Published in final edited form as:

Int Immunopharmacol. 2008 May ; 8(5): 624–633. doi:10.1016/j.intimp.2008.01.004.

L.E.A.P.S. heteroconjugate is able to prevent and treat experimental autoimmune myocarditis by altering trafficking of autoaggressive cells to the heart

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Abstract

We evaluated the efficacy of the Ligand Epitope Antigen Presentation System (L.E.A.P.S.[™]) in preventing or treating experimental autoimmune myocarditis (EAM) in A/J mice. L.E.A.P.S. (here, J-My-1) is a conjugate of the myocarditogenic peptide of cardiac myosin MyHC $\alpha_{334-352}$ (My-1) and J peptide, derived from the sequence of human β -2 microglobulin. Remarkably, early prophylactic (J-My-1 injected on days –14 and –7 before EAM induction), late prophylactic (J-My-1 injected on days 0, 7, 14, and 21), and therapeutic (J-My-1 injected on days 7, 14, and 21 or 10, 17 and 24) administration of J-My-1 significantly decreased the incidence and severity of EAM. However, extended therapeutic treatment was associated with anaphylaxis and death, corresponding with global immune activation associated with J-My-1 treatment. In J-My1-treated animals, we observed expanded numbers of activated CD69⁺ and CD44⁺ CD4⁺ and CD8⁺ T cells in the spleens. J-My-1 treatment also increased the proportion of CD11c⁺ dendritic cells in spleens and induced strong production of anti-J-My-1 specific antibodies. J-My-1 injections resulted in decreased levels of chemokines MIP-1 α and IP-10 in hearts. We propose that J-My-1 treatment interferes with trafficking of auto-aggressive immune cells to the heart.

Keywords

Autoimmunity; Myocarditis; experimental autoimmune myocarditis (EAM); altered peptide ligand (APL); antigen specific therapy

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Introduction

Myocarditis is a major cause of dilated cardiomyopathy that can lead to heart failure and sudden death in people under 40 years of age (1,2). Current treatments for myocarditis only address the symptoms and not the underlying pathophysiology of the disease process. Experimental autoimmune myocarditis (EAM) offers a unique opportunity to evaluate therapeutic vaccines since it is one of the few autoimmune conditions where the autoantigen is well defined. Cardiac myosin was recognized as a major autoantigen in human heart disease as well as in mouse and rat models (3,4). In A/J mice, EAM can be induced by immunization with murine or porcine cardiac myosin or a myocarditogenic peptide derived from the sequence of murine cardiac myosin, here denoted as My-1 (MyHC $\alpha_{334-352}$) (5) with adjuvant. After immunization, mice develop pathology similar to that seen in humans, including mononuclear cell infiltration of the myocardium, and serum autoantibodies specific for cardiac myosin.

Here we show that a LEAPS heteroconjugate, J-My-1 (DLLKNGERIEKVEGGGAFDVLSTAEKAGVYK) is effective for both prevention and treatment of EAM. J-My-1 was derived by a novel technology, Ligand Epitope Antigen Presentation System (L.E.A.P.S.TM); it is a conjugate of the myocarditogenic My-1 peptide, and the J peptide, derived from a sequence of human β -2 microglobulin (aa38–50). Similarly designed L.E.A.P.S. peptides have been shown to successfully protect mice from herpes simplex virus infection (HSV) (6).

Others have shown that covalent attachment of the entire β -2 microglobulin protein, either chemically or as recombinant fusion proteins, to an epitope can enhance antigen presentation of that particular epitope (7,8). The original rationale behind the mechanism of L.E.A.P.S. was that the J-My-1 heteroconjugate would assist binding to CD8 molecules on the T cell and thus enhance binding of J-peptide-loaded MHC I to the TCR complex (9,10). However, the β -2-microglobulin sequence corresponding to the J peptide has been shown not to be involved in binding of Class I to the CD8 $\alpha\alpha$ homodimer (11). Therefore, we investigated an alternative hypothesis; namely that J-My-1 might have an effect similar to antagonist altered peptide ligands (APLs), thereby ameliorating severity of myocarditis by inducing anergy (12). We show here that J-My-1 is remarkably successful in preventing and even treating EAM; however, the mechanism of J-My-1 action appears to be through altering trafficking of auto-aggressive immune cells into the heart by reducing levels of chemokines MIP-1 α and IP-10 in the heart.

Materials and Methods

Mice

6–8 week-old female A/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in The Johns Hopkins University School of Medicine AALAC-approved SPF animal facility. All methods and protocols involving mice were approved by the Animal Care and Use Committee of Johns Hopkins University. Invasive procedures were performed under Avertin (Sigma-Aldrich, St. Louis, MO) anesthesia.

Peptides

For the induction of EAM, we used the myocarditogenic My-1 peptide, derived from the sequence of the murine cardiac myosin heavy chain (MyHC $\alpha_{334-352}$, AFDVLSTAEKAGVYK) (5) synthesized by Fmoc chemistry and purified by HPLC (Global Peptide, CO). The patented CEL-SCI L.E.A.P.S. constructs are heteroconjugates of β -2 microglobulin (aa38–50), J-peptide, My-1 peptide and a triglycine spacer (DLLKNGERIEKVEGGGAFDVLSTAEKAGVYK). J-My-1 and My-1 peptides were commercially synthesized by Fmoc chemistry, purified by preparative reverse-phase HPLC

and lyophilized (Global Peptide, CO). Both analytical RP-HPLC (for purity) and mass spectrophotometry (for molecular weight verification) were done by Global Peptides. We independently verified purity on a different proprietary RP-HPLC chromatography.

