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Ethanol and Vitamin D Receptor in T cell Apoptosis

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Abstract

Ethanol has been demonstrated to cause T cell apoptosis. In the present study, we evaluated the role of VDR and the renin angiotensin system (RAS) in oxidative stress-induced T cell apoptosis. Ethanol-treated human T cells displayed down regulation of vitamin D receptor (VDR) and the activation of the RAS in the form of enhanced T cell renin expression and angiotensin II (Ang II) production. The silencing of VDR with siRNA displayed the activation of the RAS, and activation of the VDR resulted in the down regulation of the RAS. It suggested that ethanol-induced T cell RAS activation was dependent on the VDR status. T cell ROS generation by ethanol was found to be dose dependent. Conversely, ethanol-induced ROS generation was inhibited if VDR was activated or Ang II was blocked by an angiotensin II type 1 (AT1) receptor blocker (Losartan). Furthermore, it was observed that ethanol not only induced double strand breaks in T cells but also attenuated DNA repair response, whereas, VDR activation inhibited ethanol-induced double strand breaks and also enhanced DNA repairs. Since free radical scavengers inhibited ethanol-induced DNA damage, it would indicate that ethanol-induced DNA damage was mediated through ROS generation. These findings indicated that ethanol-induced T cell apoptosis was mediated through ROS generation in response to ethanol-induced down regulation of VDR and associated activation of the RAS.

Alcoholics are prone to develop bacterial infections (1–3). In addition, the course of infection in alcoholics is more severe and often associated with increased morbidity and mortality (3, 4). Alcohol dependent patients displayed a decrease in peripheral CD4+ve T cells, which recovered after alcohol withdrawal (5–10). Chronic ethanol administration in animals also resulted in reduction of both peripheral and splenic CD4 +ve T cells (11–18). On account of these observations, it has been suggested that alcohol may be modulating the immune status both directly as well as through associated complications such as cirrhosis, malnutrition and neurological abnormalities (2, 19).

Lymphocytes are the major components of the host defense system (20). Both quantitative and qualitative alterations of lymphocytes may contribute to decreased host resistance to infections. Lymphopenia has been reported in most alcoholic patients with leucopenia (21, 22). Ethanol-induced T cell apoptosis might have contributed to lymphopenia to some extent (23). Because apoptotic cells are functionally compromised, ethanol might have affected immune function directly in these patients.

Naive human T cells have been reported to display very low expression of vitamin D receptors (VDR) (24). However, T cells with activated T cell receptors (primed T cells as

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Conflict of Interest

The authors declare they have no conflict of interest.

well as Jurkat T cells) exhibit moderate VDR expression (25). Vitamin D receptors (VDR) mediate most of the biologic effects of 1, 25(OH)₂D₃ (active product of vitamin D) (26, 27). 1, 25(OH)₂D₃ stabilizes the VDR and inhibits its degradation (28). Depending on its cross-linking with vitamin D, VDR shuttles constantly between the nucleus and cytoplasm. Since VDR has been shown to be a negative regulator of renin transcription and NF- κ B signaling, its cellular status depicts indirectly the activity of the RAS (29–31).

Although oxidative stress has been demonstrated to play a role in T cell apoptosis yet the role of ethanol-induced ROS generation in T cell apoptosis has not been studied to date. We hypothesized that ethanol-induced T cell RAS activation contributed to ethanol-induced ROS generation and associated apoptosis. In the present study, we have evaluated the involved mechanisms for T cell loss in ethanol milieu as well as attempted to develop strategies to prevent T cell loss. Ethanol-induced down regulation of VDR led to the activation of the RAS, generation of ROS, and apoptosis of T cells. On the other hand, treatment with a VDR agonist restored T cell expression of VDR, down regulated the RAS as well as ROS generation and thus preserved survival phenotype.

Methods and Material

Human T cells

T cells were isolated from the human blood obtained from healthy volunteers (New York Blood Bank, New York). In brief, peripheral blood mononuclear cells (PBMC) were harvested by the standard technique. T cells were isolated from PBMCs by passing through the T cell column (Invitrogen, Oslo, Norway). T cells were primed before their use (IL-2, 20 units/ml and PHA-P, 5.0ug/ml). Since Jurkat cells (human T cell line, with intact T cell receptor, NIH AIDS Research and Reference Reagent Program, Bethesda, MD) are comparable to primed T cells and they constitutively express PLC-gamma1 (25), we have used them in place of primary primed human T cells. In all experiments, media was replaced with fresh ethanol, at 6 hourly intervals.

TUNEL Assay

TUNEL assay was performed using Apoptosis Detection Tacs TdT Kit (R&D System, Minneapolis, MN) (32). Briefly, the cells were fixed in 3.7% formaldehyde in 1 X PBS. The cells were permeablized in Cytonin solution for 15 min at room temperature and then quenched in 3% H₂O₂ in methanol for 5 min. The cells were washed and treated with TdT labeling buffer followed by PBS washing again. The cells were incubated in Streptavidin-HRP solution for 10 min followed by washing in PBS and incubation in DAB substrate solution for 10 min. The cells were rinsed with H₂O and counterstained with nuclear fast Red. The cover slips were mounted on the slide using aqueous mounting media and observed under light microscope.

