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# Histamine $H_1$ receptor signaling regulates effector T cell responses and susceptibility to coxsackievirus B3-induced myocarditis

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## Abstract

Susceptibility to autoimmune myocarditis has been associated with histamine release by mast cells during the innate immune response to Coxsackievirus B3 (CVB3) infection. To investigate the contribution of histamine $H_1$ receptor ($H_1R$) signaling to CVB3-induced myocarditis, we assessed susceptibility to the disease in C57BL/6J (B6) $H_1R^{-/-}$ mice. No difference was observed in mortality between CVB3-infected B6 and $H_1R^{-/-}$ mice. However, analysis of their hearts revealed a significant increase in myocarditis in $H_1R^{-/-}$ mice that is not attributed to increased virus replication. Enhanced myocarditis susceptibility correlated with a significant expansion in pathogenic Th1 and $V\gamma4^{+}\gamma\delta$ T cells in the periphery of these animals. Furthermore, an increase in regulatory T cells was observed, yet these cells were incapable of controlling myocarditis in $H_1R^{-/-}$ mice. These data establish a critical role for histamine and $H_1R$ signaling in regulating T cell responses and susceptibility to CVB3-induced myocarditis in B6 mice.

### Keywords

CVB3; autoimmune; myocarditis; histamine receptor; Th1; γδ T cells; Tregs; mice

## 1. Introduction

Myocarditis typically results from a microbial infection of the myocardium with either viruses, bacteria, or protozoa [1], and enteroviruses, including coxsackievirus B3 (CVB3), represent the major viral etiologic agents of this disease [2]. In both the clinical and experimental settings, myocarditis predominates in males and can result in acute, resolving, or chronic forms of the disease. Myocarditis frequently consists of a mononuclear cell infiltration of the myocardium and multiple pathogenic mechanisms contribute to cardiac injury, including direct virus infection, virus-specific immunity, and virus-induced autoimmunity [3].



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CD8 T cells are the main contributors to autoimmune-mediated cardiac injury during CVB3 infection, which target cardiocytes through antigenic mimicry[4, 5]. The activation of the autoreactive CD8 T cells requires innate effector CD1d-restricted γδ T cells expressing the Vγ4 receptor[6]. Following CVB3 infection, CD1d is up-regulated on cardiac cells, which leads to the recruitment of Vγ4 cells into the heart[7]. Vγ4 cells produce high levels of proinflammatory cytokines, including IFN-γ, that effectively bias the virus-specific adaptive immune response to the pathogenic Th1 phenotype, which in turn leads to the activation of auto reactive CD8 effector T cells[6, 8, 9]. The CD8 T cells effectively kill uninfected cardiocytes resulting in cardiac injury. In addition to CD8 T cells, Vγ4 can selectively target and kill uninfected CD1d expressing cardiocytes and further contribute to cardiac injury[10]. Vγ4 cells also promote autoimmunity and inflammation by inhibiting regulatory T (Treg) cell activity. CD1d expressing Tregs are highly immunosuppressive compared to CD1d negative Tregs in the CVB3 myocarditis model. However, Vγ4 cells actively kill the $CD1d^+$ Tregs in a CD1d and caspase dependent manner, thereby further facilitating autoimmune disease[11, 12].

In addition to γδ T cells, mast cells represent another innate effector cell type that has been implicated in the induction of myocarditis. Mast cells act as regulators of the innate and adaptive immune systems through the secretion of immune mediators, including histamine and an array of cytokines [13]. Studies have shown that elevated mast cell numbers, which undergo degranulation within 6 hours of CVB3 infection, correlate with enhanced myocarditis susceptibility [14, 15]. Furthermore, when using encephalomyocarditis virus (EMCV) as an inducer of myocarditis, mast cell deficient mice developed significantly less myocarditis than control littermates[16]. Treating mice after EMCV infection with histamine $H_1$ receptor ($H_1R$) antagonists substantially reduced myocarditis by inhibiting inflammatory cytokine expression[16, 17]. Therefore, histamine and $H_1R$ signaling may play a pivotal role in regulating the adaptive immune responses associated with viral myocarditis.