Induction of EAM and Histopathology

EAM was induced in 6–8 weeks old female A/J mice by s.c. immunization with 100 nmol of My-1 peptide in 50µL of 1xPBS emulsified in 50µL CFA (Sigma, Saint Louis, MO), supplemented with *Mycobacterium tuberculosis* strain H37Ra (Difco, Detroit, MI) to total concentration of 5 mg/ml, on days 0 and 7. Mice also received 500 ng pertussis toxin *ip* (List Biological Laboratories, Campbell, CA) on day 0. Mice were evaluated for the development of EAM at the peak of disease on day 21, or on day 28 with later treatment schedule initiation. Heart tissues were fixed in 10% phosphate-buffered formalin; 5 µm thick sections were cut longitudinally and stained with hematoxylin and eosin. Myocarditis severity was evaluated by histopathologic approximation of the percent area of myocardium infiltrated with mononuclear cells and fibrosis, measured microscopically in five sections from each heart. We used the following scoring system: grade 0 – no inflammation; grade 1: less than 10% of the heart section is involved; grade 2: 10–30%; grade 3: 30–50%; grade 4: 50–90%; grade 5: more than 90%. Two independent researchers scored all slides separately in a blinded manner. Additionally, we used heart weight (mg)/body weight(g) ratio as another indicator of myocarditis severity, since hearts with severe disease tend to be enlarged and severe myocarditis is associated with cachexia (13).

Prophylactic or therapeutic injections of J-My-1 to prevent or treat EAM

6–8 weeks old female A/J mice were injected s.c. with 100nmol of J-My-1 in 50µL of 1xPBS emulsified in 50µL of ISA-51 adjuvant on days –14 and –7 *prior to* EAM induction and sacrificed on day 28 (*prophylactic*), or injected on days 0, 7, 14, 21 *during* EAM induction and sacrificed on day 28 (*late prophylactic*) or injected *after* EAM induction on days 7, 14, 21 or 10, 17, 24 and sacrifice on day 28 (*therapeutic*). Control groups were vaccinated with PBS emulsified in ISA-51, using the same schedule. Each experiment was repeated twice with 10–14 mice per group.

My-1 and J-My-1 specific antibodies

Mice were bled on days 0, and on sacrifice day (day 21 or 28) from the retroorbital venous plexus using heparinized capillary tubes. Sera were collected and stored frozen until evaluated for antibodies. Serum levels of anti-My-1 or anti-J-My-1 specific IgG and its subclasses were determined using microtiter plates coated with appropriate peptide (0.5 µg of My-1 or J-My-1/well). Adjusted optical density (OD) was calculated as follows: adjusted OD = (mean OD of a sample – mean OD of a negative control).

Cytokine and chemokines analysis by ELISA

Half of the hearts and spleens were snap frozen on dry ice immediately after resection and stored at –80°C until homogenized. Both homogenized supernatants were stored at –80°C until used in enzyme-linked immunosorbent assays (ELISAs). Cytokines, chemokines and histamine levels were measured in homogenized heart and spleen supernatants using Quantikine cytokine ELISA kits (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The limit of detection for the kits were as follows: IFN-γ, 2 pg/mL; TGF-β1, 1.6 pg/mL; IL-1β, 3 pg/mL; ; TNF-α, 5.1 pg/mL; IL-4, 2 pg/mL; IL-13, 1.5 pg/mL; IL-1α, 2.5 pg/mL; IL-2, 3 pg/mL; IL-10, 4 pg/mL; histamine, 1.5 ng/mL, MIP-1α 1.5 pg/mL and IP-10, 2.2pg/mL. Intracardiac cytokine and chemokine levels were expressed as pg/g; histamine is expressed as ng/g. All values were normalized to wet heart weight.

My-1- and J-My-1-specific lymphoproliferation of splenocytes from immunized mice *in vitro*

Splenocytes were collected on day 21, red blood cells were lysed, and live cells were counted by trypan blue exclusion (13, 1.5). The proportions of dead cells did not vary between groups. Splenocytes were then cultured for 48 hours at an initial cell density of 5×10^6 cells/mL in complete RPMI 1640 medium (Life Technology) in the presence of media alone or with different concentrations of My-1 or J-My-1 in 96-well format in triplicate. Cells were pulsed 24 hours prior to harvest with 1 μ Ci/well [3 H]-methyl thymidine (Amersham), harvested onto glass filters and specific radiouptake was detected on a Matrix-96 direct beta counter (Packard).

FACS analysis

Mouse spleens were extracted into single cell suspension in 1x PBS. RBCs were lysed by <5 min incubation in ACK lysis buffer (Biofluids). Cells were washed and Fc γ RII/III blocked with α CD16/32 Fc block (eBiosciences). Surface markers were stained with fluorochrome-conjugated mAbs to CD4, CD8 α , CD25, CD44, and CD62L (eBiosciences, BD Pharmingen). Treg cells were further stained by intracellular staining of Foxp3 according to manufacturer's instructions (eBiosciences). Samples were acquired on a four-color dual-laser FACScalibur cytometer running CellQuest software (BD Immunocytometry).

