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Chronic ethanol fed mice increase Treg activity and inhibit HCV core-specific cellular immune responses

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Abstract

Introduction—Chronic ethanol consumption is associated with persistent hepatitis C viral (HCV) infection. This study explores the role of the host cellular immune response to HCV core protein in a murine model and how chronic ethanol consumption alters T-cell regulatory (Tregs) populations.

Methods—Balb/c mice were fed an isocaloric control or ethanol liquid diet. Dendritic cells (DCs) were isolated after expansion with a hFl3tL-expression plasmid and subsequently transfected with HCV core protein. Core-containing DCs (1×10^6) were subcutaneously injected (X3) in mice every 2 weeks. Splenocytes from immunized mice were isolated and stimulated with HCV core protein to measure generation of viral antigen specific Tregs, as well as secretion of IL-2, TNF- α , and IL-4. Cytotoxicity was measured by lactate dehydrogenase (LDH) release from HCV core-expressing syngeneic SP2/19 myeloma cells.

Results—Splenocytes from mice immunized with ethanol-derived and HCV core-loaded DCs exhibited significantly lower *in vitro* cytotoxicity compared to mice immunized with HCV core-loaded DCs derived from isocaloric pair fed controls. Stimulation with HCV core protein triggered higher IL-2 TNF- α and IL-4 release in splenocytes following immunization with core-loaded DCs derived from controls as compared to chronic ethanol fed mice. Splenocytes derived from mice immunized with core-loaded DCs isolated from ethanol-fed mice exhibited a significantly higher CD25⁺FOXP3⁺ and CD4⁺FOXP3⁺ Treg population.

Conclusions—These results suggest that immunization with HCV core-containing DCs from ethanol-fed mice induces an increase in the CD25⁺FOXP3⁺ and CD4⁺FOXP3⁺ Treg population and may suppress HCV core-specific CD4⁺ and CD8⁺ T cell immune responses.

Keywords

Hepatitis C virus; immune responses; T-regulatory cells; chronic ethanol

Introduction

Hepatitis C virus (HCV) is a single-stranded RNA virus of the *Falviviridae* family (1) that affects 2-3% of the world's population, causing more than 350,000 deaths per year (2). Since the discovery of this virus in 1989 (3), vaccine development has been difficult and its mechanism of action with respect to producing hepatocyte injury remains to be clarified. Ribavirin and pegylated IFN- α as well as Telaprevir and Boceprevir are currently used as therapy for this disease (4-7). Of the HCV genotype-1-infected patients treated, however, about 20-40% will continue to exhibit viral persistence, aggravating liver injury and accelerating progression to cirrhosis and hepatocellular carcinoma (8, 9). Further studies regarding the biology of host-viral interactions are needed in order to better understand the pathogenesis of persistent viral infection.

Hepatitis C virus has a genome size of 9.6 Kb that codes for structural (core and envelope) and nonstructural (NS2, NS3, NS4 and NS5) proteins (10). Of interest, HCV core protein plays a pathogenic role in hepatocyte apoptosis, alters lipid metabolism and activates cellular immune responses (11, 12). Its association with the ER and the mitochondria is known to increase reactive oxygen species (ROS) production, oxidation of glutathione pools in the mitochondria, ER stress, and to inhibit the JAK/STAT signal transduction pathway that is crucial in generating immunity (13, 14). The combination of HCV infection with chronic ethanol abuse, however, has a more deleterious and even synergistic adverse effect. Alcoholics with HCV infection are more likely to evolve viral persistence following exposure, and this interaction accelerates fibrosis development compared to abstainers with chronic HCV infection (15, 16). Liver biopsies derived from alcoholic and persistently infected HCV patients have demonstrated accelerated micro- and macrosteatosis, cell dropout, fibrosis and lymphocyte infiltration (17). Biochemical studies have revealed high level proteasome activity, lipid peroxidation, and cell injury as a consequence of oxidative stress and glutathione pool depletion; all of which appear to be enhanced by the combination of ethanol abuse and HCV infection (18-22). Other investigations have shown that generation of helper T cell (Th1) and cytotoxic T lymphocyte (CTL) activity to structural and non-structural proteins are reduced in mice consuming a chronic ethanol-containing diet (23), which can be rescued by immunizing with interleukin-2 or granulocyte macrophage colony-stimulating factor (GM-CSF) expression plasmids. These studies have suggested that ethanol's adverse effects on HCV infection are due, in part, to alteration of immune regulation that may particularly affect the function of antigen presenting cells (APCs).

