral responses.^{20,21} By analogy, it is possible that female CAM 406-425-specific CD4⁺ T cells, although producing abundant Th1-like cytokines before adoptive transfer (Figure 2, top), may not contain myocarditogenic clones and thus may lack the intrinsic ability to induce EAM and DCM. Alternatively, although female and male autoreactive T cells persist *in vivo* at similar frequencies (Figure 3A), it may be possible that transferred Thy1.1⁺ female T cells undergo selective deletion of high avidity autoreactive T-cell clones.²² Thus, we determined whether female T cells were intrinsically capable of mediating DCM on *in vivo* recovery from anergy.

CAM 406-425-primed female LNCs were transferred into female hosts that were subsequently given daily injections of IL-2 to ablate anergy *in vivo*. Sham-treated female controls did not develop DCM (Figure 5A, left), were relatively unresponsive to CAM 406-425, and recovered autoreactivity after *in vitro* pretreatment with IL-2 (Figure 5A, right). In contrast, IL-2-treated females showed a marked increase in the incidence of DCM (7 of 13; Figure 5A, middle) and showed responsiveness to CAM 406-425 that was ablated after *in vitro* pretreatment with IL-2 (Figure 5A, right). Thus,

Figure 5. Anergy is sufficient for providing a protective autoimmune outcome. EAM was induced in male and female recipients by transfer of sex-matched CAM 406-425-specific Thy1.1⁺ T-cell lines. **A:** Compared to sham-treated EAM female recipients that fail to develop DCM (0 of 3, **left**) and show compensatory hypertrophy (**arrow**) and clonal anergy (**right**), IL-2-injected females develop a high incidence of DCM (7 of 13, **middle**) accompanied by loss of spontaneous anergy and ablation of recall responsiveness to CAM 406-425 after *in vitro* treatment with IL-2 (**far right**). Cells were pooled from several recipient mice for final analysis. Data are representative of three separate experiments yielding similar results. **B:** In contrast, compared to sham-treated EAM male recipients that develop DCM (four of seven, **left**) and show sustained autoreactivity that is ablated after *in vitro* treatment with IL-2 (**right**), males injected intravenously with CAM 406-425 develop a low incidence of DCM (two of nine, **middle**), compensatory hypertrophy (**arrow**), and concurrent clonal anergy that is ablated after *in vitro* treatment with IL-2 (**right**). Cells were pooled from several recipient mice for final analysis of proliferation. Data are representative of two separate experiments yielding similar results. **C:** CAM 406-425-specific Thy1.1⁺ T cells were adoptively transferred into sex-matched and sex-mismatched recipients. Five weeks after adoptive transfer, the transferred Thy1.1⁺ T cells recovered from female (**top left**) and male (**top right**) hosts show hypoproliferative responses to CAM 406-425 that are potentiated after *in vitro* IL-2 pretreatment. Conversely, retrieved male Thy1.1⁺ T cells from both female (**bottom left**) and male (**bottom right**) hosts show sustained recall responses to CAM 406-425 that are ablated after *in vitro* IL-2 pretreatment. Asterisks indicate ventricular dilatation, and all error bars show $\pm$ SD. In all conditions, significant differences occurred at 100 μ g/ml ($P < 0.05$).

adoptively transferred female T cells contained sufficient numbers of inherently myocarditogenic T cells capable of mediating EAM and DCM.

To determine whether induction of anergy was sufficient for skewing the cardiac phenotype toward compensatory hypertrophy rather than DCM, anergy was induced in males by three intravenous injections of CAM 406-425 after adoptive transfer of peptide-activated male Thy1.1⁺ T cells. Compared to sham-injected males (Figure 5B, left) that developed a high incidence of DCM (four of seven) with sustained T-cell memory that was ablated