Statistical Analyses

Statistical analysis was performed by one-way ANOVA analysis. Normally-distributed data were analyzed by two-tailed Students' *t* test. Values of $p < 0.05$ were considered statistically significant. The Mann Whitney U test was used for evaluation of histopathology scores.

Results

J-My-1 conjugate used as a vaccine injected to mice *before* EAM induction (*prophylactic treatment*) significantly reduced the severity of EAM

In this experiment, we tested whether preventative administration of J-My-1 peptide prior to the induction of EAM reduced disease severity at day 21. Groups of 10 mice were injected *s.c.* with J-My-1 peptide emulsified in ISA-51 (or with PBS in ISA-51) 14 and 7 days prior to myocarditis induction (days -14 and -7). The severity of the myocarditis was significantly reduced on day 21 in the group that was pretreated with J-My-1 (*t*-test for J-My-1 v PBS is $p = 0.014$) (Figure 1A, C). Pretreated mice that developed inflammation had less than 10% of heart section inflamed, but were not completely protected from myocarditis. The amelioration of disease was also associated with decreased heart weight/body weight ratios ($p = 0.047$ by *t*-test), compared to control mice (Figure 1B). Thus, J-My-1 is able to prevent myocarditis when injected prior to EAM induction.

Prophylactic treatment with disease inducing My-1 peptide in ISA-51 is not able to reproduce the results achieved with J-My-1 in ISA-51

In addition to pretreatment with J-My-1 or PBS+ ISA-51 control, we included a second control group to determine if the pretreatment with myocarditogenic My-1 in ISA-51 would be sufficient to ameliorate EAM in a manner similar to J-My-1. The mice pretreated with My-1 developed myocarditis with comparable severity to PBS-treated mice (*T*-test, My-1 v PBS-treated, $p = 0.2$) (Figure 1A, B), in marked contrast to decreased disease in J-My-1-pretreated mice (Figure 1A,B). Therefore, My-1 in ISA-51 administered in the same manner as J-My-1, prior to EAM induction, is not sufficient to prevent myocarditis.

J-My-1 conjugate used as a *late prophylactic* agent at the time of EAM induction (day 0, 7, 14 and 21) also significantly reduced the severity of myocarditis

In the next experiment, we tested the protection provided by J-My-1 pretreatment if J-My-1 injections were started on the day of EAM induction. Mice received J-My-1 on days 0, 7, 14, and 21 and were sacrificed on day 28. During treatment, 2 of 14 mice in the PBS-treated group and 1 of 14 mice treated with J-My-1 died. The deaths were not related to myocarditis and did not appear to be due to an allergic reaction to injections, because the deaths occurred several days after any immunization. Hearts of dead mice were examined by histology and showed no inflammation. The severity of myocarditis in surviving J-My-1-treated mice was significantly reduced, as assessed by histology ($p = 0.002$) (Figure 2A, left). Only one mouse from this group developed severe myocarditis. Mice treated with J-My-1 also had significantly lower heart weight/body weight ratios than control mice with EAM ($p = 0.006$) (Figure 2A, right). Thus, J-My-1 is able to prevent EAM when the treatment is started concurrent with the induction of myocarditis.

J-My-1 used as a *therapeutic* agent *after* EAM induction reduced myocarditis, but caused a fatal anaphylactic reaction in some mice

We proceeded to evaluate if further delaying J-My-1 treatment after the induction of EAM could also reduce cardiac inflammation. In two experiments, we initiated the J-My-1 treatment on day 7 or 10 post-immunization (*pi*) respectively. In the first experiment, mice were treated with J-My-1 on days 7, 14, and 21 and sacrificed on day 28. In the second experiment, mice were treated with J-My-1 on days 10, 17, 24 and sacrificed on day 28. In the first experiment, during the third injection of J-My-1 on day 21, three out of ten J-My-1 treated mice developed what appeared to be an anaphylactic reaction. Within 5 minutes after J-My-1 injection these animals showed increased respiratory rate with cyanosis, no response to pain and all other clinical signs of an anaphylactic reaction (14) and died rapidly. The dead mice had no signs of myocarditis when their hearts were examined by histology. The remaining 7 mice from the J-My-1 treated group were sacrificed on day 28 and had only minimal myocarditis compared to the control group (Figure 2B). There was no difference in HW/BW ratio (*data not shown*).

In the second treatment regiment when J-My-1 was injected on days 10, 17 and 24, six of ten mice developed anaphylactic reactions to the third J-My-1 injection on day 24. The dead mice had no signs of myocarditis by histology (*data not shown*). The surviving 4 mice from this group also did not develop any myocarditis (Figure 2C). There was no difference in HW/BW ratio (*data not shown*). Thus, therapeutic treatment with J-My-1, started as late as day 10 after immunization, is able to suppress myocarditis development. However, later treatment was associated with a significant risk of anaphylactic reaction and death during the third injection of J-My-1 in both experiments.

J-My-1 treatment change Th1/Th2 balance slightly towards Th2

The LEAPS treatment that prevents herpes simplex infection was associated with the activation of Th1 responses (6,26). To examine whether J-My-1 treatment changes Th1/Th2 balance in EAM, we examined the cytokine profile in the hearts and spleens homogenates of treated mice with EAM by ELISA for individual cytokines. When mice were treated with J-My-1 prior to EAM induction – early prophylactic treatment (day –14, –7), we detected increased IL-13 levels in the sera and increased levels of IL-1 α in sera (Figure 3C). There were no differences in the levels of IL-13, IL-4, IFN- γ or TGF- β 1 in the heart. IL-1 β was lower in the hearts of J-My-1 treated mice; however, these differences did not reach statistical significance (Figure 3A).