Dendritic cells (DCs) are professional APCs that take up, process and present peptides to T cells via major histocompatibility complexes (MHC) (24). Their robust function, with respect to activation of T cell mediated immune responses, makes them ideal for delivery of HCV viral proteins (25, 26). In this context, Aloman et al, utilizing a DC based immunization approach in chronically ethanol-fed mice observed a reduced cellular immune response against HCV NS5 protein compared to animals fed a control diet (18). In the present study, we have extended this approach to investigate the possible mechanism(s) for inhibition by using DC-based immunization against HCV core derived from ethanol-fed mice, to explore T cell mediated cytotoxicity, cytokine release and regulatory cell activation.

Materials and Methods

Media and Reagents

Dendritic cells were cultured under conditions composed of HEPES-buffered RPMI-1640 medium, supplemented with 2 mM L-Glutamine, 100 micro (μ)g/ml streptomycin, 100 mM sodium pyruvate, 10mM non-essential amino acids, 10 mM essential amino acids, 50 mg/ml gentamicin, and 5×10^{-5} M β -2-mercaptoethanol (Sigma-Aldrich St Louis, MO). HCV core was purchased from Austral Biologicals (San Ramon, CA). Ovalbumin protein and the Lactate Dehydrogenase kit were obtained from Sigma Aldrich. Chariot (Active Motif, Carlsbad, CA) was used to deliver HCV core protein into DCs. Anti-mouse-FITC, anti-CD11c-PE, anti-CD4- Cy5.5, anti-CD25-CD25, anti-FITC-iso, anti-PE-iso, anti-PercyP Cy5.5-iso were purchased from eBioscience (San Diego, CA). Monoclonal HCV core antibody (mAb) was prepared as described (27). rIL-2 was purchased from eBioscience (San Diego, CA). Neuronal thread protein (NTP) was produced and used as a control (28).

Animals and Diet Regimen

Six-week old female Balb/c mice were purchased from Harlan Laboratory. They were placed on a control or ethanol liquid diet regimen for 8 weeks (Bioserv-Liquid Diet; Bioserv, Frenchtown, NJ) prepared according to manufacturer's instructions as previously described (18). During the first week, mice received 1.25 % ethanol (wt/vol) in their diet, which was gradually increased to 2.5% in the second week, and to 3.75% in the third week, and maintained at this concentration for the duration of the feeding regimen. A separate group of six-week old female Balb/c mice that received DC-based immunization were maintained on a standard chow diet. The spleen was collected upon sacrifice for isolation of DCs in all experiments. Animal protocols were reviewed and approved by the Lifespan Animal Care and Use Committee.

Generation of DCs

Twelve and seven days before sacrifice, mice receiving their respective liquid diets were hydrodynamically injected with 10 micro (μ) of a Flt3L expressing plasmid in 2ml saline via their tail vein to expand the splenic DC population as previously described (29). Spleens were collected upon sacrifice and DCs separated magnetically using CD11c+microbeads (Myltenyi Biotech, Auburn, CA).

Immunizations

DCs were isolated from chronic ethanol fed and isocaloric pair fed controlled mice following *in vivo* expansion, and subsequently transfected with HCV core protein for 2 hours via the Chariot system in culture media as described (25). The DCs were collected (1×10^6), resuspended in 200 micro (μ) of Hanks balanced salt solution (HBSS), and injected subcutaneously in the flank of mice receiving chow diet to generate T cell responses. Vaccination was performed 3 times at two weeks interval, and mice receiving immunization generated from control-fed mice were kept separate from ethanol-fed mice. As controls, a separate group of mice fed a chow dietary regimen were immunized in the same manner

with either HCV soluble core or non-relevant neuronal thread protein(NTP) (2 μ g)proteins dissolved in HBSS.