Figure 6. Sex-defined EAM phenotypes are attributable to differential frequencies of IL-17-producing T cells. **A:** Ten days after immunization with CAM 406-425, frequencies of IFN- γ -producing T cells are consistently higher in females than males ($P < 0.003$) but frequencies of IL-17-producing T cells are consistently higher in males than females ($P < 0.03$). Data show actual spot numbers in 3×10^5 LNCs per well in a representative experiment of several showing similar results. **B:** Before transfer into female SWXJ recipients, 10-day CAM 406-425-primed female LNCs were cultured in the presence of peptide plus 10 ng/ml of IL-23 or peptide alone. Eight weeks after transfer, female recipients of IL-23-treated donor LNCs show significantly increased HW:BW ($P = 0.006$) with heightened frequency of DCM (**left**) and increased production of both IL-17 and IL-2 in splenocyte recall responses to CAM 406-425 (**right**) compared to mice receiving transferred LNCs not cultured in the presence of IL-23 (**middle**). **C:** Male SWXJ mice injected with 100 μ g of IL-17-neutralizing monoclonal antibody every day for 5 weeks starting 3 weeks after immunization with CAM 406-425 show significantly reduced HW:BW ($P = 0.03$) with attenuated DCM incidence (**middle**) and decreased production of both IL-17 and IL-2 in splenocyte recall responses to CAM 406-425 (**right**) compared to control mice similarly treated with an IgG isotype control monoclonal antibody. All male and female mice were euthanized 8 weeks after adoptive transfer of primed LNCs. Asterisks indicate ventricular dilatation, and all error bars show $\pm$ SD.

after *in vitro* pretreatment with IL-2 (Figure 5B, right), peptide-injected male hosts showed a decreased incidence of DCM (two of nine) and preferentially developed compensatory hypertrophy (Figure 5B, middle) accompanied by hypoproliferative responsiveness to CAM 406-425 that became enhanced after *in vitro* pretreatment with IL-2 (Figure 5B, right). Thus, active *in vivo* induction of anergy to cardiac self was sufficient to provide DCM protection to males.

Studies have suggested that sex-specific differences related to the end-target organ may differentially modulate the immune effector response.²³ To test this possibility, we transferred female or male CAM 406-425-specific Thy1.1⁺ T-cell lines into sex-matched and sex-mismatched recipients and recovered Thy1.1⁺ T cells 5 weeks after transfer (Figure 3, F and G). Recovered female Thy1.1⁺ T cells, whether transferred into female hosts (Figure 5C, top left) or male hosts (Figure 5C, top right), showed hypoproliferative responses to CAM 406-425 that became enhanced after IL-2 preculture. In contrast, retrieved male Thy1.1⁺ T cells showed recall responses to CAM 406-425 regardless of transfer into female recipients (Figure 5C, bottom left) or male recipients (Figure 5C, bottom right). We concluded that programming for anergy in transferred Thy1.1⁺ T cells occurred during T-cell priming and persisted independently of end-organ modulation of the effector response. More specifically, female host hearts do not have any selective ability to anergize myocarditogenic T cells.

Sex-Defined EAM Phenotypes Are Attributable to Differential Frequencies of IL-17-Producing T Cells

We next examined the underlying basis for the enhanced ability of male T cells to transfer DCM into either male or female hosts. Since recent studies have shown that severe autoimmune outcomes occur after transfer of a subpopulation of T cells expressing IL-17,²⁴ we compared ELISPOT frequencies of IL-17-producing effector T cells in male and female SWXJ mice 10 days after immunization with CAM 406-425. We observed a consistent differential sex-defined cytokine profile with females showing higher LNC frequencies of IFN- γ -producing T cells ($P < 0.003$, Figure 6A) but much lower frequencies of IL-17-producing T cells than males ($P < 0.03$, Figure 6A). We hypothesized that the higher frequencies of IL-17-producing T cells in males may account for both their failure to anergize and their severe autoimmune outcome. To test this hypothesis, we boosted frequencies of IL-17-producing T cells in female recipients by treating CAM 406-425-primed LNCs with IL-23 before adoptive transfer.²⁴ Similarly, we neutralized IL-17 in males by treatment with 100 μ g of IL-17-neutralizing monoclonal antibody every other day for 5 weeks starting 3 weeks after immunization with CAM 406-425. IL-23 treatment of female LNCs increased IL-17-producing T-cell frequencies (data not shown) resulting in more severe cardiac pathology and a higher incidence of DCM (Figure 6B, middle) with

Figure 7. Cytokine levels of heart-infiltrating T cells and Treg frequencies. **A:** Eight weeks after transfer of sex-matched CAM 406-425-primed LNCs, heart infiltrating T cells were activated with peptide, and cytokines concentrations in 48-hour supernatants were determined by ELISA. Male cardiac-infiltrating T cells produced substantially higher levels of the Th1 cytokines IFN- γ and IL-2 ($P > 0.05$), but male and female infiltrating T cells produced similar low level concentrations of IL-4 and TGF- β ($P > 0.05$). **B:** Ten days after immunization with CAM 406-425, primed LNCs from male and female SWXJ mice were activated with peptide for 3 days and examined by flow cytometry for Treg frequencies. In three experiments, similar low frequencies of FoxP3-FITC $^{+}$ CD4-PerCP $^{+}$ Tregs were found in the gated CD25-PE $^{+}$ T-cell population of males and females ($P > 0.05$).