To investigate whether $H_1R$ signaling influences T cell responses and susceptibility to CVB3-induced myocarditis, we compared various disease parameters between infected wild-type C57BL/6J (B6) and $H_1R^{-/-}$ mice. We show that the absence of $H_1R$ signaling increases susceptibility to CVB3-induced myocarditis and results in an increase in pathogenic $CD4^+IFN\text{-}\gamma^+$ and $V\gamma4^+$ peripheral T cells. Furthermore, infected $H_1R^{-/-}$ mice also exhibit an increase in Tregs, which are clearly incapable of controlling disease. Therefore, as opposed to EMCV-induced myocarditis, $H_1R$ signaling helps protect CVB3-infected B6 mice from developing myocarditis by regulating T cell responses and autoimmunity associated with disease.

## 2. Materials and Methods

### 2.1. Mice

C57BL/6J(B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129P-*Hrh1*$^{tm1Wat}$($H_1R^{-/-}$) mice were generated in the vivarium of the Given Medical Building at the University of Vermont [18, 19]. $H_1R^{-/-}$ mice were backcrossed onto the B6 background for more than 10 generations. Only male mice were used in the experiments. Male B6 mice 12 weeks of age or older were used in the histamine EIA experiments. Mice were maintained in the animal facility at the University of Vermont. The experimental procedures used in this study were approved by the Animal Care and Use Committee of the University of Vermont.

### 2.2. Virus and infection

The H3 variant of CVB3 was made from an infectious cDNA clone as previously described [20]. Mice were infected by intraperitoneal (i.p.) injection of 0.5 ml of PBS containing 100 PFU CVB3 and euthanized 7 days after infection. Controls were uninfected mice or PBS-injected mice that were euthanized at the same time as the infected animals.

### 2.3. Histamine EIA assay

Male B6 mice were injected i.p. with either PBS alone or 2, 20, 200 PFU CVB3 and euthanized 3 hours after injection. Histamine concentrations were assessed using an EIA histamine kit according to the manufacturer's instructions (Beckman Coulter, Brea, CA). Briefly, spleens were homogenized in 10ul of 0.2N $HClO_4$ per mg of tissue and neutralized using an equal volume of 1M $Na_2B_4O_7$ (Sigma, St. Loius, MO). Supernatants were acylated and added to the histamine-coated plate plus conjugate and incubated overnight at 4°C. The wells were washed and the substrate was added for 30 minutes at RT while shaking. The reaction was stopped and the plate read at 405nm.

### 2.4. Evaluation of myocarditis

Myocarditis was quantified according to the method of Knowlton et al. [20]. Briefly, the heart is fixed in 10% formalin, paraffin embedded, ventricle sections taken at ~ 400 micron intervals and stained with hematoxylin and eosin. Sections are captured in transmitted light mode using a Nikon Eclipse 50i microscope fitted with a Nikon Digital Camera DXM1200F using a 4× objective lens and the images analyzed using the Meta Morph Series 6.1 program (Molecular Devices Co., Downingtown, PA). The area of the whole heart (square micrometers) is determined on a binary image with the areas of injury determined on the color image using the true color feature of the software. Final percent cardiac injury is calculated by dividing the area of injury by the total area of the heart.

### 2.5. Cardiac virus titers

Hearts are asceptically removed, weighed, homogenized and the cellular debris removed by centrifugation at 1500 × g for 15 min. The supernatants are diluted serially (10-fold) in RPMI 1640 with 5% FBS and incubated for 45 min on HeLa cell monolayers which are then overlaid with agar. After two days, HeLa cell monolayers are fixed with 10% formalin and stained with crystal violet. Plaques are counted and data expressed as plaques/g tissue.

