Published in final edited form as:

Clin Immunol. 2009 January ; 130(1): 74–82. doi:10.1016/j.clim.2008.06.015.

A Locus on Chromosome 1 Promotes Susceptibility of Experimental Autoimmune Myocarditis and Lymphocyte Cell Death¹

Davinna L. Ligons^a, Mehmet L. Guler^a, Haiyan S. Li^a, and Noel R. Rose^{a,*}

^a Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, MD 21205

[†]Feinstein Department of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, MD 21205

Abstract

We previously identified by linkage analysis a region on chromosome 1 (*Eam1*) that confers susceptibility to experimental autoimmune myocarditis (EAM). To evaluate the role of *Eam1*, we created a congenic mouse strain, carrying the susceptible *Eam1* locus of A.SW on the resistant B10.S background (B10.A-*Eam1* congenic) and analyzed three outcomes: 1) the incidence and severity of EAM, 2) the susceptibility of lymph node cells (LNCs) to Cy-enhanced cell death, and 3) susceptibility of lymphocytes to antigen-induced cell death. Incidence of myocarditis in B10.A-*Eam1* congenic mice was comparable to A.SW mice, confirming that *Eam1* plays an important role in disease development. Caspase 3, 8 and 9 activation in LNCs following Cy treatment and in CD4⁺ T cells after immunization with myosin/CFA was significantly lower in A.SW than B10.S mice whereas B10.A-*Eam1* congenic mice exhibited an intermediate phenotype. Our results show that *Eam1* reduces lymphocyte apoptosis and increases susceptibility to EAM.

Keywords

autoimmune; apoptosis; gene; regulation; rodent; caspase; susceptibility; myocarditis; chromosome; locus

Introduction

Myocarditis can be triggered by a number of different infectious agents. In susceptible individuals, infectious myocarditis can progress to autoimmune myocarditis and dilated cardiomyopathy which account for 25% of the cases of heart failure in the United States [1, 2,3]. Autoimmune myocarditis is thought to be due to an immune response to cardiac-specific antigens expressed during the infectious process. Supporting this view, experimental autoimmune myocarditis (EAM) can be induced by immunizing susceptible mouse strains with purified murine cardiac myosin in the absence of viral infection.

¹This work was supported in part by National Institutes of Health Grants HL077611 and HL067290

*Corresponding author: Noel R. Rose Departments of Pathology and of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions 1721 E. Madison St. Ross Building 659, Baltimore, MD 21205. Email address: nrrose@jhsph.edu. Phone: 410-955-0330. Fax: 410-955-0105.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We have previously determined that susceptibility to autoimmune myocarditis is dependent in part on the H-2 haplotype of the mice, but our early data also clearly established the importance of non-H-2 background genes [4,5]. The roles of these non-H-2 genes have not been clearly defined [6]. The overall goal of this research is to better understand the background genes that contribute to susceptibility to autoimmune myocarditis. To accomplish this goal we have compared A.SW and B10.S mice, which share the H-2^s haplotype, and discovered that A.SW mice are susceptible to EAM defined by a marked mononuclear cell infiltrate, whereas B10.S mice develop little or no myocardial infiltration [4,7]. Genetic linkage analysis strongly suggested that a proximal region on chromosome 1 plays a role in EAM susceptibility [7]. Interestingly, regions overlapping *Eam1* have been associated with susceptibility to several other autoimmune diseases of mice like type 1 diabetes, autoimmune orchitis and systemic lupus erythematosus, suggesting that this region may contain one or more genes with a common immunoregulatory role in different autoimmune disorders [8,9,10].

Regulation of cell death in lymphocytes is critical for maintaining homeostatic balance in the immune system [11]. Caspase 8 is thought to be central role in this process [12]. Initially T lymphocytes undergo cell death following antigen stimulation via the activation of the death receptor pathway, a process mediated by caspase 8 activation [11]. FLIP can inhibit the activation of caspase 8. In addition, death stimuli, like decreased trophic support following clearance of antigen and DNA damage, can also lead to the activation of an intrinsic mitochondrial cell death pathway, which is mediated by the activation of caspase 9. Both activated caspases 8 and 9 activate effector cysteine proteases, like caspase 3. Failure of either of these pathways has been shown to influence susceptibility to autoimmune diseases and autoimmune manifestations [13,14].

Sensitivity to cyclophosphamide (Cy)-enhanced cell death has been mapped in the NOD mouse to the *Idd5* diabetes susceptibility locus. It overlaps with the EAM susceptibility locus (*Eam1*) on chromosome 1 which was identified in our study of the A.SW strain [7,15]. We previously reported that A.SW mice, like NOD mice, exhibit an immunologic defect in that the lymph node cells (LNCs) of these mice are less sensitive to Cy-enhanced cell death compared to the EAM resistant B10.S mouse [7;16]. This finding suggested to us that a gene in this region regulates cell death of LNCs.

To study the function of *Eam1* in EAM and cell death, we developed the B10.A-*Eam1* congenic mouse. This mouse, of B10.S origin, contains a 50 cM segment of chromosome 1 of A.SW origin. Thus, we could examine how the presence of *Eam1* alters the susceptibility to autoimmune myocarditis and affects lymphocyte cell death on a resistant background. We report now that B10.A-*Eam1* congenic mice have increased susceptibility to autoimmune myocarditis, confirming that this region on chromosome 1 contains a susceptibility locus. We further found that this chromosomal region alters activation of caspases 3, 8 and 9 in lymphocytes in response to Cy-enhanced cell death and decreases caspase activation in effector CD4⁺ T cells after antigen stimulation.