enhanced production of both IL-17 ($P = 0.0000001$) and IL-2 ($P = 0.002$) in splenocyte recall responses to CAM 406-425 (Figure 6B, right). In a complementary manner, IL-17 neutralization in males resulted in a decreased incidence of DCM (Figure 6B, middle) as well as abrogated production of both IL-17 ($P = 0.03$) and IL-2 ($P = 0.03$) in splenocyte recall responses to CAM 406-425 (Figure 6C, right). Thus, the severe male non-nergic DCM phenotype and the protected female anergic DCM phenotype may be interchanged through manipulation of frequencies of IL-17-producing T cells.

Analysis of Cardiac-Infiltrating T Cells and Treg Frequencies

Recent reports have implicated enhanced TGF- β production and a Th2 phenotype with the development of cardiac fibrosis and DCM and that IFN- γ reduces cardiac fibrosis by transcriptional down-regulation of IL-4.²⁵ Although we found that male mice with severe DCM had increased frequencies of IFN- γ -producing T cells 8 weeks after transfer (Figure 2, lower right), we examined the possibility that the apparent absence of IFN- γ -induced cardio-protection in male mice may have been attributable to decreased local production of IFN- γ by cardiac-infiltrating T cells. Heart cytokine levels were measured by ELISA 8 weeks after transfer of activated sex-matched CAM 406-425-primed LNCs. We found that when compared to females, T cells that infiltrated male hearts produced substantially higher levels of the Th1 cytokines IFN- γ and IL-2 ($P > 0.05$) and that male and female cardiac-infiltrating T cells produced similar low level concentrations of both IL-4 and TGF- β ($P > 0.05$, Figure 7A).

Tregs have been shown to regulate and confer experimental autoimmune encephalomyelitis resistance in SJL/J males,⁶ the parental male partner for SWXJ mice used in the current study, and may possibly provide a source for TGF- β required in Th17 lineage selection.^{26–31} Therefore, we examined Treg frequencies and found that the percentage of CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$ T cells in CAM 406-425-primed LNCs were similar in both male (3.2 to 4.6%) and female (3.94 to 4.53%) SWXJ mice ($P > 0.05$, Figure 7B). Our results are consistent with the view that male and female SWXJ mice have similar frequencies of Tregs and that male mice de-

velop severe DCM in the context of a cardiac milieu involving high levels of IFN- γ and IL-2 and low levels of IL-4 and TGF- β .

Discussion

Our data show sex-defined responses to a singular self-antigen are capable of dictating differential autoimmune disease outcomes. Predisposition to the severe autoimmune outcome of DCM is linked directly to the ability of male SWXJ T cells to maintain conventional autoimmune memory to cardiac self whereas protection from DCM and predisposition to develop protective compensatory hypertrophy is linked to the ability of female T cells to undergo spontaneous clonal anergy and aborted self memory. Most notably, differential sex-defined disease outcomes are innately determined by the primed T cells per se and are unaffected by and independent of any postpriming events including host-induced shaping of effector responses by endogenous hormones or sex-distinct target organ susceptibility to autoimmune-mediated tissue destruction.

The observed sex-defined autoimmune outcomes are directly related to differential CAM 406-425 priming events that predominantly favor a Th1-like response in female SWXJ mice and a Th17-like response in male SWXJ mice. Th1 lineage development is highly dependent on IL-12 whereas the Th17 lineage associated with severe autoimmune outcome has only recently been shown to be induced by IL-6 and TGF- β up-regulation of IL-23 receptors on T cells.^{26–31} However, the source of enhanced TGF- β in SWXJ males does not appear to be increased numbers of Tregs because males and females showed similar frequencies of CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$ T cells in response to CAM 406-425. This latter observation stands in contrast to the increased Treg frequencies implicated in protection of male SJL/J mice from experimental autoimmune encephalomyelitis susceptibility⁶ and the decreased Treg frequencies implicated in the enhanced myocarditis co-observed in BALB/c males infected with coxsackievirus B3.³² It remains currently unclear whether the selected target antigen or the strain of mice analyzed is more important in determining the role of Tregs in the enhanced severity of autoimmune disease often observed in males.