### 2.6. Intracellular cytokine staining and flow cytometry

The details for the intracellular cytokine staining have been previously published [21]. Briefly, peripheral blood leukocytes (PBLs) were isolated from EDTA treated blood by centifugation on Histopaque (Sigma, St. Louis, MO). PBLs and red blood cell depleted splenocytes were cultured for 4 hrs in RPMI 1640 medium containing 10% FBS, antibiotics, 10 ng/ml phorbolmyristate acetate, 500 ng/ml ionomycin, and 10 μg/ml brefeldin A (Sigma, St. Louis, MO). The cells were washed, fixed with 2% paraformaldehyde, permeablized with 0.5% saponin in PBS containing 1% BSA and intracellularly stained with Alexa647-anti-IL-4 and PE-anti-IFN-γ and surface stained with PerCP-Cy5.5-anti-CD4. A second set of cells was stained using FITC-anti-Vγ4, PerCP-Cy5.5-anti-CD69, APC-Cy7-anti-CD8α, and Alexa647-anti-CD4 (BD Pharmingen, Franklin Lakes, NJ). FoxP3 labeling was performed as previously described using Alexa-647-anti-CD4, PerCP-Cy5.5-anti-CD25, FITC-anti-IL-10 and PE-anti-FoxP3[12]. The cell population was classified for cell size (forward scatter) and complexity (side scatter). At least 10,000 cells were evaluated. Positive staining was determined relative to isotype controls. The percentages of the different T cell subsets were obtained from the gated lymphocyte population using Flowjo Experiment-Based Analysis for flow cytometry (Tree Star Inc., Ashland, OR).

### 2.7. Statistics

Statistical analyses were performed using GraphPad Prism version 5.0c (GraphPad Software, San Diego, CA). The specific tests used are detailed in the figure legends. A *p*-value of ≤0.05 was considered significant.

## 3. Results and Discussion

### 3.1. CVB3 infection induces an innate histamine response in B6 mice

To date, the studies investigating the influence of histamine on CVB3-induced myocarditis have been performed using susceptible BALB/c mice. However, CVB3-infected B6 mice, although often thought of as myocarditis resistant, are also susceptible to myocarditis when inoculated with increased virus titers [22]. Therefore, to determine whether histamine and $H_1R$ signaling regulates CVB3-induced myocarditis susceptibility in B6 mice, we assessed whether infection induces a histamine response in these animals.

Our model of experimental myocarditis is induced in mice via i.p. injection of the virus making the resident immune cells within the spleen among the first to encounter the virus [23]. Therefore, to determine whether CVB3 can induce an innate histamine response, we measured histamine by EIA in the spleens of B6 mice 3 hours after i.p. injection of PBS alone or 2, 20, and 200 PFU CVB3. Importantly, this experiment revealed a significant decrease in splenic histamine that is dependent on the viral dose administered to the mice (Fig. 1). Although the mechanism behind the CVB3-induced decrease in histamine remains to be determined, it may be due to rapid splenic mast cell degranulation following infection resulting in the release of histamine into the periphery and/or the migration of these cells from the spleen in response to infection. These findings signify that CVB3 infection induces a rapid histamine response in B6 mice leading us to hypothesize that $H_1R$ signaling may contribute to disease susceptibility in this strain.

### 3.2. $H_1R$ deficiency increases susceptibility to CVB3-induced myocarditis

To assess the role of $H_1R$ signaling during CVB3-induced myocarditis, we utilized a traditional knockout approach using B6 mice. B6 and $H_1R^{-/-}$ mice were infected with 100 PFU CVB3 and monitored for survival up to 7 days after infection. The surviving animals were then analyzed for myocarditis and cardiac virus titers. Although there was no difference in survival between B6 and $H_1R^{-/-}$ mice (Fig. 2), histological analysis of the hearts from $H_1R^{-/-}$ mice revealed a significant increase in myocarditis ($p<0.01$) (Fig. 3). This difference in disease is not due to an increase in virus replication, since the hearts from $H_1R^{-/-}$ mice showed no difference in cardiac virus titers by plaque assay compared to controls (Fig. 3). Therefore, histamine signaling through the $H_1R$ assists in protecting B6 mice from CVB3-induced myocarditis.

Our results are in contrast to studies utilizing the EMCV-induced myocarditis model, where it has been shown that the treatment of DBA/2 mice with $H_1R$ antagonists significantly reduced myocarditis [16, 17]. Similarly, other differences in immune pathogenesis exist between the EMCV versus CVB3-induced myocarditis models. For example, CD1d dependent innate immunity is important in protecting mice from EMCV-induced disease but enhances myocarditis in the CVB3 model[10, 24]. These opposing results likely reflect viral specific differences underlying immunopathology and may reflect differences in the antiviral strategies used by the host during picornavirus infections. Therefore, our results accentuate the importance of determining the viral etiologic agent in patients with myocarditis before the regulation of histamine and/or $H_1R$ signaling could be used as therapeutic strategies.