Mice treated with J-My-1 at the time of EAM induction – late prophylactic treatment (days 0, 7, 14, 21) had significantly decreased intracardiac IFN- γ levels ($p = 0.03$), and highly decreased

levels of IL-1 β ($p = 0.003$) in the spleen (Figure 4A, B). There were no differences in IL-13, IL-4, IL-1 α , IL-1 β , TGF- β , IL-2, IL-10 or TNF- α in the heart (Figure 4A). Nor were there changes in levels of IL-13, IL-1 α , IL-4, IFN- γ or TGF- β in the spleen (Figure 4B). Thus, J-My-1 treatment appears to deviate Th1/Th2 responses slightly towards decreasing Th1 response and increasing Th2 response in the heart. Interestingly, J-My-1 was associated with reduced levels of proinflammatory IL-1 β in all treatment regimens in both the hearts and spleens of mice treated with J-My-1 although those differences did not always reach significance.

J-My-1 treatment did not decrease pathologic IgG1 anti-My-1 response but initiated increased production of anti-J-My-1 antibodies

We have previously reported that the levels of IgG1 anti-myosin peptide antibodies correlate well with EAM severity (15). Therefore, we tested whether J-My-1 treatment resulted in changes in antigen-specific antibodies. We examined serum samples collected on the day of sacrifice from J-My-1 treated mice for antibodies reactive to myocarditogenic peptide (My-1), but we did not observe significant differences in total anti-My-1 IgG or IgG1 after J-My-1 treatment compare to PBS treated groups in any of the treatment regimens. There were changes in IgG2a and IgG2b subclasses in some experiments (decreased levels of IgG2a in mice treated prophylactically on days -14 and -7; decreased IgG2b and a tendency towards decreased IgG2a in mice treated on days 0, 7, 14, and 21). (Figure 5A). IgG2a antibody levels are often taken as an indicator of Th1 responses. However, IgG2a or IgG2b anti-myosin antibodies generally do not correlate with EAM severity in A/J mice (15).

Additionally, we examined the immunogenicity of the treatment peptide in both J-My-1 and ISA-51 treated groups by examining their sera for antibodies reactive to J-My-1. All groups of mice with EAM pretreated or treated with J-My-1 in different regimens developed elevated levels of anti-J-My-1 antibodies compared to PBS treated controls. These differences were significantly higher in total anti-J-My-1 antibodies and in all IgG subclasses. Representative data for mice treated with J-My-1 at the time of EAM induction (days 0, 7, 14, 21) are shown in Figure 5B ($p < 0.05$). Thus, J-My-1 treatment did not decrease pathologic IgG1 anti-My-1 antibody response. It was, however associated with significantly increased production of anti-J-My-1 antibodies.

Mice treated with J-My-1 had increased histamine in spleen

It has been previously shown that IgE and histamine as well as IgG1 and platelet-activating factor (PAF) can induce allergic reactions in mice (16). Therefore, we assayed for the levels of histamine in spleens of J-My-1- and PBS-treated mice. Histamine levels in spleens of mice treated with J-My-1 were consistently higher than histamine in spleens of PBS-treated animals, irrespective of J-My-1 treatment regimen. Representative figure for animals treated on days 0, 7, 14 and 21 is shown ($p = 0.009$) (Figure 5D).

Spleen cells from J-My-1 treated mice with EAM have slightly decreased proliferative responses to My-1 peptide *in vitro*

Spleen cells from J-My-1-treated mice were collected at day 21 *pi* and stimulated *in vitro* with either the myocarditogenic My-1 peptide or J-My-1. Antigen-specific proliferative responses were measured by incorporation of [3 H]methyl thymidine. We did not observe appreciable differences in proliferative responses to stimulation with J-My-1 (Figure 6A). Repeated injections of J-My-1 (days 0, 7, 14, 21) slightly decreased the proliferation of splenocytes in response to My-1 *in vitro* although the difference did not reach statistical significance ($p = 0.066$) (Figure 6A). Thus, J-My-1 treatment did not significantly reduce proliferative responses to myocarditogenic peptide My-1.

J-My-1 treatment increased proportions of CD3⁺, CD4⁺ or CD8⁺ T cells and CD11c⁺ dendritic cells in spleen on day 28

We analyzed proportions of CD3⁺, CD4⁺, CD8⁺, CD11c⁺, CD45R⁺ (B220), F4/80⁺, DX5⁺, and Foxp3⁺ cells in the spleen to determine whether J-My-1 treatment changes proportions of T cells, dendritic cells, B cells, macrophages, NK cells, or regulatory T cells, respectively. Primary spleen cells collected from mice with EAM that were treated with J-My-1 on days 0, 7, 14 and 21 and sacrificed on day 28 were analyzed by flow cytometric staining. The cells received no stimulation *in vitro*. There were no differences in the proportions of DX5⁺ NK cells or F4/80⁺ macrophages. However, we observed increased proportions of CD3⁺, CD4⁺, and CD8⁺ T cells and CD11c⁺ dendritic cells (Table 1, Figure 6B, D). Additionally, we analyzed activation of CD4 and CD8 cells by determining expression of CD44, CD62L, CD69, and CD71. The proportions of CD4⁺ and CD8⁺ cells expressing the activation markers CD69 and CD44 were increased in the spleens of J-My-1-treated animals (Figure 6C and *data not shown*). We have not seen any differences in levels of CD62L and CD71 activation markers on CD4⁺ or CD8⁺ T cells. Furthermore, we did not observe any changes in the proportions of CD4⁺CD25⁺ Foxp3⁺ regulatory T cells (Figure 6E); thus, J-My-1 treatment did not expand or induce regulatory T cells. However, J-My-1 treatment increased the proportions of total CD3⁺ cells, total CD4⁺ and CD8⁺ T cells, activated CD4⁺ and CD8⁺ T cells, and DCs.