Materials and Methods

Mice

A.SW and B10.S were purchased from the Jackson Laboratory and were bred and maintained in the conventional housing facilities at The Johns Hopkins University (Baltimore, MD). All protocols have been reviewed and approved by the Johns Hopkins Animal Care and Use Committee.

Generation of B10.A-Eam1 congenic mice

The B10.A-*Eam1* congenic mouse was created by the speed congenic approach [17]. Briefly, genomic DNA was prepared from tail tissue as described previously [18]. Mice were genotyped with 81 SSLP markers to determine inheritance pattern of A.SW and B10.S alleles. A.SW mice were crossed to the B10.S mouse to generate the heterogeneous F₁ generation. Next, the F₁ generation was backcrossed to the B10.S mouse for six generations. After every generation each mouse was screened for inheritance of A.SW at markers that define the *Eam1* locus while all other markers were screened for inheritance of B10.S (Fig. 1) [7]. Mice that meet these requirements at generation six were mated to create the homozygous B10.A-*Eam1* congenic mouse. The following chromosome 1 SSLP markers are of A.SW origin: *D1Mit65*, *D1Mit373*, *D1Mit213*, *D1Mit435* and *D1Mit334*. The following SSLP markers *D1Mit26*, *D1Mit15* and *D1Mit407* are of B10.S origin. The remainder of the genome was of B10.S origin, as determined by previously defined SSLP markers [7]. NCBI Blastn was used to identify the approximate physical locations for *D1Mit435* and *D1Mit334*. Blastn search was performed with primer sequences used to amplify these SSLPs which are reported by Mouse Genome Informatics (<http://www.informatics.jax.org/>).

Induction and phenotype quantification of EAM

EAM was induced in male and female 8 – 12 week old A.SW, B10.S and B10.A-*Eam1* congenic mice by subcutaneous injections of 200 to 250 µg of purified murine cardiac myosin emulsified in complete Freund's adjuvant (CFA) (Sigma Aldrich, St. Louis, MO), supplemented with 5 mg/ml of H37Ra extract (Difco, Lawrence, Kansas), (1:1 ratio) on days 0 and 7 as previously described [7]. Pertussis toxin (Sigma Aldrich, St. Louis, MO) (500 ng in 100 µl of PBS) was also administered intraperitoneally (i.p.) on day 0. Hearts were collected on day 21 post injection, bisected coronally along any major lesion that could be identified by visual inspection, and fixed in 10% formaldehyde and embedded in paraffin. Coronal sections (5 µM thick) at different levels throughout the heart were cut and stained with hematoxylin and eosin. The degree of myocardial infiltrations was blindly determined histologically as previously described [19]. Since B10.S mice sometimes developed minimal lesions following immunization with cardiac myosin, we used a conservative threshold of 5% infiltration in determining incidence and severity of disease [7].

Cy-enhanced peripheral lymphocyte cell death

Male and female mice 8 – 12 week old were injected i.p with 7.5 mg of Cy (Sigma Aldrich, St. Louis, MO) dissolved in 300 µl of sterile PBS. Control mice were injected with 300 µl of sterile PBS. Mice were sacrificed 16 hours after injection and single cell suspensions of the lymph nodes were prepared using standard immunological techniques. Three x 10⁵ LNCs were stained with fluorescent labeled caspase 3, 8 or 9 irreversible inhibitors, FITC-DEVD-FMK, rhodamine-IETD-FMK or rhodamine-LEHD-FMK (BioVision, Mountain View, California), respectively. Lymph node cells of each mouse were then incubated at 37°C for 45 min or 1 hr. The cells were then washed twice with PBS, fixed in 1% paraformaldehyde and stored at 4°C until analyzed with flow cytometry. Four to 5 mice per strain per treatment group were used. Five - 6 experiments performed for each of the caspase activity assays with an n = 4 – 5 mice per group.

Real time quantitative-RT-PCR for caspase 3, 8 and 9

Spleens were isolated from mice injected with Cy or, as controls, with PBS only and single cell suspensions of the splenocytes were prepared as previously described [7]. Total RNA was extracted from 7 × 10⁶ splenocytes using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacture's instructions. DNase 1 was also used in the preparation according to manufacture's instructions (Qiagen, Valencia, CA). First strand cDNA synthesis was

performed with M-MLV RT transcriptase (Invitrogen, Carlsbad, California) according to manufacture's instructions. Mouse specific caspase 3, 8 and 9 proprietary primer and probe (FAM) sets were purchased from Applied Biosystems. Mouse specific GAPDH primer and probe set (Applied Biosystems, Foster City, CA) was used as internal control. Gene expression of GAPDH did not change following Cy treatment. Real time quantitative-RT-PCR was performed in triplicates with 1 x Taqman universal RCR master mix, 1 x Taqman gene expression assay (primer and probe set) and 1 – 10 ng of cDNA in a total volume of 20 μ l. Each primer and probe set was run separately for each sample. Thermal cycle conditions were performed according to manufacture's instructions. No template controls were used for each primer and probe set. Four experiments were performed with four mice per strain per treatment group.

DNA sequencing of candidate genes

Genomic DNA was isolated from the tails of A.SW and B10.S mice as described previously [18]. Primers flanking the promoter (–200 to ATG) and exon regions of each gene were designed. Standard PCR conditions were used to amplify these regions. PCR products were electrophoresed on an agarose gel and extracted using Qiagen gel extraction kit. Isolated PCR products were then sequenced by the Core Facility, Johns Hopkins University (Baltimore, MD). Sequences were aligned and analyzed with BioEdit Alignment Editor.