Annexin V-PE Staining

Control and experimental T cells were incubated in serum-free media (SFM) for 24 hours. Subsequently, cells were harvested and stained with annexin V-PE and 7-AAD as described in the kit (Annexin V-PE apoptosis detection kit, BD Biosciences Pharmingen, San Diego, CA). The Cells were analyzed by flow cytometry using a FACS-Vantage flow cytometer (BD Biosciences). Apoptotic cells were identified by the presence of annexin V staining in the absence of 7-AAD staining, and necrotic cells were identified by the presence of 7-AAD staining (33).

Immunofluorescence detection of oxidative stress In T cells

The trafficking of 2, 3, 4, and 5, 6-pentafluorodihydroxytetramethylrosamine (PF-HTMRos or Redox Sensor Red CC-1, Molecular Probes, Eugene, OR) was used to detect reactive oxygen intermediates in T cells (19, 20). Redox Sensor Red CC-1 is oxidized in the presence of O_2^- and H_2O_2 . In brief, control and experimental T cells were loaded at 37°C for 20 min with Redox Sensor Red CC-1 (0.5 μ M) and a mitochondria-specific dye, MitoTracker greenFM (100 nM; Molecular probes, Eugene, OR). Culture slides were washed and mounted with PBS buffer and visualized with Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and charge-coupled device (CCD) camera (Nikon DXM1200). The staining was performed in quadruplicate for each group and 10 random fields were studied in replicate. Images were captured using Nikon ACT-1 (version 1.12) software and combined for publishing format using Adobe Photoshop 6.0 software.

Determination of ROS Kinetics in T Cells

The kinetics of ROS metabolism in T cells was determined by measuring the intensity of the fluorescent signal from the redox-sensitive fluoroprobe 2', 7'-dichlorofluorescein diacetate (DCFDA) at multiple time points. Briefly, cells were incubated in phenol red-free media containing 10mM DCFDA for 30 minutes at 37°C. Cells were washed with phenol red-free media and treated with either vehicle of ethanol and then DCF fluorescence was detected by a Fluorescence Multi-Well Plate Reader CytoFluor 4000 (PerSeptive Biosystems) set for excitation of 485 nm and emission of 530 nm. The intensity of the fluorescent signal was calculated with Microsoft excel using equation $\{(F_t - F_0)/F_0 \times 100\}$.

Immunofluorescence detection of VDR, DNA strand breaks and repairs

Control and experimental T cells were fixed and permeabilized with a buffer containing 0.02% Triton X-100 and 4% formaldehyde in PBS. Fixed cells were washed three times in PBS and blocked in 1% BSA for 30 min at 37°C. Phospho-histone H2AX (γ H2AX) was detected by a monoclonal antibody that recognizes phosphorylated serine within the amino acid sequence 134–142 of human histone H2A.X (UBI) and FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes). KU80, a DNA repair protein was detected by rhodamine-conjugated anti-KU80 antibody (Cell Signaling, Danvers, MA). Double labeling was indicated by orange color. Negative controls were performed in the presence of non-specific isotype antibodies in place of primary antibody. In all variables, DNA was counterstained with 4',6'-diamidino-2-phenylindole (DAPI).

Specific staining was visualized with an inverted Olympus IX 70 fluorescence microscopes equipped with a Cook Sensicom ER camera (Olympus America, Melville, NY). Final images were prepared with Adobe Photoshop to demonstrate subcellular localization of γ H2AX (32).

Silencing of VDR

Jurkat cells were transfected with 100 nM VDR siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) with Siport Neofax transfection reagent and left in optiMEM media for 24 hrs. Control and transfected cells were used under control and experimental conditions.

Ang II ELISA

Ang II levels were determined in control and experimental cells using commercial ELISA kits (Peninsula Laboratories, Belmont, CA) as described by the manufacturer. Briefly, Ang II was extracted with 20 mM Tris buffer, pH 7.4 and partially purified and concentrated after filtering through Centricon Filters (MW cut off 10,000, Millipore, Billerica, MA).

Western blotting studies

T cells were lysed in RIPA buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.25% Deoxycholate, 0.1% SDS, 1X protease inhibitor cocktail I (Calbiochem, EMD Biosciences, Gibbstan, NJ), 1mM PMSF, and 0.2mM sodium orthovanadate. Protein concentration was measured with the Biorad Protein Assay kit (Pierce, Rockford, IL). Protein lysates (20 µg) were separated on a 15 % polyacrylamide gels (PAGE, Bio-Rad, Hercules, CA) and transferred onto a nitrocellulose membrane using Bio-Rad miniblott apparatus. Nitrocellulose membranes were then subjected to immunostaining with primary antibodies against VDR (mouse monoclonal, Santa Cruz) and Renin (rabbit polyclonal, Santa Cruz), and subsequently with horseradish peroxidase labeled appropriated secondary antibodies. The blots were developed using a chemiluminescence detection kit (PIERCE, Rockford, IL) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY). Equal protein loading was confirmed by stripping the blot and reprobing it for actin protein using a polyclonal α -Actin antibody (Santa Cruz Technology, Santa Cruz, CA) on the same Western blots.