Chemokines MIP-1α/CCL3 and IP-10/CXCL10 are decreased in hearts of J-My-1 treated animals

J-My-1 treatment upregulated immune responses in the spleens of treated animals but did not result in increased of myocarditis severity. In contrast, J-My-1-treated mice were protected against myocarditis. One possible explanation is that autoreactive cells were not trafficking to the heart. Therefore, we tested levels of chemokines in heart and spleen on day 28 of EAM after pretreatment or treatment with J-My-1 (days -14, -7 or days 0, 7, 14, 21). We found that levels of IFN-γ-inducible protein of 10 kDa (IP-10/CXCL10) and macrophage inflammatory protein-1α (MIP-1α/CCL3) were significantly reduced in hearts of J-My-1-treated animals (Figure 7A). In contrast, there were no changes in the levels of these chemokines in spleen (Figure 7B). Thus, it is possible that J-My-1 treatment protects against myocarditis by altering trafficking of autoreactive cells to the heart.

Discussion

We report here that the J-My-1 peptide reduced the incidence and severity of EAM when given *before* (on day -14 and -7), *at the time of* (on day 0, 7, 14, 21) and even *after* (on days 7, 14, 21 or on days 10, 17, 21) the induction of EAM. In all experiments, J-My-1 treatment significantly lowered the severity of myocarditis, as assessed by histology. The protection against myocarditis delivered by J-My-1 was not absolute, since some mice developed low-grade heart inflammation. Pretreatment with unconjugated My-1 peptide in ISA-51 did not induce similar protection against myocarditis.

We have observed occasional mortality in both J-My-1-treated and PBS-treated mice that did not follow immediately upon an injection when the treatment was started *before* or *at the time* of EAM induction. However, in the two experiments that started treatment *after* disease induction on day 7 and 10, respectively, some mice developed anaphylactic reactions shortly after J-My-1 treatment, resulting in death. Hypersensitivity has also been described following treatment for other autoimmune diseases with altered peptide ligands (APL) in both mice and humans. For example, SJL/J mice with myelin proteolipid protein (MPP)-induced EAE developed anaphylaxis after they were challenged with MPP three or more weeks after the EAE induction (17). Additionally, 13% of participants in a clinical trial receiving an APL derived from myelin basic protein (MBP) as a treatment for MS experienced some level of

allergic reaction (18). The hypersensitivity was mainly observed in patients who received the highest dose of APL. In another study, insulin β -chain peptide administered in PBS *s.c.* to NOD mice caused anaphylaxis after repeated injections (16). The anaphylaxis was only observed at higher doses of the β -chain peptide. The *i.v.* injection of the myelin-derived peptides, PLP_{139–151}, MBP_{84–104}, MOG_{35–55}, and MBP_{Ac1–11}, administered to mice with established EAE (after day 15), led to anaphylaxis in 80–100% mice (19).

Both IgE and IgG1 can be responsible for anaphylaxis in mice and histamine is the main mediator of IgE-induced allergy (16). We found high levels of anti-J-My-1 IgG1 antibodies as well as increased levels of histamine in the spleens and sera (*data not shown*) of J-My-1 treated mice in all treatment regimens, which may explain the high potential of J-My-1-induced anaphylactic reactions. We have never observed any anaphylactic reaction earlier than the third treatment with J-My-1 and only when treatment was initiated after disease induction. However the increased histamine levels in other treatment regimens indicated that there is a potential for anaphylactic reaction in all treatment regimens. It is unclear whether the anaphylactic reaction is specific to our situation when EAM is induced with the same peptide that is used as part of J-My-1, since no allergic reactions were observed before when using L.E.A.P.S. technology (26).

One potential mechanism by which J-My-1 treatment could ameliorate the severity of myocarditis may be through the induction of clonal anergy. Anergic T cells are unresponsive to antigen, failing to proliferate or produce IL-2 (20). We observed tendency to decreased T cell proliferation in response to My-1 after J-My-1 treatment; however the reduction was not statistically significant. We did not observe decreased levels of IL-2 in sera or hearts of J-My-1-treated animals, which contradicts the possibility that J-My-1 treatment induced anergy. Nor did we see any changes in IgG1 anti-My-1 specific antibodies after J-My-1 treatment. Cardiac myosin-specific IgG1, but not any other IgG subclass, correlates with the severity of myocarditis in EAM (15). Thus, we conclude that J-My-1 does not protect against EAM by inducing T cell anergy.

