Amelioration of myocarditis by HVEM-overexpressing dendritic cells through induction of IL-10-producing cells

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Aims Herpes virus entry mediator (HVEM) is considered to be a molecular ‘switch’ for immune responses, and a role in immune modification has been reported. The aim of this study was to assess whether HVEM-mediated immune suppression could protect against experimental autoimmune myocarditis (EAM) induced by myosin.

Methods and results We constructed HVEM-expressing adenovirus (AdHVEM) and fusion protein HVEM-Ig and evaluated their roles in immunoregulation in vitro and in vivo. Immunoregulation of dendritic cells (DCs) infected with recombinant virus or treated with HVEM-Ig was then studied. DCs transfected with AdHVEM (DC-AdHVEM) were protected against EAM, whereas HVEM-Ig had no protective effect. Further study showed that DC-AdHVEMs produced a regulatory cytokine, IL-10, which had further effects on induction of IL-10 producing CD4⁺ T cells. This subset of T cells was then responsible for the protection against EAM.

Conclusion Myosin-DC-AdHVEM cell gene therapy appears to be a safe and effective way of inhibiting the development of EAM. The signal induced by HVEM seems to play different roles in different cells.

KEYWORDS Experimental autoimmune myocarditis; Herpes virus entry mediator; Immune regulation; Dendritic cells; IL-10

1. Introduction

Myocarditis and dilated cardiomyopathy are strongly associated with infection by Coxsackie virus B3 (CVB3). Both diseases are major causes of heart failure and heart transplantation.¹ Several lines of evidence now suggest that local heart tissue damage associated with this viral infection is mediated by primary T cells.²,³ The damage results from antiviral immune responses but also from an autoimmune response against cardiac myosin. However, despite the known involvement of immune reactions, little progress has been made in treating myocarditis by immunosuppression. The reason for this is due to a lack of understanding of key factors that regulate the pathogenic autoimmune reactions in myocarditis.

Experimental autoimmune myocarditis (EAM) can be generated in susceptible animals by immunization with purified cardiac myosin or by a specific pathogenic cardiac myosin peptide in adjuvant. As such, EAM represents a good model for investigating the pathogenesis of myocarditis, and is considered to be a T-cell-mediated disease.⁴,⁵

Optimal T-cell activation requires T-cell receptor (TCR) engagement of a cognate MHC-peptide complex, in conjunction with a costimulatory signal from antigen presenting cells (APCs), such as dendritic cells (DCs). Costimulatory molecules, such as CD28, ICOS, CD154, have been shown to play important roles in initiation, maintenance, and regulation of immune responses. Most costimulatory molecules can be expressed diverse immunocytes.⁶,⁷ The signals that are mediated by these molecules may have different effects on different immunocytes in different phases of the immune response. Therefore, these molecules may significantly influence the final manifestation of an immune response.

The B and T lymphocyte attenuator (BTLA) is the most recently identified inducible costimulator CD28 homologue.⁸ It is unusual in that its ligand is not one of the classic B7 family members, but rather is a tumour necrosis factor receptor (TNFR) superfamily member that has been designated as the herpes virus entry mediator (HVEM).⁹,¹⁰ Thus, HVEM can promote T-cell activation by propagating positive signals from the TNF superfamily member ligand, LIGHT, a lymphotoxin-related inducible ligand that competes for glycoprotein D with HVEM on T cells.¹¹ However, the interaction between HVEM and BTLA has been shown to mediate downregulation of T-cell response.⁹,¹⁰ HVEM, BTLA, and LIGHT are
expressed at different active phases of T cells, B cells, and DCs. They constitute a complex signal network that accurately regulates the immune response.

In the current study, we proposed a new model in which HVEM-overexpressing DCs are protected against EAM by a mechanism that is dependent on IL-10. Increasing the IL-10 produced by HVEM overexpression in DCs supported the generation of IL-10 producing CD4+ T cells in vivo and consequently regulated the autoimmune response.