In vivo antigen-induced cell death

For these experiments, A.SW, B10.S and B10.A-*Eam1* congenic mice were immunized subcutaneously with 200 μ g of myosin in CFA supplemented with 5 mg/ml of H37Ra extract (Difco, Lawrence, Kansas) at the back of neck (50 μ l) and base of tail (50 μ l). Pertussis toxin (500 ng in 100 μ l of PBS) was administered i.p. Nonimmunized mice served as controls. Draining lymph nodes were removed on day 9 after immunization and pooled from 2–4 mice/group. LNCs were incubated with FITC-DEVD-FMK, FITC-IETD-FMK or FITC-LEHD-FMK (BioVision, Mountain View, California) to detect activation of caspases 3, 8 and 9 respectively as described above. Days 7 – 9 are commonly used to study primary T cell responses. Cells were then labeled with anti-CD3, anti-CD4 and/or anti-CD8 antibodies and anti-CD19 (BD Biosciences, San Diego, CA). Flow cytometry was used to determine the percentage of CD4⁺, CD8⁺ T cells and CD19⁺ B cells with activated caspases 3, 8 or 9. Activation of lymphocytes was determined by co staining with anti-CD3, anti-CD4, anti-CD8, anti-CD19 and anti-CD69 antibodies.

Microarray analysis

Male A.SW and B10.S mice, 6–8 w-old, were immunized subcutaneously with 200 μ g of cardiac myosin in CFA. Eight days later, draining lymph nodes were harvested and processed for GeneChip analysis at the Malaria Research Institute Gene Array Core Facility at the Johns Hopkins Bloomberg School of Public Health (Baltimore, MD). Assay was performed in accordance with the methods described in the Affymetrix GeneChip Expression Analysis Technical Manual. Data analysis was performed with GeneSpring version 7.2 (Silicon Genetics).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. To compare the incidence of disease we used the Fisher's exact test. To analyze difference in EAM severity in A.SW and B10.A-*Eam1* congenic mice as well as to compare the percentage of cells with caspase activation before and after immunization within each strain, we used the Mann-Whitney test. To compare differences in the percentage of CD4⁺, CD8⁺ T cells and CD19⁺ T cells with

activated caspase 3, 8 or 9 among the three groups (B10.S, A.SW and B10.A-*Eam1* congenic mice), we used the non-parametric Kruskal-Wallis test to generate an overall P value test followed by Dunn's multiple pairwise comparison test.

Results

Eam1 plays a role in the development of EAM

We previously reported that A.SW mice have a significantly higher incidence of EAM compared to B10.S mice following immunization with cardiac myosin in CFA [4,7]. We identified a non-MHC susceptibility locus, *Eam1*, located on chromosome 1. To study the role of the *Eam1* susceptibility locus, we created the B10.A-*Eam1* congenic mouse, where the *Eam1* locus is derived from the A.SW mouse, while the remainder of chromosome 1 and the rest of the genome are of B10.S origin (Fig. 1). We immunized B10.S, A.SW and B10.A-*Eam1* congenic mice with cardiac myosin in CFA and compared disease incidence and severity.

The incidence of disease (mice with myocarditis greater than 5%) in the A.SW mouse (12 of 28, 43%) was higher than the B10.S mouse (3 of 20, 15%) (Fig. 2B). Yet, the incidence of disease in the B10.A-*Eam1* congenic mouse (17 of 29, 59%) resembled the A.SW mouse, but differed significantly from the parental B10.S strain ($p < 0.01$; Fig. 2B). However, the severity of disease in mice that developed a myocardial infiltrate was significantly lower in B10.A-*Eam1* congenic mice ($20.56 \pm 11.54\%$) than in A.SW mice ($32.69 \pm 13.94\%$) ($p < 0.01$; Fig. 2B). Males and females of all strains were equally affected. Thus, although the LOD score we previously reported for this region was statistically marginal, these data confirm that a gene in the *Eam1* region influences susceptibility to EAM in resistant B10.S mice.

Eam1 regulates Cy-induced cell death by regulating caspase activation

It has been previously determined that a region on chromosome 1 modulates Cy- induced cell death of lymphocytes in the NOD mouse [15]. Since we also found that A.SW mice are significantly less sensitive to Cy-enhanced cell death than B10.S mice [7], we next wanted to determine if the locus on chromosome 1 regulates Cy-enhanced cell death in LNCs in the A.SW mouse. We used activation of caspase 8 and 9 as markers of the death receptor and the mitochondrial death inducing pathways, respectively. Caspase 3 activation was used as an overall marker for the ability of LNCs to activate either death inducing pathway.

Even in groups not treated with Cy, we found caspase 3, caspase 8, but not caspase 9 activities differed among the three groups (Fig. 3A–C). Pairwise comparisons showed that A.SW mice had significantly lower caspase 3 and caspase 8 ($p < 0.01$ and $p < 0.05$, respectively) activity than did the B10.S (Fig. 3A–B). LNCs of the B10.A-*Eam1* congenic with activated caspase 3 and caspase 8 ($p < 0.05$ and $p < 0.01$, respectively) was also significantly higher than A.SW mice prior to Cy treatment (Fig. 3A–B). Following Cy treatment, the three groups showed even greater differences in caspase 3, 8 and 9 activation (Fig. 3A–C). Following pairwise comparison, we found that the percentage of LNCs expressing activated caspase 3, 8 and 9 (all $p < 0.001$) after Cy treatment in the EAM susceptible A.SW mouse was significantly less than the EAM resistant B10.S mouse (Fig. 3A–C). B10.A-*Eam1* congenic mice also had a significantly lower percentage of cells with caspase 3, 8 and 9 (all $p < 0.05$) activity than the parental B10.S strain following Cy treatment (Fig. 3A–C). The percentage of LNCs of the B10.A-*Eam1* congenic mouse also had significantly higher levels of caspase 3, 8 and 9 (all $p < 0.01$) than the levels found in the A.SW mouse (Fig. 3A–C). Since the B10.A-*Eam1* congenic mouse revealed an intermediate phenotype, these data suggest that *Eam1* in part regulates caspase activation of lymphocytes. To determine if *Eam1* modulates caspase activation by regulating transcription, we performed quantitative real time PCR for caspases 3, 8 or 9. We observed no differences in the gene expression of caspases 3, 8 or 9 following Cy treatment

between all three groups (data not shown). These data suggest that genomic variations at *Eam1* likely regulate post translational activation of caspases rather than transcriptional modification of caspase genes.