The dual link to TGF- β in both regulating autoimmunity through Treg function and causing severe autoimmune outcome through Th17 lineage induction implies that a fine balance may exist between these two opposing TGF- β -dependent immune effects and subsequent disease outcomes. This fine balance may be tipped selectively toward Treg-mediated regulation or Th-17-mediated enhanced disease by any of several candidate factors. Bone marrow progenitors, like thymocytes, contain estrogen response elements³³ raising the possibility that hormones may govern release from bone marrow or lineage commitment of non-T-cell immune precursors. Sex-disparate terminal differentiation of these precursors may lead to altered ratios of antigen-presenting cell subsets such as the plasmacytoid and myeloid dendritic cells that in humans exert diametrically opposed effects on Th1 and Th2 function.³⁴ In addition, sex hormones may shape sex differential expression of Treg and co-stimulatory molecules^{3,35} as well as DC maturation, endocytosis, and antigen processing,³⁶ thereby potentially selecting different epitopes and/or altering avidity of the peptide-T cell receptor (TCR) synapse and the subsequent strength of signal that forms the basis for lineage fate selection of several different functional T-cell populations.^{37–39} Indeed, differential cytokine production may alter intracellular protease function so that differentially-cleaved antigens become alternatively presented during inflammatory responses.^{40–42} Thus, differentially-processed CAM 406-425 fragments in males and females during T-cell priming may subsequently lead to differential TCR engagement, a process that may fundamentally alter the T-cell response, as has been shown with altered peptide ligands containing point-specific substitutions of TCR contact residues.⁴³

In addition to inducing effects on established T-cell repertoires, sex hormones also have the potential to shape selection of the native repertoire. T-cell repertoire shaping through thymic selection processes occurs prenatally around E17 and E18 in mice coincident with the sex hormone burst that governs formation of the external genitalia and genitourinary tracts. This hormonal burst promotes thymic medullary survival *in vivo*^{44,45} and may additionally mold nascent CD4 and CD8 single-positive T-cell ratios postnatally.^{46,47} Thus, differential autoimmune responses in adult males and females may be attributable in part to sex-defined modulation of thymopoiesis. Prenatal shaping of thymic ontogeny by sex hormones may provide differential sex-based central selection and formation of holistically divergent T-cell repertoires with distinct cellular constituents and/or activation thresholds. It is not difficult to imagine that these different repertoires, in turn, may lead to highly disparate sex-specific responses to a given antigen—whether self or foreign—independent of any postnatal hormonal perturbation of the effector response as we have observed.

It is possible that the observed sex differences in response to cardiac self may represent to some extent the consequence of sex differential responsiveness to the *M. tuberculosis* adjuvant. However, there are several arguments against this perspective: 1) as a ubiquitous positive control in virtually all of our mouse and human experimen-

tation, we have never observed any differences between males and females with respect to the immune response to *M. tuberculosis*, including data obtained in the present study (data not shown). 2) We have been unable to find any reputable published reports detailing male and female differences in the immune response to *M. tuberculosis*. 3) Our model system involves induction of passive disease after adoptive transfer of primed LNCs and/or T-cell lines. Therefore, experimental mice with differential sex-biased development of DCM were never exposed to antigenic challenge with *M. tuberculosis*.

It is worth noting that our current study and those previously published^{7,8} support the view that myocarditis, whether induced by virus or autoimmunity, seems to be more severe in males than in females. At the same time, it is less clear that the increased disease severity in males is attributable to a consistently similar qualitative immune response because a variety of immunological conditions have been shown to contribute to the severe DCM outcome in males including a predominant Th2 response,²⁵ a predominant Th17 response independent of Th1/Th2 polarization,⁴⁸ decreased Treg frequencies,³² decreased T-cell Ig mucin (Tim-3) expression,³² and, as shown in the current study, failure to anergize Th1 and Th17 responses.

In conclusion, our study indicates that male and female mice engage cardiac self in distinctly different manners predisposing females to Th1 lineage selection and T-cell anergy and males to Th17 lineage responses and sustained self-memory. These sex-defined self-recognition patterns ultimately dictate clinical outcome and account directly for the relative protection afforded to females and the enhanced disease severity observed in males. The direct linkage of sex-defined autoimmune responses to differential autoimmune outcomes reveals the potential for optimizing treatment of immune-mediated diseases by developing sex-tailored therapeutic strategies.

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