HCV Core and Regulatory T cell Staining

Intracellular and cell surface staining was performed to detect HCV core transfection efficiency and the generation of Tregs respectively. After HCV core transfection using the Chariot system, 1×10^6 DCs were stained for CD11c+ using anti-cd11c-PE. Intracellular staining of HCV core was then performed with an anti-HCV core mAb and detected with anti-mouse-FITC using the Cytofix/Cytoperm Kit (BD Pharmingen, San Jose, CA). For Tregcell identification, 1×10^6 splenocytes isolated from the spleen of immunized mice were stained for CD4+ or CD25+ with anti-CD4-PercP Cy5.5 and anti-CD25-Alexa Fluor 488. Anti-FOXP3-PE was then used for intracellular identification of FOXP3 expression. Isotype staining was performed correspondingly as controls. All experiments were evaluated using FACS Calibur(BD Biosciences, San Jose, CA) and analyzed with Flowjo software.

Cytokine Measurement

Splenocytes isolated from core-loaded DC-immunized mice were incubated with 1 μ g/ml of HCV core protein for 24 hours in culture media. Splenocytes were then collected, spun down, and the supernatant collected for quantification of IL-2, TNF- α and IL-4 cytokine secretion via enzyme-linked immunosorbent assay (ELISA). The ELISAs were purchased from eBioscience(San Diego, CA) and the protocol was followed according to manufacturer's instructions

Cytotoxicity Assay

DCs isolated from control- and ethanol-fed mice, were transfected with HCV core protein, and co-incubated with splenocytes derived from mice that received the core loaded DC immunization at 1:100 ratio in a re-stimulation step, followed by incubation with rIL-2 for 3 days. The co-incubation was matched so that core loaded DCs derived from ethanol-fed mice were incubated with the splenocytes from the mice immunized by the core loaded DCs derived from ethanol-fed mice, for example. Likewise, for the negative controls, DCs derived from control-fed mice were used for the co-incubation step prior for the performance of the CTL assay. Splenocytes were then collected, counted and incubated with stable transfected HCV core-expressing SP2/19 cells (1×10^4) at 1:10, 1:50 and 1:100ratio (target cell:splenocyte) for 4 hours in a 96-well plate. After this procedure, cells were spun down, and the supernatants used to measure LDH release from SP2/19 cells (30).

Statistical Analysis

All results were analyzed using the GraphPad Prism program, where individual categories were compared using a non-paired Student *t* test. Statistically significant results were considered if the $p < 0.05$.

Results

HCV coretransfection of DCs

DCs were isolated from control and ethanol-fed mice, and cultured with HCV core protein for 4 hours using the HCV core-Chariot delivery system (25). Intracellular staining of HCV core was performed and analyzed by flow cytometry to determine if HCV core was being transfected efficiently into DCs for subsequent immunization. Both control and ethanol derived DCs were successfully transfected with and exhibited comparable levels of HCV coreexpression (Figure 1).

Mice immunized with HCV core loaded DCs derived from ethanol fed mice exhibit reduced CTL activity

Previous studies performed by Kuzushita et al. demonstrated a specific CTL response in chow-fed mice immunized with HCV core when compared to mice that were immunized with DCs alone or with medium as controls (25). Similarly, Aloman et al. demonstrated a reduced CTL response to HCV NS5 after genetic immunization with a HCV NS5 expression plasmid to mice fed with ethanol for 8 weeks compared to animals consuming an isocaloric control diet (18). Therefore, we hypothesized that HCV core-loaded DCs derived from ethanol-fed mice might also produce a reduced CTL immune response following immunization as compared to mice immunized with HCV core-transduced DCs derived from control liquid diet fed animals.

To evaluate this possibility, Balb/c mice were pair-fed control and ethanol containing liquid diet for 8 weeks. DCs were then isolated, transfected with HCV core protein using the Chariot system, and used for immunization of chow-fed mice. Mice that received the HCV core immunization delivered by DCs derived from ethanol-fed mice exhibited a significantly lower HCV core-specific CTL activity compared to mice that received the DC-core immunization prepared from isocaloric liquid diet fed control mice. (Figure 2) This observation was consistent at different SP2/19 target cell:splenocyteratios. Immunizing mice with core-loaded DCs prepared from either control or ethanol-fed mice demonstrated the efficacy and immunogenicity of this HCV viral structural protein as well as the vaccine delivery method, and subsequent generation of an antigen-specific immune response.