### 3.3. $H_1R$ signaling regulates effector T cell responses during CVB3 infection

Susceptibility to CVB3-induced myocarditis is dependent on γδ and αβ T cell responses. γδ T cells are innate effector cells that regulate the developing adaptive immune response following viral infection. During CVB3 infection, Vγ4 expressing γδ T cells produce immunomodulatory cytokines, including IFN-γ, known to promote Th1 responses, which in turn induce the activation of autoimmune CD8 T cells[6, 8, 9]. To determine whether CVB3-induced $H_1R$ signaling regulated peripheral effector T cell populations, B6 and $H_1R^{-/-}$ mice were infected with 100 PFU CVB3 and both splenocytes and PBLs were analyzed 7 days later by flow cytometry. In CVB3-infected $H_1R^{-/-}$ mice, $V\gamma4^+\gamma\delta$ T cells were significantly increased in the peripheral blood compared to infected B6 mice (Fig. 4A,E). Furthermore, these mice exhibited an increase in the percentage of pathogenic $CD4^+$IFN-$\gamma^+$ Th1 cells in the spleen and peripheral blood ($p<0.01$) (Fig. 4B,E). However, we did not observe a difference in the protective Th2 response, as $H_1R^{-/-}$ mice possessed a similar percentage of $CD4^+$IL-$4^+$ PBLs as controls, and this population of cells was nearly undetectable in the spleen of both strains of mice (Fig. 4C). Lastly, no difference was detected in the percentage of activated $CD8^+$T cells between CVB3-infected B6 and $H_1R^{-/-}$ mice (Fig. 4D). Of note, the absence of $H_1R$ does not alter the T cell profiles of naïve mice indicating that these differences are a result of CVB3 infection (Data not shown).

These data show that the absence of $H_1R$ signaling leads to an increased bias towards the Th1 phenotype in CVB3-infected $H_1R^{-/-}$ mice. To our knowledge, this is the first report describing a role for $H_1R$ signaling during the differentiation of T helper cell responses following viral infection. Nonetheless, we have previously shown that $H_1R$ activation directly in CD4 T cells can lead to Th1 differentiation in experimental allergic encephalomyelitis (EAE), a Th1-mediated autoimmune model of multiple sclerosis[25]. However, as opposed to CVB3 infection, $H_1R^{-/-}$ mice exhibit a decrease in IFN-γ producing Th1 cells and an immune bias towards the protective Th2 response in EAE. Therefore, either $H_1R$ signaling does not modulate the T helper cell response equivalently across different models of autoimmune and infectious diseases or $H_1R$ signaling in CD4 T cells is not responsible for the Th1 bias in CVB3-infected $H_1R^{-/-}$ mice.

An alternative mechanism that may result in enhanced Th1 differentiation in CVB3-infected $H_1R^{-/-}$ mice may be due to the increase observed in peripheral Vγ4 cells. We have previously shown that IFN-γ produced by Vγ4 cells is necessary for the differentiation of Th1 cells following CVB3-infection[8, 9]. Therefore, an increase in Vγ4 cells could lead to an increase in IFN-γ, thereby leading to an exaggerated Th1 bias in $H_1R^{-/-}$ mice. Th1 cells contribute to myocarditis susceptibility by activating autoimmune CD8 T cells that cause cardiac injury. However, in CVB3-infected $H_1R^{-/-}$ mice, the increased severity of myocarditis may not be due to CD8 T cells since there was no difference detected in their peripheral cellularity compared to infected B6 mice. This suggests that the increase in myocarditis may be due to the increase in Vγ4 cells themselves, as these cells are also known to contribute to cardiac injury through killing infected cardiocytes using Fas-dependent mechanisms[10]. Therefore, Vγ4 cells may be the primary contributor to the severity of myocarditis in infected $H_1R^{-/-}$ mice by influencing Th1 polarity and cardiac injury. Little is known about the effect of histamine and $H_1R$ signaling on the γδ T cell population and further studies are needed to understand whether this may be a direct effect of $H_1R$ activation in γδ T cells or a downstream event.