A second possible mechanism by which J-My-1 may protect against EAM could be the induction of suppressive regulatory responses. There is some evidence for this mechanism in tolerogenic studies using the same antigen for treatment as is used for disease induction. An ovalbumin peptide administered orally and also injected intravenously induced CD4⁺CD25⁺ regulatory T cells (21). However, we did not observe any changes in the proportions of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in spleen after J-My-1 treatment, nor did we observe increased levels of the immunoregulatory cytokines IL-10 or TGF- β in the hearts, spleens, or sera of J-My-1 treated mice. Thus, the expansion of other cell types in the spleen and lack of changes in TGF- β or IL-10 levels appear to contradict the possibility that the J-My-1 protects against EAM through increasing regulatory T cell responses.

A third possibility by which J-My-1 treatment may protect against autoimmune disease is changing the Th1/Th2 balance (22,23). Similarly designed L.E.A.P.S. peptides protected mice from herpes simplex virus infection by eliciting Th1 response (HSV) (6,26). J-My-1 treatment resulted in an increased level of IL-13 in sera when J-My-1 was used prophylactically. J-My-1 treatment resulted in decreased levels of IFN- γ in the heart when J-My-1 was given together with induction of EAM. Furthermore, myosin-reactive Th1-associated subclass IgG2a was decreased after J-My-1 treatment. IL-13 is an important protective cytokine in EAM (*Cihakova et al, manuscript submitted*). However, we have only observed increased levels of IL-13 in serum during prophylactic treatment with J-My-1. Although we cannot exclude the possibility that an increase of IL-13 or deviation to a Th2 cytokine profile might contribute to the therapeutic role of J-My-1 treatment in EAM, the shift towards Th2 response after J-My-1

treatment was very subtle, and not consistent and did not accord with the marked reduction in myocarditis inflammation.

The increased proportion of total CD3⁺ cells, CD8⁺ and CD4⁺ T cells, that were also significantly more activated as indicated by increase proportion of CD4⁺ and CD8⁺ cells expressing the activation markers CD69 and CD44, plus increased CD11c⁺ dendritic cells (DCs), high production of anti-J-My-1 antibodies, and susceptibility to anaphylactic reactions in J-My-1-treated animals, indicates that J-My-1 upregulated immune responses, compared to already increased cellular and humoral responses during EAM. Therefore, J-My-1 induced stronger cell- and antibody-mediated responses than My-1 alone, yet it protected against autoimmune disease. One possible explanation is that J-My-1 decreased expression of MIP-1 α /CCL3 and IP-10/CXCL10 in the heart and thus interfered with trafficking of autoreactive cells to the heart. In other studies, we have shown that chemokines MCP-1 and MIP-1 α are important in the induction of EAM and that inhibition of MCP-1 significantly reduced disease severity (24). Similar to our current study, Fischer, *et al.* (25) showed that protection against experimental autoimmune encephalomyelitis (EAE) by an altered peptide ligand was accompanied by reduced expression of selected chemokine ligands and receptors (CCR1, CCR5, CCR8, CXCR2 and CXCR3). The mechanism by which J-My-1 alters chemokine expression in the heart is presently unknown.

In conclusion, the J-My-1 L.E.A.P.S. construct generates a strong immune response, without reducing myosin-specific immunity in the periphery, but still is able to protect against experimental autoimmune myocarditis. It has been generally assumed that for a treatment against an autoimmune disease to be effective, it must induce anergy, change the Th1/Th2 balance or induce regulatory responses. We have presented evidence indicating that J-My-1 probably does not protect against myocarditis by any of these mechanisms but rather that treatment with J-My-1 is accompanied by a reduction in expression of MIP-1 α and IP-10 in the heart and thus possibly alters trafficking of autoaggressive cells to the heart.

Acknowledgments

The work was supported by NIH grants **R01 HL67290** and **RO1 HL077611** (JHU) and **1R43HL71352-01A1** (CEL-SCI)

L.E.A.P.S. is a patented technology of CEL-SCI Corporation, Vienna VA 22182.

References

1. Rose NR, Hill SL. Autoimmune myocarditis. *Int J Cardiol* 1996;54(2):171–5. [PubMed: 8803682]
2. Rose NR, Afanasyeva M. From infection to autoimmunity: the adjuvant effect. *ASM News* 2003;69(3):132–137.
3. Neu N, Beisel KW, Traystman MD, Rose NR, Craig SW. Autoantibodies specific for the cardiac myosin isoform are found in mice susceptible to Coxsackievirus B3-induced myocarditis. *J Immunol* 1987;138(8):2488–92. [PubMed: 3031159]
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(11):3630–6. [PubMed: 3680946]
5. Donermeyer DL, Beisel KW, Allen PM, Smith SC. Myocarditis-inducing epitope of myosin binds constitutively and stably to I-Ak on antigen-presenting cells in the heart. *J Exp Med* 1995;182(5):1291–300. [PubMed: 7595200]
6. Rosenthal KS, Mao H, Horne WI, Wright C, Zimmerman D. Immunization with a LEAPS heteroconjugate containing a CTL epitope and a peptide from beta-2-microglobulin elicits a protective and DTH response to herpes simplex virus type 1. *Vaccine* 1999;17(6):535–42. [PubMed: 10075159]
7. Uger RA, Chan SM, Barber BH. Covalent linkage to beta2-microglobulin enhances the MHC stability and antigenicity of suboptimal CTL epitopes. *J Immunol* 1999;162(10):6024–8. [PubMed: 10229842]