2. Methods

2.1 Animals

Male 6- to 8-week-old Balb/c (H-2b) mice, DO11.10 transgenic mice (Balb/c), and 11O-/- mice (Balb/c) were all purchased from Nanjing Model Animal Center (Nanjing, China). All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2 Reagents and antibodies

Phorbol 12-myristate 13-acetate (PMA), ionomycin (IO), lipopolysaccharide (LPS), ovalbumin (OVA), protein A column, and porcine cardiac myosin were from Sigma (Saint Louis, MO, USA). Neutralized monoclonal antibody (mAb) to IL-10, and fluorescein-labelled mAb, including PE-anti-IL-10, PE-anti-IL-17, PE-anti-HVEM, APC-anti-IFN-γ, APC-anti-IL-4, APC-anti-CD11c, and FITC-anti-CD4 were from eBioscience (San Diego, CA, USA). Biotin-anti-TGF-β and PE-anti-Biotin were from BD Pharmingen (San Jose, CA, USA) and Miltenyi Biotech (Bergisch Gladbach, Germany), respectively.

2.3 Protein expression and purification

The murine HVEM-Fc recombinant protein (HVEM-Ig) was expressed in CHO cells and purified on a protein A column. A brief protocol is described in the online Supplementary material. The purity of the protein was more than 90% (data not shown).

2.4 Virus preparation

The E1/E3-deleted recombinant adenovirus Ad-HVEM was constructed with the AdEasy system (Stratgene, La Jolla, CA, USA) according to the manufacturer's instructions. A brief protocol is described in the online Supplementary material. The concentrations of plaque forming units (pfu) of individual stocks were determined by plaque assays on 293 cells, as described previously. The adenovirus expressing HVEM contained, as an additional feature, an expression cassette for green-fluorescent protein (GFP) as a reporter gene. A control adenovirus, which had only the gfp gene expressed, was also prepared in this experiment.

2.5 Isolation of DCs and CD4+ T cells

Dendritic cells were generated as described previously. Briefly, bone marrow mononuclear cells were prepared from mouse femur and tibia bone marrow suspensions by depletion of red cells. The cells were then cultured in dishes in complete RPMI 1640 medium (HyClone, Logan, UT, USA) in the presence of 10 ng/mL of recombinant mouse granulocyte-monocyte colony-stimulating factor (GM-CSF, R&D, Minneapolis, MN, USA) and 1 ng/mL of recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ, USA). After 5 days, the loosely adherent clusters were positively selected with CD11c magnetic microbeads (Miltenyi Biotech) according to the manufacturer's recommendations. The recovered cells showed more than 95% CD11c+. The CD4+ T cells were collected from spleen and lymph nodes with CD4+ magnetic microbeads (Miltenyi Biotec), and the purity of CD4+ cells was more than 95%.

2.6 Transfection of DCs with Ad

Immature DCs were generated as described above. The DCs were transfected with AdHVEM or AdGFP at 50 pfu/cell of each virus system for 1 h. After washing, the DCs were cultured for 12 h with cardiac myosin (10 ng/mL) and LPS (10 ng/mL), then washed again. For in vivo analysis, the DCs were intraperitoneally transferred into mice (5 x 10^6 cells/mouse).

2.7 Induction of active EAM and treatment

Myosin was dissolved in 0.01 M phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (CFA). On Day 0 and 7, mice were injected twice subcutaneously in the inguinal groove with 0.2 mL of emulsion, which yielded an immunizing dose of 0.2 mg cardiac myosin per mouse. Mice subjected to immunization with PBS and CFA were included as a control group. EAM mice were treated with Ad transfected DCs (5 x 10^6 cells/mouse), HVEM-Ig treated DCs (5 x 10^6 cells/mouse), or HVEM-Ig (10 μg/mouse) twice a week for 2 weeks following the first immunization of myosin. In some experiments, myosin antigen-loaded DCs were treated with 100 μg/mL HVEM-Ig for 48 h before being transferred into mice.