### 3.4. $H_1R$ signaling regulates Treg responses during CVB3 infection

It has previously been shown that $V\gamma4^+\gamma\delta$ T cells can modulate Treg activity and further promote autoimmunity and inflammation following CVB3 infection and exposure to histamine has been reported to down regulate CD25 and Foxp3 expression on Treg cells

[26]. To determine whether the absence of $H_1R$ signaling influenced Treg populations in CVB3-infected mice, we analyzed IL-$10^+$ cells, as well as $CD4^+$IL-$10^+$, $CD4^+CD25^+$, and $CD4^+FoxP3^+$ splenocytes and PBLs by flow cytometry (Fig. 5). Interestingly, we found that CVB3-infected $H_1R^{-/-}$ mice possess an overall increase in IL-$10^+$ peripheral Tregs compared to infected B6 mice (Fig. 5A,C). Furthermore, we observed an increase in $CD4^+$IL-$10^+$, $CD4^+CD25^+$, and $CD4^+FoxP3^+$ in the spleen and an increase in$CD4^+$IL-$10^+$ in peripheral blood (Fig.5B,C).

Although an increase in Tregs was observed in the periphery of CVB3-infected $H_1R^{-/-}$ mice, these cells are clearly incapable of suppressing the effector T cell response. This may be due to the antagonizing role of Vγ4 cells on Treg activity in these animals, whereby the highly immunosuppressive $CD1d^+$ Tregs are actively killed[11, 12]. Another possibility is that the Tregs cannot effectively outcompete the high number of effector T cells also present in CVB3-infected $H_1R^{-/-}$ mice for antigen presentation. Lastly, these findings may result from the inability of the peripheral Tregs to home to the heart and prevent resident effector cells from causing cardiac injury. Despite the unknown mechanism, we can conclude that the suppressive activity of the peripheral Tregs is insufficient in preventing myocarditis in CVB3-infected $H_1R^{-/-}$ mice.

## 4. Conclusion

$H_1R$ signaling plays a significant role in regulating T cell responses and susceptibility to myocarditis during CVB3 infection. While it was known that $H_1R$ signaling could influence the polarity of T helper cells, our mouse model revealed a previously uncharacterized link between $H_1R$ signaling and Vγ4 T cell responses to infection. Understanding the effector functions that are directly regulated by $H_1R$ signaling in CVB3-induced myocarditis may aid in delineating the role that the $H_1R$ plays in modulating T cell responses to infection. Lastly, the differing results obtained from our work with CVB3 and those obtained using the EMCV-induced myocarditis model emphasizes the importance of determining the viral etiologic agent of myocarditis when considering pharmacological targeting the $H_1R$ as a therapeutic strategy in the treatment of myocarditis. Therefore, these findings suggest that the routine use of anti-histamines by atopic patients has the potential, depending upon the viral etiologic agent, to significantly influence the clinical course of myocarditis.

### Highlights

- $H_1R$ deficient mice are more susceptible to CVB3-induced myocarditis
- Susceptibility correlated with expansion in pathogenic Th1 and $V\gamma4^+\gamma\delta$ T cells
- Tregs are incapable of controlling myocarditis in CVB3-infected $H_1R$ deficient mice

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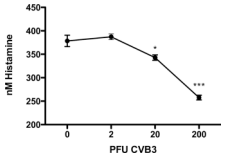


**Figure 1.**

CVB3 infection induces an innate histamine response in B6 mice. Mice were either PBS-injected or infected with 2, 20, or 200 PFU CVB3. Spleens were harvested 3 hours after injection and the histamine concentration measured by EIA. Data is representative of three separate experiments; n=7 mice per group; *, $p<0.05$, ***, $p<0.005$. The observed significance of differences was determined by One-way ANOVA followed by Dunnett's Multiple Comparison Test.