Eam1 modulates differences in caspase activation in CD4⁺ T cells following myosin immunization

Since *Eam1* modulates Cy-induced cell death in LNCs, we next wanted to determine if *Eam1* modulates antigen-induced cell death in CD4⁺ or CD8⁺ T cells or both. A.SW, B10.S and B10.A-*Eam1* congenic mice were challenged once with murine cardiac myosin in CFA and cell death was measured by caspase activation on day 9 following challenge. We found that the percentage of CD4⁺ T cells (Fig. 4) and CD8⁺ T cells (Fig. 5) with caspase activation was similar prior to immunization in all strains of mice. Although T cell activation was slightly higher in the B10.S mouse, T cell activation did not differ significantly among the three groups (data not shown). The percentage of CD4⁺ T cells (Fig. 4), but not CD8⁺ T cells (Fig. 5) with caspase 3, 8 and 9 activation was different among the three groups 9 days following immunization with myosin in CFA. Pairwise comparison revealed that the percentage of CD4⁺ T cells of the A.SW mouse had significantly less activated caspase 3, 8 and 9 (all $p < 0.05$) than B10.S mice (Fig. 4A–C). Also we found that the percentage of CD4⁺ T cells with activated caspase 3, 8 and 9 in the B10.A-*Eam1* congenic mouse was not significantly different from A.SW strain or the B10.S mouse (Fig. 4A–C). B10.S mice were the only group that significantly increased the percentage of CD4⁺ T cells with caspase 3, 8 and 9 (all $p < 0.05$) activity following immunization (Fig. 4A–C and Table 1). These data suggest that allelic variations at the *Eam1* susceptibility locus could regulate caspase activity in CD4⁺ T cells.

Eam1 modulates activation of caspases in B cells

The role of B cells in autoimmune myocarditis remains controversial [20,21], but defects in B lymphocyte cell death can influence autoimmune manifestations [22,23]. Therefore, we questioned whether B cells could also be defective in antigen-induced caspase activation. Mice were injected with myosin in CFA and caspase 3, 8 and 9 activation and B cell activation was determined 9 days later. The percentage of B cells with caspase 3, 8 and 9 activation was similar in nonimmunized A.SW, B10.S, and B10.A-*Eam1* congenic mice (Fig. 6A–C). B cell activation did not differ in A.SW, B10.S or B10.A-*Eam1* congenic mice following immunization (data not shown). Although not statistically significant, the percentage of B cells of the A.SW and B10.A-*Eam1* congenic mice with caspase 3 activation was somewhat less than the B10.S mice following immunization (Fig. 6A). Although all three groups were statistically similar following immunization, the percentage of B cells of A.SW mice with caspase 8 and caspase 9 activity was lower than B10.S while B10.A-*Eam1* congenic mice showed an intermediate phenotype for caspase 3 (Fig. 6A). However, B10.S was the only group of mice to significantly increase the percentage of B cells with caspase 3, 8 and 9 (all $p < 0.05$) activity following immunization (Fig. 6A–C and Table 1). B10.A-*Eam1* Congenic mice demonstrated marginal increases in the percentage of B cells with caspase 9 activity following immunization which approached but did not achieve statistical significance ($p = 0.06$; Fig. 6C and Table 1). These data imply that *Eam1* may have a greater role in regulating caspase activation in CD4⁺ T cells than in B cells.

Genes that are differentially regulated between A.SW and B10.S mice

The susceptibility locus on chromosome 1 contains several genes reported to modulate apoptosis including caspase 8 and Flip, an inhibitor of caspase 8. However, we found no polymorphisms in the basal promoter or coding regions of any of these genes, when we compared B10.S and A.SW mice (data not shown). Next we wanted to identify genes in the *Eam1* locus that can potentially modulate T cell responses in the antigen priming phase which

could influence susceptibility to EAM or cell death. Microarray analysis was then used to discover genes within the *Eam1* region with differential expression in myosin-primed lymph nodes of susceptible A.SW and resistant B10.S strain. We found two genes with differential expression following myosin stimulation on day 8 in the lymph node: IL-1 receptor II (Il1r2) and inducible T cell co-stimulator (Icos) (Supplementary Table 1). Il1r2 and Icos mRNA expression following myosin challenge was 2.4 and 3 times higher, respectively, in the lymph nodes of B10.S mice compared to those of the A.SW mice (Supplementary Table 1). These data suggest that Icos and Il1r2 may modulate lymphocyte death and susceptibility to EAM.