2.8 Histology of EAM hearts

Mice were sacrificed at Day 21 by cervical dislocation, after blood samples were collected. Hearts were fixed in 10% buffered formalin and embedded in paraffin. Five micrometre sections were cut and stained with haematoxylin and eosin (H&E) for microscopic histological examination. Myocardia were blindly scored for the presence of histopathological myocarditis according to the scale: 0, normal; 1, mild (<5% of heart cross-section involved); 2, moderate (5–10% of cross-section involved); 3, marked (10–25% of cross-section involved); and 4, severe (>25% of cross-section involved). The level of cTnI in plasma was evaluated with sandwich fluorimunoassay according to the manufacturer's instructions (Access2, Beckman Coulter).

2.9 In vitro analysis of DCs

The infection rate of Ad into DCs was determined as the percentage of CD11c+CD4+ cells. Expression of HVEM in the DCs was evaluated by flow cytometry with anti-HVEM mAb (LH1). The impacts of Ad-transfected DCs on T cells were determined using DO11.10 transgenic CD4+ T cells. Immature DCs were transfected with Ad at a dose of 50 pfu/cell for an hour, and then activated by 10 ng/mL LPS and 2 μg/mL OVA for 24 h. Irradiated or non-irradiated mature DCs and OVA233-259-specific CD4+ T cells were co-cultured for 5 days at a ratio of 1:10. Cell proliferation, cytokine production, and differentiation were determined by [3H]-thymidine ([3H]-TdR) incorporation, ELISA, and flow cytometry, respectively. The impacts of HVEM-related signals on DC were determined using intracellular staining and ELISA.

2.10 Anti-cardiac myosin antibodies

The titre of anti-myosin antibodies in plasma was determined by sandwich ELISA as described previously.

2.11 Proliferation assay

The proliferative response of splenocytes from EAM mice to myosin were determined by [3H]-thymidine ([3H]-TdR) incorporation, as described previously. To determine the degree of suppression mediated by HVEM, purified CD4+ T cells were co-cultured for 5 days with AdHVEM or control virus transfected DCs, followed by another 18 h of culture in the presence of 1 μCi/well [3H]-thymidine. The cell proliferation was determined by [3H]-thymidine incorporation.

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proliferation level was determined by radioactivity counting (cpm) in a liquid scintillation system.

### 2.12 Intracellular cytokine staining

CD4⁺ T cells were collected and either left unstimulated or were stimulated for 5 h with PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (750 ng/mL, Sigma-Aldrich) in the presence of either Golgiplug (IFN-γ and IL-17) or Golgistop (IL-4 and IL-10) at the recommended concentrations (BD Pharmingen). Standard intracellular cytokine staining was done as described previously.16 Cells were stained extracellularly with anti-CD4 (NK1.1), fixed, permeabilized with Cytofix/Cytoperm solution (BD Pharmingen), and then stained intracellularly with anti-IFN-γ (XMG1.2), anti-IL-17 (eBio17B7), anti-IL-4 (11B11) or anti-IL-10 (JE55-16E3). Sample fluorescence was measured by flow cytometry with a FACSCalibur (BD) and data were analysed with CellQuest software (BD). For analysis of intracellular IL-10 in CD11c⁺ DCs, cells were first blocked for non-specific binding with unlabelled CD16/CD32 Ab, and then treated with the same protocol as CD4⁺ T cells. For analysis of TGF-β, it was indirectly labelled with a PE-anti-Biotin mAb following staining with biotin-anti-TGF-β.