T cell activation is impaired when receiving core loaded DC immunization derived from ethanol-fed mice

Previous studies have shown that chronic ethanol consumption is associated with reduced IL-2 expression and secretion in mice due to abnormalities in the DC antigen presentation pathway, thereby decreasing T cell activation(31). To determine whether CD4+ and CD8+T cells function was altered by chronic ethanol in mice receiving HCV-core loaded DCs, splenocytes from immunized mice were stimulated with 1 micro (μ) g/ml HCV-core in vitro for 24 hours, and supernatants harvested and analyzed for IL-2 levels. Immunizing mice with core loaded DCs from control-diet fed mice produced higher amounts of IL-2 levels compared to mice receiving core loaded DCs derived from ethanol-fed animals, which had similar levels to mice immunized with saline or irrelevant NTP protein, indicating that ethanol impairspriming of T cells against this HCV structural protein(Figure 3). Moreover,

T cell activation was antigen specific because incubating splenocytes derived from both ethanol and control derived DC-core-immunized mice with 1 micro (μ) g/ml OVA protein did not influence levels in culture supernatants.

Mice receiving HCVcore loaded DC immunization from ethanol fed mice exhibit reduced pro-inflammatory cytokine levels

TNF- α and IL-4 are cytokines have been associated with induction of a T_{H1} and T_{H2} immune response, which activate macrophages and induce B and T cell proliferation(32, 33). Expression of both of these cytokines has been proposed to play a role in both ethanol-induced as well as HCV-induced liver injury(34, 35). We determined if the DC-based immunization approach would also produce inhibitory effects in mice immunized with core loaded DCs derived from ethanol or isocaloric pair fed (control) mice. The secretion of both of these cytokines was impaired in mice immunized with ethanol-derived and HCV core loaded DCs following stimulation of splenocytes *in vitro* with core protein; however, there was no difference in TGF β and IL-10 secretion (data not shown)(Figure 4). In addition, there was no significant difference in secretion of both TNF- α and IL-4 between control and ethanol-derived core loaded DCs when stimulated *in vitro* with OVA, indicating that the antigen specific immune sensitization was provided by the HCV core-loaded DCs *in vivo*. These results suggest that chronic ethanol consumption inhibits the host's inflammatory response following core loaded DC immunization.

Increased levels of CD25⁺FOXP3⁺ and CD4⁺FOXP3⁺ T regulatory cells are observed following core loaded DC immunization derived from chronic ethanol fed mice

The reduced CD4⁺ and CD8⁺ activity in mice immunized with HCV core loaded DCs derived from ethanol fed mice may be due to induction of Tregs. To examine this possibility, the Treg levels were evaluated in the total splenocyte population isolated from mice immunized with core- loaded DCs derived from control and ethanol-fed mice as well as groups receiving saline and irrelevant NTPantigen. Analysis was performed following after a24 hour incubation restimulation with HCV core protein (1 μ g/ml). Mice that received the core loaded DCs derived from ethanol-fed mice exhibited an expanded population of both CD25⁺FOXP3⁺ (Figure 5) and CD4⁺FOXP3⁺ Tregs (Figure 6) by FACS analysis compared to mice receiving core loaded DC immunization derived from control-diet fed animals, or those injected with saline or irrelevant NTPprotein. This finding suggests a potential mechanism(s) for the observed reduced CD4⁺ and CD8⁺ immuneresponses following core loaded DC immunization and may suggest an additional effect produced by ethanol on DC function other than reduced cytokine secretion(18, 31).