Discussion

The congenic mouse provides a valuable tool for understanding the immunogenetics of autoimmune diseases. Its use allowed us to study functional allele(s) located in the *Eam1* susceptibility locus in an EAM resistant B10.S background. To investigate the role of the *Eam1* locus, we replaced a 50 cM portion at the centromeric end of chromosome 1 in the B10.S mouse with one of A.SW origin. First we confirmed that our previously identified *Eam1* locus on chromosome 1 has an important role in determining susceptibility to EAM because the incidence of EAM in the B10.S mouse was significantly increased when the region on chromosome 1 was replaced with the *Eam1* susceptibility locus of A.SW origin. This finding shows that the *Eam1* locus controls a key immunologic phenotype which promotes disease even in a relatively resistant strain. On the other hand, since the severity of disease was significantly less severe in B10.A-*Eam1* congenic mice than A.SW, our data support the observation that other non-MHC loci also play a role in determining disease susceptibility [7, 21,24]. In seeking a plausible basis for the difference, we found that CD4⁺ T cells (and, to a lesser extent, B cells) of the EAM susceptible A.SW mouse were less sensitive to antigen-induced caspase activation in vivo compared to EAM resistant B10.S mice. EAM is known to depend primarily on CD4⁺ T cells [25].

Defects in caspases has been implicated in several human autoimmune diseases including autoimmune thyroiditis, autoimmune polyendocrine syndrome-2 and type 1 diabetes mellitus [26,27]. Mutations in caspase 10 have been reported in patients with autoimmune lymphoproliferative syndrome type II [28]. Here caspase 3 served as an overall marker of the triggering of death-inducing pathways. We used activation of caspase 8 and 9 as additional markers of the ability of lymphocytes to activate the death receptor or mitochondria pathway, respectively. There are, however, several positive feedback loops; for example, caspase 3 can activate caspase 8 and 9. Caspase 8 can also activate caspase 9 via a pro-apoptotic Bcl-2 family member, Bid. We found that A.SW and B10.A-*Eam1* mice have impaired caspase 3, 8 and 9 activation following Cy treatment, showing that the *Eam1* locus modulates caspase activation. However, we found that transcription of caspases did not change in A.SW, B10.S and B10.A-*Eam1* congenic mice following Cy-enhanced apoptosis. Together these data imply that the *Eam1* locus likely modulates post translational activation of these caspases, rather than their direct transcription. Cy has been shown to induce cell death via the death receptor pathway [29]. These data thus suggest that the death receptor pathway is less activated in the A.SW mice compared to B10.S mice.

Antigen-induced cell death, a phenomenon that occurs in T lymphocytes following T cell activation, has been shown to be mediated by the death receptor pathway and plays an important role in peripheral tolerance [30]. Although there are conflicting data about role of CD8⁺ T cells [25,31], it is generally accepted that autoimmune myocarditis is predominately mediated by CD4⁺ T cells. Here we show that CD4⁺ T cells of the EAM susceptible A.SW mouse fail to activate caspases following antigen challenge in vivo as fully as EAM resistant B10.S mice. These data imply that CD4⁺ T cells of the A.SW mouse are less likely to die in response to antigen stimulation than are those of B10.S mice, suggesting a plausible mechanism to explain

why A.SW mice are more susceptible to EAM. Our data further demonstrate that the *Eam1* susceptibility locus modulates the activation of caspases in CD4⁺ T cells, following myosin challenge, since CD4⁺ T cells with activated caspases is intermediate in the B10.A-*Eam1* congenic. The observation that *Eam1* regulates the cell death of CD4⁺ T cells, which are the key mediators of autoimmune myocarditis, provides an explanation as to why the susceptibility of the B10.S mouse is profoundly increased in the presence of the *Eam1* susceptibility locus. However, it is important to note that the *Eam1* susceptibility locus affects CD4⁺ T cell death after cell death pathways has been strongly activated by immunization with antigen or by Cy, since lymphocyte cell death of nonimmunized, and untreated B10.A-*Eam1* congenic mice resembled that found in nonimmunized and untreated B10.S mice. Caspases are paradoxical in nature in that they are involved in activation of T cells [32]. Since we did not observe significant differences in T cell activation following immunization, differences in caspase activation were not be due to decreased T cell activation. Furthermore, a control experiment showed that caspase activation following immunization with ovalbumin in CFA is similar between A.SW and B10.S mice, suggesting that differences in caspase activation are likely due to CD4⁺ T cell responses specific to myosin and not CFA (unpublished data).

Mice that contain a mutation in death receptors, or in the pro-apoptotic Bcl-2 family member, accumulate autoreactive B cells [22,23], suggesting that cell death is also an important mechanism for modulating the fate of self reactive B-cells. The role of B lymphocytes and antibody in the development of autoimmune myocarditis is not clear and may be strain specific [20,21]. BALB/c mice depleted of B cells developed disease comparable to wild type mice [20]. Although it is possible that failure to remove autoreactive B cells influences susceptibility to autoimmune disease as shown several mice lupus models [33], this phenomenon may be secondary to the involvement of the CD4⁺ T lymphocyte in EAM.

It remains uncertain that the same polymorphism that regulates susceptibility to EAM also regulates cell death. Further narrowing of the locus by congenic strain based positional cloning and haplotype mapping is required to determine if the two phenotypes are closely linked. We were not able to identify any polymorphisms in the basal promoter or coding regions of caspase 8 or Flip. However, it is possible that these genes contain polymorphisms outside the regions that we sequenced. The *Eam1* locus probably contains several genes that are polymorphic and may have differential effects on susceptibility to autoimmune myocarditis. We found that ICOS and IL-1 receptor II gene expressions are differently regulated during the antigen priming phase. ICOS-ICOSL signaling pathway has been reported to be important for EAM susceptibility in rats [34]. Others have shown that the ICOS-ICOSL signaling pathway negatively regulates autoimmune diseases such as EAE and crescentic glomerulonephritis [35,36,37]. ICOS has been proposed to modulate cell death of immune cells [38]. IL-1 receptor II is a decoy receptor for IL-1 [39]. Alterations in IL-1 receptor II expression can potentially influence susceptibility to EAM, since B10.A mice develop myocarditis when immunized with myosin in the presence of IL-1 [40]. Further work is needed to determine if putative polymorphisms in these genes regulate autoimmune myocarditis and lymphocyte death.