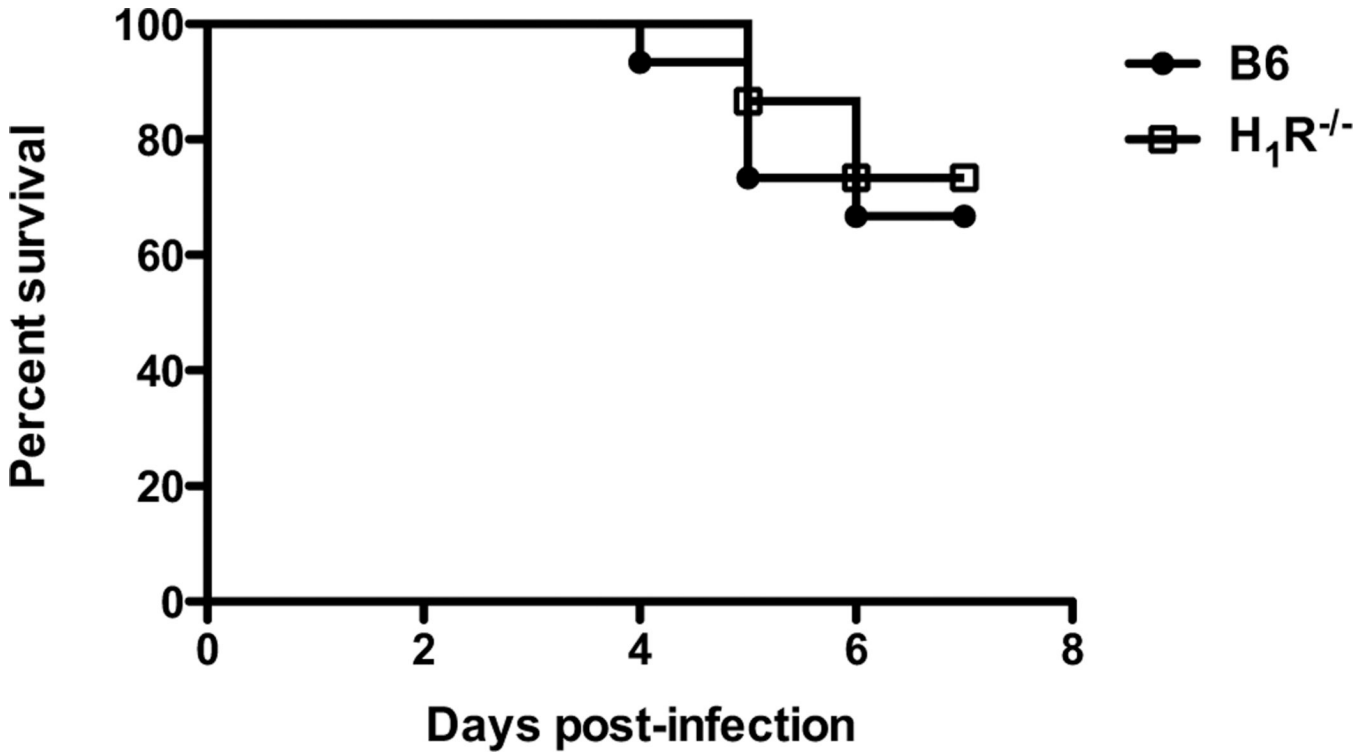


**Figure 2.**

$H_1R$ signaling does not affect the survival of CVB3-infected B6 mice. Mice were infected with 100 PFU and monitored over 7 days for survival. n=15 mice per strain combined from 2 independent experiments; p=0.615 as determined by Log-rank (Mantel-Cox) test.

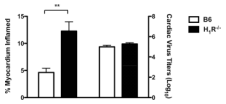


**Figure 3.**

$H_1R^{-/-}$ mice are more susceptible to CVB3-induced myocarditis irrespective of virus replication. Surviving mice from Figure 2 were euthanized at day 7. Hearts were evaluated by image analysis to quantify the percent myocardium inflamed (left). Cardiac virus titers were determined by plaque assay (right). n ≥ 10 mice per strain; **, $p<0.01$. The observed significance of differences was determined by Student's t-test.