Discussion

The mechanism(s) of HCV-mediated liver injury and potential synergistic adverse effects in combination with ethanolabuse on the host immune response have not been well elucidated. Previous studies have proposed a role of HCV core protein in combination with chronic ethanol consumption to produceoxidative stress, lipid oxidation, increased apoptosis, altered regulation of the MAPK signaling pathway(36), and subsequent inhibition of anti-viral host immune response(36, 37)(37, 38). The latter is of particular interest because

alcoholics appear to be predisposed to persistent viral infection, a finding believed to be linked to a blunted host immune response initiated by DCs through reduced viral peptide presentation(31). This action by ethanol is amplified during HCV infection since it interferes with DC mediated T_H1 (12) and T_H17 activation by upregulating anti-inflammatory molecules such as TGF- β and IL-10 (39), while reducing IFN- α levels (34, 40). Therefore, understanding the possible mechanisms of ethanol effects on host immune responses to HCV core could help define immunologic factors important in viral clearance from the liver.

Past investigations have revealed potent inhibitory effects of ethanol on viral-specific CD4⁺ and CD8⁺ T cell responses against HCV nonstructural proteins that were capable of being partially reversed by ethanol abstinence (41) as well as by genetic immunizations through amplified secretion of GM-CSF and IL-2, at the site of vaccination; such cytokines may improve activation of APCs(23) to induce viral specific immune responses. Further studies have confirmed the importance of DCs in promoting anti-viral immune responses by utilizing them as vehicles for delivering viral structural and non-structural proteins to activate CD4⁺ and CD8⁺ T cells. This DC based vaccine approach takes advantage of their phagocytic and antigen presenting properties to sensitize host CD4⁺ and CD8⁺ T cells against HCV structural and non-structural proteins. Indeed, such methods have been shown to increase viral-specific CTL response against HCV NS5 and core proteins(25, 26, 42). In this context, Aloman et al. demonstrated that the transfer of DCs from ethanol-fed mice immunized with an NS5-expressing plasmid to other ethanol-fed mice did not improve the NS5-specific response whereas immunized mice receiving the DCs derived from control-fed mice did. Such findings illustrate ethanol's inhibitory effects on DC function and the subsequent ability to generate appropriate anti-viral activity to HCV core protein; however, the mechanisms involved in this altered immune response are largely unknown.

In this study, we suggest a potential role for Tregs in ethanol-fed mice that may contribute to depressed cellular immune responses to HCV core protein. Utilizing the DC-based immunization approach, we evaluated the immune response in chow-fed mice to HCV core protein after being immunized with DCs loaded with HCV core that were derived from either control or ethanol fed mice. Ethanol-derived core loaded DCs produced lower IL-2 levels after *in vitro* stimulation compared to core loaded DCs derived from control diet fed animals. In addition, there was impaired release of IL-4 and TNF- α . Furthermore, ethanol derived DC-core induced significantly less CTL activity at various lymphocyte target cell ratios. These experiments evaluated the evolution of host immune response by using the entire splenocyte T cell populations for all *in vitro* stimulation experiments, as well as employing HCV core loaded DC immunizations when activating a CTL response in the presence or absence of prior chronic ethanol consumption. These results were HCV-core specific since *in vitro* stimulation with non-relevant NTP did not elicit a CTL response or IL-2 release. Similarly, *in vitro* stimulation with OVA albumin did not induce IL-2 and TNF- α release in either control or ethanol derived HCV core loaded DCs immunized groups confirming antigen specificity.

These observations suggest that DCs isolated from ethanol-fed mice are unable to fully support generation of T cell responses compared to core loaded DCs immunization derived from isocaloric pair fed control animals. The possible mechanism(s) of this effect was