### 2.13 Real-time PCR

RNA was isolated from cardiac tissue (Qiagen RNeasy kit; Qiagen) and then transcribed into cDNA using random hexamers (Invitrogen Life Technologies) as primers and Superscript II RNase H⁻ reverse transcriptase (Invitrogen). The resulting cDNA was normalized for expression of the constitutively expressed gene Gapdh (5′-TG TAGACCATGTAGGACTCA-3’ and 5′-AGGTCCGTTGAACCGATT TG-3’) and then used as a template for PCR. The following primers were used (Sigma-Genosys): Mip-1α (5′-CCATGACACTCTGCAAACCA AGT-3’ and 5′-TCGGCTGTAGGAGAAAGCA-3’); Ifn-γ (5′-TCAGAGTGCCA TAGATGGAGAAAG-3’ and 5′-TGCCCTGCAAGGATTTCTG-3’), and Il-17 (5′-GCTCCAGAGGGCCCTCAGA-3’ and 5′-AGGTTGTCCG CATTGA-3’).

### 2.14 Statistics

Statistical significance was evaluated using one-way ANOVA test in all experiments, except for those depicted in Figures 2 and 5. A P-value of <0.05 was considered significant. A Wilcoxon rank test was used to assess the severity of myocarditis and the concentration of cTnl.

### 3. Results

#### 3.1 Both HVEM-overexpressing DCs and HVEM-Ig inhibit CD4⁺ T-cell proliferation

We first examined the effects of HVEM overexpression mediated by the recombinant virus on murine myeloid DCs and its impact on CD4⁺ T cell proliferation. After 48 h culture with the recombinant virus, more than 90% of the Ad-HVEM and control virus (Ad-GFP) transfected DCs were found to be GFP⁺. Ad-HVEM transfected DCs showed much higher inducible expression of HVEM than was seen with constitutive expression. Ad-GFP transfected DCs did not show differential expression (Figure 1A).

The HVEM-overexpressing DCs could specifically inhibit DO11.10 transgenic CD4⁺ T-cell activation in the presence of OVA (Figure 1B). The fusion protein HVEM-Ig was inhibited T-cell activation mediated by anti-CD3 in a dose-dependent manner (see Supplementary material online Figure S1). This HVEM-mediated inhibition might represent T cells that have been arrested but were not apoptotic, because very little annexin V positive cells were found after culture (data not shown).

#### 3.2 Myosin-DC-AdHVEM, but not HVEM-Ig, protects against myosin-induced myocarditis

To demonstrate whether treatment with myosin-DC-AdHVEM could prevent myosin-induced myocarditis, DCs from Balb/c mice were transfected with AdHVEM and then pulsed with cardiac myosin in the presence of LPS. These DCs were then used to treat EAM mice, commencing 2 weeks after the mice had been immunized with myosin in CFA. The development of myocarditis was assessed at Day 21 after the first immunization.

On Day 21, histological examination of the hearts in the control groups showed severe myocarditis, including pronounced inflammatory cell infiltration with mononuclear cells, neutrophilic granulocytes, and lymphocytes. Degeneration, destruction, and even necrosis of cardiac myocytes were observed (Figure 2A). The most severe morphological changes were observed in the three control groups (untreated or treated with either myosin-DC-AdGFP or DC-AdGFP).

These adverse histological effects were most notably prevented in the group of mice treated with myosin-DC-AdHVEM. Myocarditis was also partially prevented in mice treated with DC-AdHVEM, which suggested that DC-AdHVEM, in the absence of a myosin antigen pulse, can also diminish the severity of myocarditis. Compared with the EAM-no treatment group, there was a significant decrease in the severity score of mice treated with DC-AdHVEM (P < 0.05) or with myosin-DC-AdHVEM (P < 0.01) (Figure 2B). Control groups treated with myosin-DC-AdGFP or DC-AdGFP had higher severity scores when compared with the EAM-no treatment group. These results confirmed that optimal inhibition of EAM occurs in the presence of DCs that have been pulsed with myosin.

Interestingly, in contrast to HVEM-overexpressing DCs, recombinant protein HVEM-Ig itself did not seem to play any role in protecting against EAM as this treatment had no significant effect on the histology of EAM. The level of plasma cTnl, an effective index of myocardiac lesion, confirmed the data of the histological evaluations (Figure 2C).