8. White J, Crawford F, Fremont D, Marrack P, Kappler J. Soluble class I MHC with beta2-microglobulin covalently linked peptides: specific binding to a T cell hybridoma. *J Immunol* 1999;162(5):2671–6. [PubMed: 10072510]
9. Zimmerman DH, Bergmann KF, Rosenthal KS, Elliott DA. A new approach to T cell activation: Natural and synthetic conjugates capable of activating T cells. *Vaccine Research* 1996;5:91–102.
10. Zimmerman DH, Rosenthal KS. The L.E.A.P.S. approach to vaccine development. *Front Biosci* 2005;10:790–8. [PubMed: 15569618]
11. Gao GF, Tormo J, Gerth UC, Wyer JR, McMichael AJ, Stuart DI, et al. Crystal structure of the complex between human CD8alpha(alpha) and HLA-A2. *Nature* 1997;387(6633):630–4. [PubMed: 9177355]
12. Nishimura Y, Chen YZ, Kanai T, Yokomizo H, Matsuoka T, Matsushita S. Modification of human T-cell responses by altered peptide ligands: a new approach to antigen-specific modification. *Intern Med* 1998;37(10):804–17. [PubMed: 9840700]
13. Afanasyeva M, Georgakopoulos D, Fairweather D, Caturegli P, Kass DA, Rose NR. Novel model of constrictive pericarditis associated with autoimmune heart disease in interferon-gamma-knockout mice. *Circulation* 2004;110(18):2910–7. [PubMed: 15505106]
14. Navuluri L, Parvataneni S, Hassan H, Birmingham NP, Kelly C, Gangur V. Allergic and anaphylactic response to sesame seeds in mice: identification of Ses i 3 and basic subunit of 11s globulins as allergens. *Int Arch Allergy Immunol* 2006;140(3):270–6. [PubMed: 16699288]
15. Afanasyeva M, Wang Y, Kaya Z, Park S, Zilliox MJ, Schofield BH, et al. Experimental autoimmune myocarditis in A/J mice is an interleukin-4-dependent disease with a Th2 phenotype. *Am J Pathol* 2001;159(1):193–203. [PubMed: 11438466]
16. Liu E, Moriyama H, Abiru N, Miao D, Yu L, Taylor RM, et al. Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9-23 and B:13-23. *J Clin Invest* 2002;110(7):1021–7. [PubMed: 12370280]
17. Pedotti R, Mitchell D, Wedemeyer J, Karpuz M, Chabas D, Hattab EM, et al. An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat Immunol* 2001;2(3):216–22. [PubMed: 11224520]
18. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med* 2000;6 (10):1176–82. [PubMed: 11017151]
19. Smith CE, Eagar TN, Strominger JL, Miller SD. Differential induction of IgE-mediated anaphylaxis after soluble vs. cell-bound tolerogenic peptide therapy of autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 2005;102(27):9595–600. [PubMed: 15983366]
20. Peakman M, Dayan CM. Antigen-specific immunotherapy for autoimmune disease: fighting fire with fire? *Immunology* 2001;104(4):361–6. [PubMed: 11899420]
21. Thorstenson KM, Khoruts A. Generation of anergic and potentially immunoregulatory CD25+CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J Immunol* 2001;167(1):188–95. [PubMed: 11418648]
22. Larche M. Inhibition of human T-cell responses by allergen peptides. *Immunology* 2001;104(4):377–82. [PubMed: 11899422]
23. Tian J, Clare-Salzler M, Herschenfeld A, Middleton B, Newman D, Mueller R, et al. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 1996;2(12):1348–53. [PubMed: 8946834]
24. Goser S, Ottl R, Brodner A, Dengler TJ, Torzewski J, Egashira K, et al. Critical role for monocyte chemoattractant protein-1 and macrophage inflammatory protein-1alpha in induction of experimental autoimmune myocarditis and effective anti-monocyte chemoattractant protein-1 gene therapy. *Circulation* 2005;112(22):3400–7. [PubMed: 16316965]
25. Fischer FR, Santambrogio L, Luo Y, Berman MA, Hancock WW, Dorf ME. Modulation of experimental autoimmune encephalomyelitis: effect of altered peptide ligand on chemokine and chemokine receptor expression. *J Neuroimmunol* 2000;110(1–2):195–208. [PubMed: 11024550]

26. Goel N, Rong Q, Zimmerman D, Rosenthal KS. A L.E.A.P.S. heteroconjugate vaccine containing a T cell epitope from HSV-1 glycoprotein D elicits Th1 responses and protection. *Vaccine* 2003;21(27–30):4410–20. [PubMed: 14505924]

Figure 1.

Myocarditis at day 21 *pi* in mice treated prophylactically on days -14 and -7 with J-My-1 (filled diamonds), control PBS (open diamonds), or control My-1 (open circles). A) EAM severity by histopathologic severity scoring. B) HW/BW ratios; bars indicate means of each group, plus/minus standard deviation. Statistics are by one-way ANOVA. Representative histopathology in a LEAPS-treated animal (C) and a PBS-treated control (D) at 5 $\times$ (left) and 40 $\times$ (right).

Figure 2.

Myocarditis at day 28 *pi* in mice treated in *late prophylactic* regimen on days 0, 7, 14 and 21 (A), *therapeutically* on days 7, 14 and 21 (B), or *therapeutically* on days 10, 17 and 24 (C) with J-My-1 (filled diamonds) or control PBS (open diamonds). Bars indicate means of each group, plus/minus standard deviation. Statistics are by Mann-Whitney *U*-test for histopathology scores, and by two-tailed Students' *t*-test for HW/BW ratios.