In summary, a centromeric locus on chromosome 1 has been shown to modulate several cell types and processes [41,42,43]. In this study we show that lymphocytes of the EAM susceptible A.SW mouse compared to those of the EAM resistant B10.S mouse have impaired caspase activation following two different modes of cell death induction: Cy-enhanced cell death and antigen-induced cell death with myosin in CFA. Specifically, the B10.A-*Eam1* congenic mouse revealed that the *Eam1* locus decreases caspase activation in CD4⁺ T cells in the B10.S mouse following myosin challenge. Additionally, replacing the region of A.SW origin in the B10.S mouse significantly increases EAM incidence such that the incidence of disease is comparable in B10.A-*Eam1* congenic mice and A.SW mice. However, the severity of disease was significantly less in the congenic mouse compared to the A.SW mouse, indicating that while

Eam1 plays a role in the development of autoimmune myocarditis, other susceptibility loci also modulate disease severity. Our findings demonstrate that allelic variations located at the centromeric end of chromosome 1 affect both development of EAM and caspase activation in the CD4⁺ T cells.

Acknowledgements

We thank Drs. Patrizio Caturegli, Dolores Njoku, Daniela Cihakova and Karl Broman for their valuable comments on the manuscript.

Abbreviations in this paper

CFA	complete Freund adjuvant
EAM	experimental autoimmune myocarditis
Cy	cyclophosphamide
LNCs	lymph node cells
<i>Eam1</i>	EAM susceptibility locus 1

References

1. Drory Y, Turetz Y, Hiss Y, Lev B, Fisman EZ, Pines A, Kramer MR. Sudden unexpected death in persons less than 40 years of age. *Am J Cardiol* 1991;68:1388–92. [PubMed: 1951130]
2. Brown CA, O'Connell JB. Myocarditis and idiopathic dilated cardiomyopathy. *Am J Med* 1995;99:309–14. [PubMed: 7653492]
3. Feldman AM, McNamara D. Myocarditis. *N Engl J Med* 2000;343:1388–98. [PubMed: 11070105]
4. Neu N, Rose NR, Beisel KW, Herskowitz A, Gurri-Glass G, Craig SW. Cardiac myosin induces myocarditis in genetically predisposed mice. *J Immunol* 1987;139:3630–6. [PubMed: 3680946]
5. Rose NR, Neumann DA, Herskowitz A, Traystman MD, Beisel KW. Genetics of susceptibility to viral myocarditis in mice. *Pathol Immunopathol Res* 1988;7:266–78. [PubMed: 2853345]
6. Wandstrat A, Wakeland E. The genetics of complex autoimmune diseases: non-MHC susceptibility genes. *Nat Immunol* 2001;2:802–9. [PubMed: 11526390]
7. Guler ML, Ligons DL, Wang Y, Bianco M, Broman KW, Rose NR. Two autoimmune diabetes loci influencing T cell apoptosis control susceptibility to experimental autoimmune myocarditis. *J Immunol* 2005;174:2167–73. [PubMed: 15699148]
8. Wicker LS, Chamberlain G, Hunter K, Rainbow D, Howlett S, Tiffen P, Clark J, Gonzalez-Munoz A, Cumiskey AM, Rosa RL, Howson JM, Smink LJ, Kingsnorth A, Lyons PA, Gregory S, Rogers J, Todd JA, Peterson LB. Fine mapping, gene content, comparative sequencing, and expression analyses support *Ctla4* and *Nramp1* as candidates for *Idd5.1* and *Idd5.2* in the nonobese diabetic mouse. *J Immunol* 2004;173:164–73. [PubMed: 15210771]
9. Meeker ND, Hickey WF, Korngold R, Hansen WK, Sudweeks JD, Wardell BB, Griffith JS, Teuscher C. Multiple loci govern the bone marrow-derived immunoregulatory mechanism controlling dominant resistance to autoimmune orchitis. *Proc Natl Acad Sci U S A* 1995;92:5684–8. [PubMed: 7777570]
10. Haywood ME, Rose SJ, Horswell S, Lees MJ, Fu G, Walport MJ, Morley BJ. Overlapping BXSB congenic intervals, in combination with microarray gene expression, reveal novel lupus candidate genes. *Genes Immun* 2006;7:250–63. [PubMed: 16541099]

11. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol* 2007;7:532–42. [PubMed: 17589543]
12. Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *Embo J* 1997;16:2794–804. [PubMed: 9184224]
13. Scatizzi JC, Bickel E, Hutcheson J, Haines GK 3rd, Perlman H. Bim deficiency leads to exacerbation and prolongation of joint inflammation in experimental arthritis. *Arthritis Rheum* 2006;54:3182–93. [PubMed: 17009248]
14. Karray S, Kress C, Cuvellier S, Hue-Beauvais C, Damotte D, Babinet C, Levi-Strauss M. Complete loss of Fas ligand gene causes massive lymphoproliferation and early death, indicating a residual activity of gld allele. *J Immunol* 2004;172:2118–25. [PubMed: 14764677]
15. Colucci F, Bergman ML, Penha-Goncalves C, Cilio CM, Holmberg D. Apoptosis resistance of nonobese diabetic peripheral lymphocytes linked to the Idd5 diabetes susceptibility region. *Proc Natl Acad Sci U S A* 1997;94:8670–4. [PubMed: 9238035]
16. Colucci F, Cilio CM, Lejon K, Goncalves CP, Bergman ML, Holmberg D. Programmed cell death in the pathogenesis of murine IDDM: resistance to apoptosis induced in lymphocytes by cyclophosphamide. *J Autoimmun* 1996;9:271–6. [PubMed: 8738973]
17. Wakeland E, Morel L, Achey K, Yui M, Longmate J. Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol Today* 1997;18:472–7. [PubMed: 9357138]
18. Gorham JD, Guler ML, Steen RG, Mackey AJ, Daly MJ, Frederick K, Dietrich WF, Murphy KM. Genetic mapping of a murine locus controlling development of T helper 1/T helper 2 type responses. *Proc Natl Acad Sci U S A* 1996;93:12467–72. [PubMed: 8901605]
19. Cihakova D, Sharma RB, Fairweather D, Afanasyeva M, Rose NR. Animal models for autoimmune myocarditis and autoimmune thyroiditis. *Methods Mol Med* 2004;102:175–93. [PubMed: 15286386]
20. Malkiel S, Factor S, Diamond B. Autoimmune myocarditis does not require B cells for antigen presentation. *J Immunol* 1999;163:5265–8. [PubMed: 10553048]
21. Kuan AP, Chamberlain W, Malkiel S, Lieu HD, Factor SM, Diamond B, Kotzin BL. Genetic control of autoimmune myocarditis mediated by myosin-specific antibodies. *Immunogenetics* 1999;49:79–85. [PubMed: 9887344]
22. Takeuchi O, Fisher J, Suh H, Harada H, Malynn BA, Korsmeyer SJ. Essential role of BAX, BAK in B cell homeostasis and prevention of autoimmune disease. *Proc Natl Acad Sci U S A* 2005;102:11272–7. [PubMed: 16055554]
23. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A. Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. *J Exp Med* 2003;198:1119–26. [PubMed: 14517273]
24. Aly M, Wiltshire S, Chahrouh G, Osti JC, Vidal SM. Complex genetic control of host susceptibility to coxsackievirus B3-induced myocarditis. *Genes Immun* 2007;8:193–204. [PubMed: 17287827]
25. Smith SC, Allen PM. Myosin-induced acute myocarditis is a T cell-mediated disease. *J Immunol* 1991;147:2141–7. [PubMed: 1918949]
26. Vendrame F, Segni M, Grassetti D, Tellone V, Augello G, Trischitta V, Torlontano M, Dotta F. Impaired caspase-3 expression by peripheral T cells in chronic autoimmune thyroiditis and in autoimmune polyendocrine syndrome-2. *J Clin Endocrinol Metab* 2006;91:5064–8. [PubMed: 16968794]
27. Vendrame F, Santangelo C, Misasi R, Dionisi S, Gizzi C, Realacci M, Grassetti D, Di Mario U, Dotta F. Defective lymphocyte caspase-3 expression in type 1 diabetes mellitus. *Eur J Endocrinol* 2005;152:119–25. [PubMed: 15762195]
28. Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, Yao X, Puck JM, Straus SE, Lenardo MJ. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell* 1999;98:47–58. [PubMed: 10412980]
29. Lee JH, Park JH, Yang MH. The effect of cyclophosphamide on Fas-mediated apoptosis. *J Korean Med Sci* 1997;12:185–9. [PubMed: 9250912]
30. Tibbetts MD, Zheng L, Lenardo MJ. The death effector domain protein family: regulators of cellular homeostasis. *Nat Immunol* 2003;4:404–9. [PubMed: 12719729]

31. Penninger JM, Neu N, Timms E, Wallace VA, Koh DR, Kishihara K, Pummerer C, Mak TW. The induction of experimental autoimmune myocarditis in mice lacking CD4 or CD8 molecules [corrected]. *J Exp Med* 1993;178:1837–42. [PubMed: 8228830]
32. Schwerk C, Schulze-Osthoff K. Non-apoptotic functions of caspases in cellular proliferation and differentiation. *Biochem Pharmacol* 2003;66:1453–8. [PubMed: 14555221]
33. Tsubata T. B cell abnormality and autoimmune disorders. *Autoimmunity* 2005;38:331–7. [PubMed: 16240466]
34. Futamatsu H, Suzuki J, Kosuge H, Yokoseki O, Kamada M, Ito H, Inobe M, Isobe M, Uede T. Attenuation of experimental autoimmune myocarditis by blocking activated T cells through inducible costimulatory molecule pathway. *Cardiovasc Res* 2003;59:95–104. [PubMed: 12829180]
35. Dong C, Nurieva RI. Regulation of immune and autoimmune responses by ICOS. *J Autoimmun* 2003;21:255–60. [PubMed: 14599850]
36. Odobasic D, Kitching AR, Semple TJ, Holdsworth SR. Inducible co-stimulatory molecule ligand is protective during the induction and effector phases of crescentic glomerulonephritis. *J Am Soc Nephrol* 2006;17:1044–53. [PubMed: 16540559]
37. Rottman JB, Smith T, Tonra JR, Ganley K, Bloom T, Silva R, Pierce B, Gutierrez-Ramos JC, Ozkaynak E, Coyle AJ. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. *Nat Immunol* 2001;2:605–11. [PubMed: 11429544]
38. Lutz J, Lu R, Strobl M, Huang H, Deng M, Wang M, Ouyang N, Heemann U. ICOS/B7RP-1 interference in mouse kidney transplantation. *Transplantation* 2007;84:223–30. [PubMed: 17667814]
39. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE, Mantovani A. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science* 1993;261:472–5. [PubMed: 8332913]
40. Lane JR, Neumann DA, Lafond-Walker A, Herskowitz A, Rose NR. Interleukin 1 or tumor necrosis factor can promote Cocksackie B3-induced myocarditis in resistant B10.A mice. *J Exp Med* 1992;175:1123–9. [PubMed: 1552283]
41. Motta V, Lejon K, Holmberg D. The NOD allele of the Idd5 locus on chromosome 1 mediates a non-cell-autonomous defect in negative selection of T cells. *J Autoimmun* 2007;28:216–23. [PubMed: 17449224]
42. Sundstrom M, Lejon K. Idd-linked genetic regulation of TACI^{high} expressing B cells in NOD mice. *J Autoimmun* 2007;29:116–24. [PubMed: 17656068]
43. Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G, Herr MH, Dahlman I, Payne F, Smyth D, Lowe C, Twells RC, Howlett S, Healy B, Nutland S, Rance HE, Everett V, Smink LJ, Lam AC, Cordell HJ, Walker NM, Bordin C, Hulme J, Motzo C, Cucca F, Hess JF, Metzker ML, Rogers J, Gregory S, Allahabadia A, Nithiyananthan R, Tuomilehto-Wolf E, Tuomilehto J, Bingley P, Gillespie KM, Undlien DE, Ronningen KS, Guja C, Ionescu-Tirgoviste C, Savage DA, Maxwell AP, Carson DJ, Patterson CC, Franklyn JA, Clayton DG, Peterson LB, Wicker LS, Todd JA, Gough SC. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 2003;423:506–11. [PubMed: 12724780]