#### 3.3 Suppression of inflammatory reactions in response to myosin-DC-AdHVEM treatment

To further investigate the impacts of myosin-DC-AdHVEM treatment on EAM mice, the expression of inflammatory cytokines was assessed by Real-Time PCR and intracellular staining.
factor mRNAs in hearts was examined by PCR, while the expression of anti-myosin IgG in sera was examined by ELISA. Levels of mRNA for interleukin (IL)-17, IFN-γ, and MIP-1α, which were enhanced by immunization, were significantly decreased in groups transplanted with HVEM-overexpressing DCs. There was no decrease seen in the groups that received HVEM-Ig injection when compared with their corresponding controls (Figure 3A). The autoantibody in sera decreased in groups that received HVEM-overexpressing DC transplantation but was maintained in the groups that received HVEM-Ig treatment (Figure 3B).

3.4 Non-irradiated DC-AdHVEM-induced production of IL-10 producing CD4⁺ T cells
The different impacts of treatment with HVEM-overexpressing DCs vs. HVEM-Ig on EAM suggested that the DC cell treatment had resulted in a stronger immunoregulation than did HVEM-Ig treatment. In subsequent experiments, the impacts on the subset of effective and regulatory T cells were analysed using OVA323–339-specific CD4⁺ T cells obtained from DO11.10 mice. After 7 days of stimulation, a decrease in IFN-γ⁺, and IL-17⁺ cells, and an increase in IL-4⁺ cells, were seen in groups receiving DC-AdHVEM or
HVEM-Ig, when compared with groups receiving DC-AdGFP or control IgG, respectively. No changes were observed in CD4<sup>+</sup>-Foxp3<sup>+</sup> cells. Treatment with DC-AdHVEM also induced a significant and unexpected production of IL-10<sup>+</sup> cells (Figure 4A).

Cytokine analysis, for cytokines other than IL-10, showed no significant differences between groups of DC-AdHVEM and DC-AdGFP, or between HVEM-Ig and control IgG (Figure 4B). These data suggested that HVEM overexpressing DCs induced the formation of IL-10 producing CD4<sup>+</sup> T cells. However, this induction did not occur through the direct ligation of HVEM and its ligands between DC and T cells. This suggestion was further confirmed by the results in which irradiated DCs inhibited CD4<sup>+</sup> T cells proliferation (data not shown) but not induce IL-10 producing T cells (Figure 4C).

3.5 HVEM overexpressing DCs produce large amounts of IL-10

To study the possible mechanisms of induction of IL-10 producing cells mediated by HVEM overexpressing DCs, we tested their phenotype and cytokine production. These DCs and their respective controls expressed comparable levels of CD40, CD80, CD86, B7RP-1, programmed cell death ligand, and I-A<sup>d</sup> (data not shown). Additionally, the overexpression of HVEM had little impact on TGF-β secretion from the DCs (Figure 5, upper). However, intracellular staining showed that IL-10 had increased significantly in AdHVEM-transfected DCs (Figure 5, lower).

3.6 HVEM-Ig-modified myosin-specific DCs produce IL-10 and protect against EAM

As both BTLA and LIGHT could be expressed in DCs, we postulated that overexpressing HVEM affected DCs through its ligands on these cells. We found that HVEM-Ig could induce DCs to produce IL-10, but not TGF-β (Figure 6A). This result confirmed our hypothesis, and suggested that it was the IL-10 from the DCs that induced the IL-10-producing T cells. Mixed lymphocyte culture using IL10<sup>−/−</sup> DCs or anti-IL-10 neutralizing antibody demonstrated that the IL-10 from DCs could induce IL-10 producing T cells in vitro (Figure 6B).