further explored. Regulatory T cells include natural Tregs developed in the thymus, or adaptive Tregs which develop in the periphery as a result of IL-2 trapping, cytokine production such as IL-10 and TGF- β , and cell-cell based interactions. Their activation attenuates effector CD4⁺ and CD8⁺ T cell activity by direct targeting or by inhibiting APCs, inducing cell cycle arrest, altering pro-inflammatory cytokine release or inducing apoptosis (43). Because of their role in suppressing anti-viral immune response(s), we hypothesized that Tregs may be involved in the poor CD4⁺ and CD8⁺ sensitization to HCV core protein in the ethanol derived core loaded DC immunized group. Mice vaccinated with core loaded DCs from ethanol fed mice exhibited a higher CD25⁺FOXP3⁺ and CD4⁺FOXP3⁺ Treg population compared to mice immunized with DC-core derived from isocaloric pair fed controls. These results suggest a potential interaction between chronic ethanol consumption and T-reg generation, thereby suppressing DC-mediated T-cell sensitization against HCV core protein; further studies will be required to determine if it is due to a direct interaction of Tregs with HCV core sensitive CD4⁺ and CD8⁺ T-cells or to reduced cytokine secretion and altered antigen presentation by DCs as previously described (18, 31, 35). It is also important to note that the levels of CD4⁺ T cells (Figure 6) are higher in mice immunized with core loaded DCs derived from control than with core loaded DCs obtained from chronic ethanol fed mice. Indeed, there is a higher number of effector CD4⁺ T cells and reduced levels of CD4⁺FOXP3⁺ T cells when comparing mice immunized with core loaded DCs derived from control compared to ethanol that may explain, in part, the reduced *in vitro* and *in vivo* generation of CD4⁺ and CD8⁺ anti-viral immune responses.

We hypothesize that immunosuppression may be mediated by a direct DC-Treg interaction, whereby Inducible Costimulatory Molecule (ICOS) on the Treg surface may interact with Inducible Costimulatory Molecule Ligand (ICOS-L) on DCs. Previous studies have shown that ICOS-L on DCs is upregulated as a consequence of chronic ethanol consumption and this phenomenon may contribute to their inability to trigger an effective T cell response (31). Moreover, other investigations have demonstrated that ICOS expression is associated with CD4⁺CD25⁺FOXP3⁺ T cell accumulation in the periphery as well (44, 45).

Taken together, these studies demonstrate that the poor sensitization against HCV core protein induced by ethanol-impaired DCs compared to that from control-fed mice resides mainly in their inability to activate CD4⁺ T cells, trigger a CTL response and induce TNF- α and IL-4 cytokine release. This immunosuppression may be due, in part, to an upregulation of CD4⁺CD25⁺FOXP3⁺ Treg population in the context of dysfunctional DCs with altered costimulatory ICOS molecule expression. These observations suggest potential new mechanisms to consider with respect to ethanol immunosuppressive effects on the host CD4⁺ and CD8⁺ anti-viral immune response, and establish a putative role for interactions between ethanol altered DCs and the generation of Tregs that may contribute to persistent HCV infection in alcoholics.

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Jack R. Wands outlined the studies, provided the research support and was involved in manuscript preparation.

Vivian Ortiz designed and performed the experiments, participated in data analysis and writing of the manuscript.

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Abbreviations

APC	antigen presenting cells
CD8+	cytotoxic cells
CTL	cytotoxic T-lymphocyte
DCs	dendritic cells
ELISA	enzyme linked immunosorbent assay
GM-CSF	granulocyte macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
Th1	helper T cell
HCV	hepatitis C virus
LD	lactate dehydrogenase
MHC	major histocompatibility complex
mAb	monoclonal antibodies
NTP	neuronal thread protein
ROS	reactive oxygen species

Tregs	T regulatory cells
CD4+	viral specific helper cells

Figure 1. HCV core transfection into DCs

The DCs were isolated from ethanol and pair fed control Balb/c mice using the anti-cd11c+ magnetic microbeads, and transfected with HCV core protein using the Chariot system for two hours. Intracellular staining, as analyzed by flow cytometry, revealed that DCs from both diet groups have similar transfection efficiencies with an average fluorescence emission of 58%.

Figure 2. Immunization with DC-core from mice fed control diet exhibit higher *in vitro* cytotoxicity against HCV core-expressing SP2/19 cells compared to DCs derived from ethanol fed animals