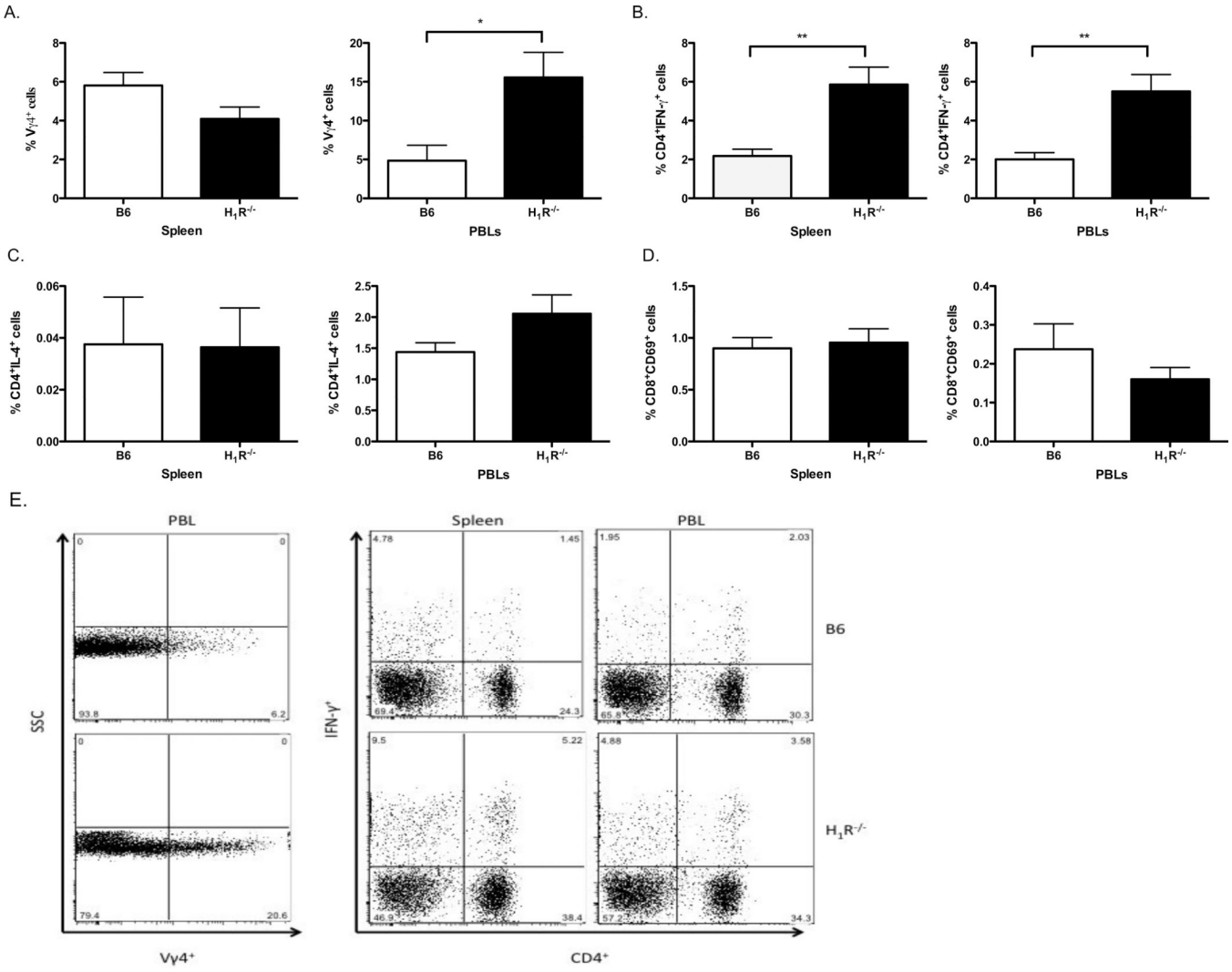


**Figure 4.**

Myocarditis susceptibility correlates with an increase in pathogenic $V\gamma4^{+}\gamma\delta$ T cells and Th1 cells in CVB3-infected $H_1R^{-/-}$ mice. Splenocytes and PBLs from the mice in Figure 3 were stained with fluorescently labeled antibodies to detect the percentage of (**A**) $V\gamma4^{+}\gamma\delta$ T cells, (**B**) Th1 effector T cells ($CD4^{+}IFN\text{-}\gamma^{+}$), (**C**) Th2 effector T cells ($CD4^{+}IL\text{-}4^{+}$), and (**D**) activated CD8 T cells ($CD8^{+}CD69^{+}$) by flow cytometry. (**E**) Representative dot plots of T cell subsets exhibiting statistical significance. n ≥ 10 mice per strain; *, $p<0.05$; **, $p<0.005$. The observed significance of differences was determined by Student's t-test.