We further investigated the immunoregulatory function of HVEM-Ig-modified DCs in vivo using an EAM model. Although a slight local infiltration of inflammatory cells could be found in mice that were injected with HVEM-Ig and treated with wild-type DCs, there was a significant amelioration of myocardial histology in these mice. Conversely, in groups of mice treated with IL10<sup>−/−</sup> DCs, the severity of myocarditis was comparable with that seen in the control groups (Figure 6C). Plasma cTnI concentration assays showed parallel results with cardiac histology (data not shown). These data collectively suggested that involvement of IL-10 is critical for the observed regulation of the immune response by HVEM-mediated DCs.

4. Discussion

Experimental autoimmune myocarditis is a well-established mouse model for the study of myocarditis induced by autoimmune mechanisms. Antigen-presenting cells, myocarditic epitopes of cardiac myosin, and their reactive T cells are known key factors in EAM induction. Many investigators have used this model to analyse the effects of either anti-T cell treatment or anti-inflammatory treatments. In the present model, we wanted to specifically inhibit the development of myocarditis through limiting autoreactive T-cell activation. Our original strategy had been to ligate a
negative costimulatory molecule of T cells, namely BTLA, with its ligand HVEM, onto DCs. However, some unexpected phenomena appeared in our experimental system. These results suggested that an HVEM-mediated network was operating in a different subset of cells.

Herpes virus entry mediator is considered to be a ‘molecular switch’ for immune regulation, because it enhances or inhibits immune responses by propagating different costimulatory molecules.11,18 However, the exact mechanism of this regulation is unclear. Cheung et al.19 demonstrated that the affinity of LIGHT-HVEM is greater than that of HVEM-BTLA, and suggested that a high-level expression of HVEM would therefore promote BTLA combination. In addition, HVEM-overexpressing regulatory T cells were shown to enhance immunosuppression of effector T cells.20 In the current study, we found that either irradiated or untouched HVEM-overexpressing DCs could inhibit T cell proliferation. This suggested that the induced HVEM expression might sufficiently propagate BTLA and that a negative signal might be transmitted between them. Therefore, DC-AdHVEM cell

Figure 4 HVEM-overexpressing DCs, but not HVEM-Ig, induced IL-10 producing T cells in vitro. (A) Flow cytometry of DO11.10 transgenic CD4+ T cells cultured for 7 days in various conditions (above plots) and stained intracellularly for IL-17, IFN-γ, IL-4, IL-10, and Foxp3. Numbers in quadrants or outlined areas indicate percentages of given positive cells. (B) ELISA of IFN-γ, IL-2, IL-4, IL-10, IL-12, TNF-α, and TGF-β in supernatant of DO11.10 transgenic CD4+ T cells cultured for 7 days in conditions of (A). (C) Flow cytometry of DO11.10 transgenic CD4+ T cells stimulated with irradiated DC and stained intracellularly for the same molecules as (A). Numbers in quadrants or outlined areas indicated percentages of given positive cells. Data are representative of four to six independent experiments.
HVEM overexpression induced DCs that produced IL-10. Myeloid DCs were transfected with Ad-HVEM or control virus for 48 h and the intracellular staining of TGF-β and IL-10 were assessed in CD11c+ cells. Shown is a representative example from four experiments. Numbers in outlined areas indicated percentages of positive cells.

Figure 5 HVEM overexpression induced DCs that produced IL-10. Myeloid DCs were transfected with Ad-HVEM or control virus for 48 h and the intracellular staining of TGF-β and IL-10 were assessed in CD11c+ cells. Shown is a representative example from four experiments. Numbers in outlined areas indicated percentages of positive cells.

A key component of the cell gene therapy studied here was the apparent ability to increase immunosuppressive specificity by pulsing DCs with antigen, in order to specifically inhibit autoreactive cells. There was a statistically significant difference in the severity of myocarditis in mice treated with DC-AdHVEM, even in the absence of a myosin pulse. Nonetheless, the optimal decrease in myocarditis took place in mice treated with myosin-DC-AdHVEM.