Figure 1.

Schematic of the *Eam1* susceptibility locus. B10.A-*Eam1* congenic are of B10.S origin, but contain genomic material of A.SW origin on chromosome 1 (arrow). Several candidate genes with immune function are located in *Eam1*. (Not drawn to scale).

The *Eam1* locus increases susceptibility to EAM. B10.S (■), A.SW (●) and B10.A-*Eam1* congenic (◆) mice were immunized with murine cardiac myosin/CFA. Hearts were collected on day 21 and histologically examined to determine percentage of myocardium affected (% myocarditis). (A) Representative histology of each group: (left panel: whole hearts (4X) and right panel: higher magnification of heart sections (64X)). (B) Shows the percentage of myocarditis for individual mice; pooled data from 3 independent experiments. Fisher's exact test was used to compare incidence of disease.

Figure 3.

Eam1 regulates activation of caspases in response to Cy-enhanced cell death. B10.S, A.SW and B10.A-*Eam1* congenic mice were injected with 7.5 mgs of Cy or PBS. LNCs were stained with fluorescent labeled caspase 3, 8 or 9 irreversible inhibitors 16 hours later. Percentage of cells with activated caspase 3 (A), 8 (B) or 9 (C) was determined by flow cytometry. Figure shows 6 experiments pooled together and each symbol represents one individual mouse. Data were normalized to the experiment with B10.S group having highest percentage of positive cells. Lines indicate means, pairwise post-hoc: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 4.

CD4⁺ T cells of A.SW mice have significantly less caspase activation following immunization in vivo. Immunized mice received 200 µg of murine cardiac myosin/CFA and 500 ng of pertussis toxin. LNCs were removed and pooled from mice immunized on day 9 and non-immunized mice. Cells were stained with fluorescent labeled caspase 3, 8 or 9 irreversible inhibitors. Representative histogram showing nonimmunized mice (gray line) and immunized mice (black line). Percentage of CD4⁺ cells with activated caspases 3 (A), 8 (B) and 9 (C) of nonimmunized mice (top numbers) and immunized mice (bottom numbers). Numbers are means ± standard deviations. ^aKruskal-Wallis test followed by pairwise post-hoc to compare immunized mice between strains (*p < 0.05). ^bMann Whitney Test to compare nonimmunized to immunized mice within mouse strain (*p < 0.05). Data pooled from 3 independent experiments.

Figure 5.

CD8⁺ T cells have similar caspase activation following immunization in vivo. Experimental design as in figure 3. Representative histogram showing nonimmunized mice (gray line) and immunized mice (black line). Percentage of CD8⁺ cells with activated caspases 3 (A), 8 (B) and 9 (C) of nonimmunized mice (top numbers) and immunized mice (bottom numbers). Numbers are means ± standard deviations. Data pooled from 3 independent experiments.

Figure 6.

CD19⁺ B cells of A.SW mice have less caspase activation following immunization in vivo. Experimental design as in figure 3. Representative histogram showing nonimmunized mice (gray line) and immunized mice (black line). Percentage of CD19⁺ cells with activated caspases 3 (A), 8 (B) and 9 (C) of nonimmunized mice (top numbers) and immunized mice (bottom numbers). Numbers are means ± standard deviations. ^aMann Whitney Test to compare nonimmunized to immunized mice within mouse strain (*p < 0.05 and †p = 0.06). Data pooled from 3 independent experiments.

Table 1

Fold increase in caspase expression in CD4⁺ and CD19⁺ lymphocytes following myosin challenge (day 9)

	B10.S	A.SW	B10.A-<i>Eam1</i>
CD4⁺			
Caspase 3	2.12	1.67	1.06
Caspase 8	2.35	1.07	1.33
Caspase 9	2.31	1.36	1.79
CD19⁺			
Caspase 3	1.86	1.24	1.13
Caspase 8	1.83	1.12	1.36
Caspase 9	1.86	1.11	1.73