Balb/c mice were immunized (X3 at 2 week interval) with DC-core from control and ethanol pair-fed mice. Splenocytes were isolated from the immunized mice, and incubated with rIL-2 and HCV core transfected DCs from control and ethanol-fed mice, (restimulation step) at a 1 to 100 ratio (DC to splenocytes) for 3 days. Splenocytes from HBSS- and control-immunized mice were incubated with HCV core DCs derived from control-fed mice. Splenocytes were subsequently harvested and incubated with HCV core expressing SP2/19 cells (1×10^4 cells/well) at 1:10, 1:50 and 1:100 ratio (SP2/10 target cell:splenocyte) for 4 hours; Lactate Dehydrogenase release was measured as an index of CTL activity and was comprised of measurements on 8 wells for each experiment which was repeated three times. Significantly higher *in vitro* cytotoxicity was observed in mice fed the control diet and immunized with HCV core transfected DCs compared to mice immunized with DCs derived from ethanol fed mice (* $P < 0.05$). Immunization with HCV core from either control-fed or ethanol-fed mice, however, exhibited higher cytotoxicity against SP2/19 cells compared to mice immunized with HBSS or irrelevant protein (# $P < 0.001$). Experiments were repeated three times with similar results.

Figure 3. Mice immunized with HCV core loaded DCs from ethanol-fed mice exhibit low IL-2 secretion

Splenocytes from mice immunized with HBSS, irrelevant protein, and core loaded DCs derived from control and ethanol fed mice were isolated and incubated with 1 micro g/ml soluble HCV core protein for 24 hours. Splenocytes were harvested and centrifuged; the supernatant was collected for measuring IL-2 via ELISA. IL-2 concentrations were higher in splenocyte culture derived from mice immunized with core loaded DCs from controls compared to splenocytes from mice immunized with core loaded DCs derived from ethanol-fed mice, HBSS, or irrelevant protein (* $P < 0.001$). Each assay was run in triplicate and the experiments were repeated three times. These observations suggest that immunizing mice with ethanol-derived, core-transfected DCs alters CD4⁺ T cell activation (18, 25) against HCV core.

Figure 4. Immunizing mice with HCV core loaded DCs from ethanol-fed mice produces an altered cytokine response

Splenocytes from mice immunized with core loaded DCs from control-fed and ethanol fed mice were incubated with soluble HCV core or OVA protein for 24 hours. The cell supernatant was collected and used for measuring TNF- α and IL-4 levels via ELISA. Mice who received immunizations with core loaded DCs from pair-fed control mice had higher levels of TNF- α and IL-4 compared to mice that received immunizations with core loaded DCs derived from ethanol fed mice (* $P < 0.05$). Splenocytes pulsed with OVA for 24 hours confirmed the HCV core-specific response. Each assay was run in triplicate and the experiments were repeated three times.

Figure 5. CD25⁺FOXP3⁺ Tregs are increased in mice immunized with HCV core loaded DCs derived from ethanol-fed mice

Splenocytes from immunized mice with core loaded DCs derived from control and ethanol-fed mice were isolated and stained for CD25⁺FOXP3⁺ expression before and 24 hours after incubation with 1 micro (μ) g/ml HCV core protein. Incubation with HCV core increased the levels of CD25⁺FOXP3⁺ Treg cells in core-DC-immunized mice from both ethanol and control diet pair fed groups. However, core-DC-immunized mice, derived from chronic ethanol fed mice, had a higher CD25⁺FOXP3⁺ population than mice immunized with HCV core loaded DCs derived from controls; both of which were found to be higher than in mice immunized with saline or irrelevant protein (NTP). Experiments were repeated three times with similar results. At 24 hours, the values were control 75.5, ethanol 85.5, saline 55.4 and irr protein 51.9.

Figure 6. CD4⁺FOXP3⁺ T cells are increased in mice immunized with HCV core loaded DCs derived from chronic ethanol fed mice

Splenocytes from immunized mice with HCV core loaded DCs derived from control and ethanol-fed mice were isolated and stained for CD4⁺FOXP3⁺ expression before (time 0) and 24 hours after incubation with HCV protein (1μg/ml). Incubation with HCV core increased CD4⁺FOXP3⁺ expressing cells in core loaded DC immunized mice derived from both diet groups. However, HCV core loaded DC immunized mice, obtained from chronic ethanol fed mice had a higher CD4⁺FOXP3⁺ expressing population than either core-DC-immunized mice derived from controls or mice immunized with saline or irrelevant protein. Experiments were repeated three times with similar results. At 24 hours, the values were control 73, ethanol 81.5, saline 44.9 and irr protein 43.0.