We propose that the DC-AdHVEM has an ameliorating effect induced by some type of non-specific inhibition of the activated T cells that are involved in the myocarditis process. The difference between DC-AdHVEM and myosin-DC-AdHVEM might have been because the latter had higher HVEM expression than the former did. In our adenovirus expressing system, the cytomegalovirus (CMV) promoter had been demonstrated to respond to inflammatory stimulation. Thus, if DCs were activated by antigen, higher expression of HVEM could be induced. In the absence of inducible HVEM, myosin-pulsed DCs increased the severity of myocarditis. The data from mice treated with myosin-DC-AdGFP further support the conclusion that the presentation of myosin-derived peptides by mature DCs was sufficient for induction of EAM.

HVEM-Ig also appeared to be involved in the EAM process. Theoretically, HVEM-Ig could ameliorate myocarditis if it inhibited T-cell activation. This would occur not only by transmitting a negative signal to BTLA, but also by blocking LIGHT-mediated activation. In the current study, HVEM-Ig was capable of blocking the activation of resting T cells in a dose-dependent manner in vitro. Nevertheless, HVEM-Ig itself failed to protect against EAM. This finding was similar to previous reports that HVEM-Ig alone did not prolong cardiac allograft survival.

The reason that HVEM-Ig failed to regulate immune response in vivo is unclear at this time. A high-molecular-weight aggregate in crude extracts of HVEM-Ig has previously been reported to potently inhibit T-cell proliferation. This is still not a convincing explanation for our observed in vivo results. Therefore, we postulate that other distinct effects on T cells take place in response to exposure to HVEM-overexpressing DCs.

Beside anti-T-cell activation, the balance among T helper (Th) subsets and expansion of regulatory T cells are always reported as outcomes of crosstalk between costimulatory molecules. This type of crosstalk was also a possible explanation of the amelioration of autoimmune diseases. For this reason, we also examined the impact of DC-AdHVEM on induction of Th subsets and regulatory T cells, comparing its action to that of HVEM-Ig. Exposure to HVEM-overexpressing DCs and to HVEM-Ig both down-regulated Th1 and Th17 subsets, and up-regulated Th2 subsets, but had no effects on CD4+ Foxp3+ Tregs. However, HVEM-overexpressing DCs induced significant numbers of IL-10 producing CD4+ T cells, whereas HVEM-Ig did not.

IL-10 is a pleiotrophic immunomodulatory cytokine that functions at different levels in the immune response. The IL-10-producing CD4+ T cells, designated as Tr1, have a role in maintaining peripheral tolerance. Therefore, the discrepancy in Tr1 induction between HVEM-overexpressing DCs and HVEM-Ig may, in part, explain their differences in protecting against EAM.

In the following experiments, we asked why HVEM-overexpressing DCs would induce Tr1. Since irradiation of HVEM-overexpressing DCs had few effects on T cells, it is unlikely that any effects would be a direct result of the interaction of the DC HVEM with its partners on T cells. It is more likely that the effects resulted from a changed property of the HVEM-overexpressing DCs themselves. A high level of IL-10 was induced in HVEM-overexpressing DCs, which supported the generation of Tr1 cells. Additionally, HVEM-Ig also induced production of IL-10 in DCs, and these DCs protected against EAM specifically, after a pulse with myosin antigen.

These results, taken together, provide new understanding of the complex impacts of HVEM-mediated networks on immune responses and immune regulation. In this regard, the HVEM-mediated network was seen to exert its influence on at least two aspects. One was through its diversity of partners, which allows transmission of either negative or positive signals. The other was through its capability of producing different effects on different cells. In present study, the HVEM-dependent IL-10 production took place in DCs, and this provided supplemental evidence that IL-10-producing DCs prevented EAM induction.
may shed light on potential development of antigen-specific immune therapy to control chronic myocarditis. The exact mechanism of HVEM-dependent IL-10 production in DCs is unclear at this time, but this novel method shown here, of using HVEM-modified DCs to protect against autoimmune myocarditis, may be worth testing in clinical trials.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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