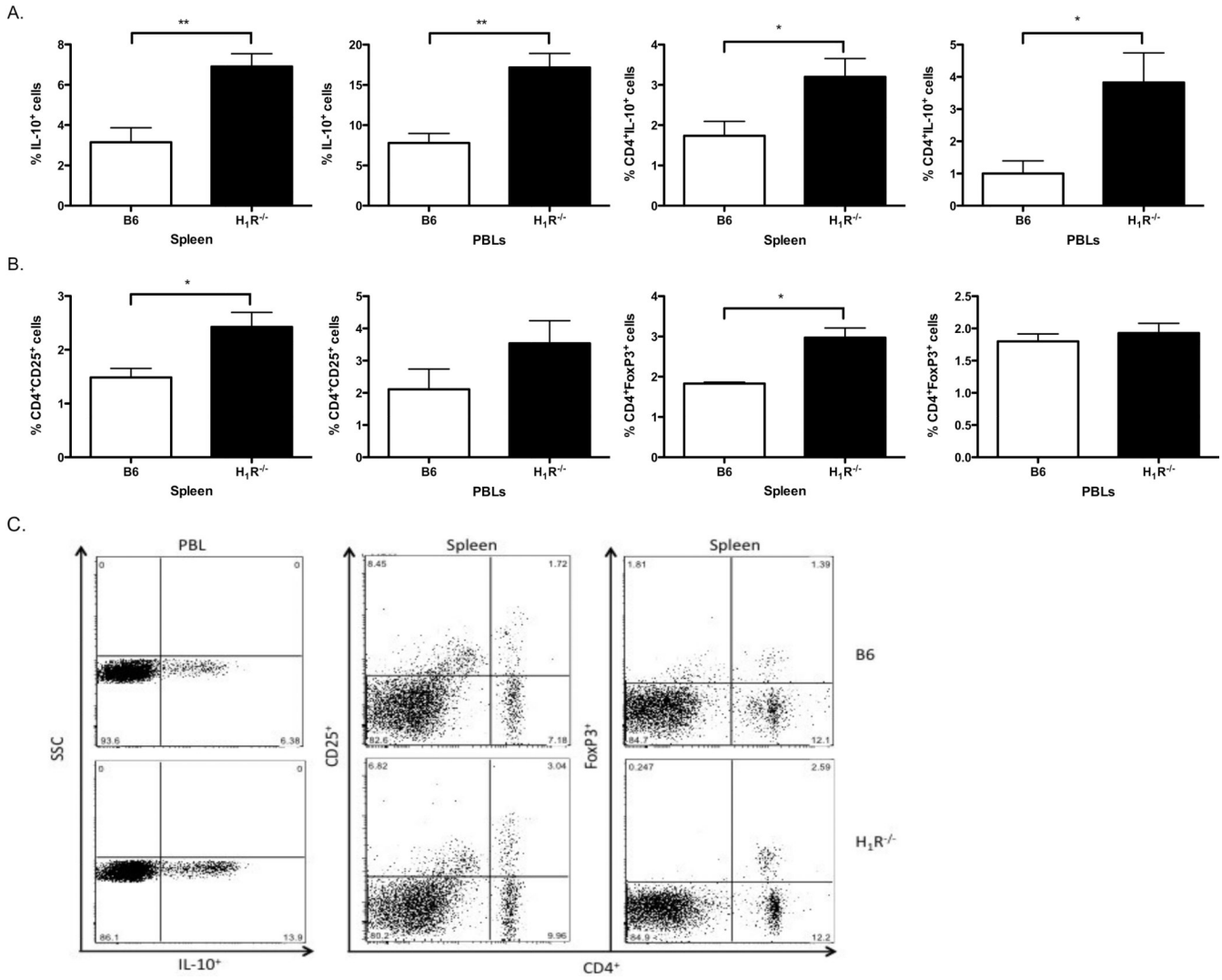


**Figure 5.**

An increase inperipheral Tregs is incapable of controlling myocarditis in CVB3-infected $H_1R^{-/-}$ mice. Splenocytes and PBLs from the mice in Figure 3 were analyzed for the percentage of Treg cells by staining for (**A**) all IL-10$^+$ cells and CD4$^+$IL-10$^+$ cells, (B) CD4$^+$CD25$^+$ cells and CD4$^+$FoxP3$^+$ cells. (**C**) Representative dot plots for each class of Treg cells. n ≥ 10 mice per strain; *, $p<0.05$; **, $p<0.01$. The observed significance of differences was determined by Student's t-test.