Figure 3.

Tissue cytokines at day 21 *pi* in mice treated prophylactically on days -14 and -7 with J-My-1 (filled bars) or control PBS (open bars) from heart homogenates (A), spleen homogenates (B) or sera (C). Data represent means of each group, plus standard deviation. Cytokines are shown as pg/g with exception of IL-1 α , IL-1 β and TGF β that are shown as ng/g of tissue. Statistics are by two-tailed Students' *t*-test.

Figure 4.

Tissue cytokines at day 28 *pi* in mice treated therapeutically on days 0, 7, 14 and 21 with J-My-1 (filled bars) or control PBS (open bars) from heart homogenates (A) or spleen homogenates (B). Data represent means of each group, plus standard deviation. Cytokines are shown as pg/g or as ng/g of tissue (IL-1 α , IL-1 β , IL-2, TGF β and TNF α). Statistics are by two-tailed Student's *t*-test.

Figure 5.

Antibodies reactive to My-1 (A), J-My-1 (B) at day 28 *pi* in mice treated therapeutically on days 0, 7, 14 and 21 with J-My-1 (filled bars) or control PBS (open bars) by total IgG, or subclass. C) Histamine levels from spleen homogenates. Data represent means of each group, plus standard deviation. Statistics are by two-tailed Students' *t*-test.

Figure 6.

T cell responses at day 21 *pi*. A) Splenocyte proliferative responses from mice treated with J-My-1 (left) or control PBS (right) in response to *in vitro* stimulation with My-1 (filled bars) or J-My-1 (open bars). Data represent means of each group, plus standard deviation. Statistics are by two-tailed Student's *t*-test. Analysis of spleen T cells by flow cytometry. Expansion of CD4⁺ and CD8 α ⁺ T cells as a proportion of lymphocytes (B), and increased activation of CD4⁺-gated cells (C), as indicated by expression of CD69 and CD44. D) Expansion of total CD11c^{hi} dendritic cells in spleen. E) Canonical CD4⁺CD25⁺FoxP3⁺ regulatory T cells in spleen.

Figure 7.

Chemokine expression in (A) hearts and (B) spleens of J-My-1-treated (filled) and PBS control (open) mice at day 28 of EAM, as determined by quantitative sandwich ELISA. Chemokine levels are normalized to wet tissue weight. Statistics are by Mann-Whitney *U*-test.

Table I**Spleen leukocyte populations after J-My-1 treatment**

Primary spleen cells collected from mice with EAM that were treated with J-My-1 on days 0, 7, 14 and 21 and sacrificed on day 28 were analyzed by flow cytometric staining. Data indicate mean of the group, plus/minus standard deviation. Statistics are by two-tailed Students' *t*-test.

	Gating	J-My-1+ISA-51 on days 0, 7, 14, 21	PBS+ISA-51 on days 0, 7, 14, 21
CD3 ϵ ⁺ T cells	% lymphoid	27.1 $\pm$ 1.0*	23.7 $\pm$ 2.1
CD8 α ⁺ T cells	% lymphoid	10.9 $\pm$ 0.78*	9.24 $\pm$ 1.50
CD4 ⁺ T cells	% lymphoid	20.1 $\pm$ 1.86*	17.7 $\pm$ 1.53
CD11c ⁺ dendritic cells	% total	3.75 $\pm$ 0.41*	3.37 $\pm$ 0.20
F4/80 ⁺ CD11c ⁻ macrophages	% total	12.3 $\pm$ 0.35	12.6 $\pm$ 1.70
CD19 ⁺ B cells	% lymphoid	57.8 $\pm$ 3.4	60.6 $\pm$ 4.0
Gr1 ⁺ granulocytes	% total	11.7 $\pm$ 2.3	10.1 $\pm$ 2.4
DX5 ⁺ CD3 ϵ ⁻ NK cells	% total	1.68 $\pm$ 0.22	1.79 $\pm$ 0.21
DX5 ⁺ CD3 ϵ ⁺ NKT cells	% total	0.50 $\pm$ 0.08	0.46 $\pm$ 0.07
CD25 ⁺ FoxP3 ⁺ Treg cells	% CD4 ⁺	0.80 $\pm$ 0.29	1.10 $\pm$ 0.32
CD69 ⁺	% CD4 ⁺	14.6 $\pm$ 1.5*	12.2 $\pm$ 0.8
CD44 ⁺	% CD4 ⁺	33.8 $\pm$ 2.9*	30.2 $\pm$ 2.4
CD62L ^{lo}	% CD4 ⁺	34.8 $\pm$ 2.7	33.7 $\pm$ 2.6
CD71 ⁺	% CD4 ⁺	17.5 $\pm$ 1.8	16.9 $\pm$ 1.9
CD69 ⁺	% CD8 α ⁺	5.85 $\pm$ 0.6	6.9 $\pm$ 2.8
CD44 ⁺	% CD8 α ⁺	43.0 $\pm$ 3.0	43.4 $\pm$ 3.0
CD62L ^{lo}	% CD8 α ⁺	7.9 $\pm$ 1.9	8.9 $\pm$ 1.6
CD71 ⁺	% CD8 α ⁺	10.3 $\pm$ 2.1	11.0 $\pm$ 0.8

p < 0.05 by two-tailed Students' *t*-test