Statistical analysis

For comparison of mean values between two groups, the unpaired t test was used. To compare values between multiple groups, analysis of variance (ANOVA) was used to calculate a p-value. Statistical significance was defined as $P < 0.05$. Results are presented and mean $\pm$ SD

Results

Ethanol down regulates T cell VDR

To determine the effect of ethanol on T cell VDR, we evaluated dose response effect of ethanol on T cell VDR expression. T cells were incubated in variable concentrations of ethanol (0, 10, 25, and 50 mM) for 24 hours. Cellular lysates were electrophoresed and probed for VDR and actin. Representative gels are shown in Fig. 1A. Ethanol down regulated VDR expression at a concentration of 25 mM and higher. Cumulative data are shown in bar graphs (Fig. 1A)

To determine the effect of ethanol on Jurkat cell VDR expression, Jurkat cells were incubated in media containing either buffer or ethanol (25 mM) for 24 hours. Immunoblots were probed for VDR and actin. Representative gels from three different sets of experiments are shown in Fig. 1B. Ethanol down regulated Jurkat cell VDR expression. Cumulative data (n=3) are shown in a bar diagram.

To determine the effect of VDR agonist (VD), Jurkat cells incubated either in buffer or ethanol + variable concentrations of EB 1089 (a VDR agonist, 0, 10, 50 nM) for 24 hours. Western blots were probed for VDR and actin. Representative gels are shown (Fig. 1C). VD at a concentration of 10 nM or higher upregulated VDR in ethanol-treated cells.

Ethanol up regulates renin angiotensin system (RAS) through down regulation of VDR

To study the effect of ethanol on T cell RAS status, T cells were incubated in media containing either buffer or variable concentrations of ethanol (0, 10, 25, and 50 mM) for 24 hours. Western blots were probed for renin. The same blots were stripped and reprobed for actin. Representative gels are shown in Fig. 2A.

To determine the effect of ethanol on T cell Ang II production, T cells were incubated in serum-free media containing either buffer or ethanol (25 and 100 mM) for 24 hours. Cellular lysates under control and experimental conditions were assayed for their Ang II levels by

ELISA. Results (means $\pm$ SD) representing 3 sets of experiments are shown in Fig. 2B. Ethanol stimulated ($P<0.01$) T cell Ang II production by four- to five-fold.

Lack of VDR is critical for T cell production of Ang II

To establish cause and effect relationship between lack of VDR and T cell Ang II production, cellular lysates of control T cells, T cells transfected with siRNA-VDR, and T cells transfected with scrambled siRNA(SCR-siRNA) were assayed for their Ang II content by ELISA ($n=4$). Cumulative data showing the effect of VDR silencing on T cell Ang II production are shown in Fig. 2C. T cells lacking VDR displayed 7-fold increase in Ang II production when compared to control cells.

Ethanol stimulates T cell ROS generation

To determine the effect of ethanol on T cell ROS generation, Jurkat cells were loaded with DCFDA and then treated with ethanol (0, 25, 50, 100 mM), followed by measurement of ROS generation at the indicated time periods. Ethanol stimulated T cell ROS generation in a dose dependent manner (Fig. 3A).

VDR down regulation is associated with T cell ROS generation

To determine role of VDR in T cell ROS generation, T cells transfected with siRNA-VDR, and scrambled siRNA (SCR-siRNA) were loaded with DCFDA, followed by indicated treatment and subsequent measurement of ROS generation at the indicated time periods. Ethanol stimulated T cell ROS generation (Fig. 3B). T cells silenced for VDR displayed mild increase in ROS generation. Interestingly, T cells silenced for VDR and treated with ethanol displayed increased ROS generation when compared to cells treated with ethanol alone (Fig. 3B).

VD diminishes ethanol-induced T cell ROS generation

We hypothesized that if down regulation of VDR causing T cell ROS generation then up regulation VDR should attenuate ethanol-induced T cell ROS generation. To test our hypothesis, Jurkat cells were incubated in media containing either buffer or EB1089 (10 nM, VD) for 4 hours and then loaded with DCFDA for 30 minutes. Subsequently, cells were treated with either vehicle or ethanol, followed by ROS generation at the indicated time periods. As shown in Fig. 3C, Ethanol stimulated ($P<0.001$) T cell ROS generation; however, this effect of ethanol was attenuated ($P<0.01$) by VD.

RAS blockade attenuates ethanol-induced ROS generation

To determine the role of the RAS, Jurkat cells were incubated in media containing either buffer or losartan (10^{-7} M) for 4 hours and then loaded with DCFDA for 30 min. Subsequently, cells were treated with either buffer or ethanol (25 mM), followed by measurement generation at the indicated time periods. Ethanol stimulated ($P<0.001$) T cell ROS generation (Fig. 3D); however, this effect of ethanol was attenuated ($P<0.01$) by losartan.

VDR activation and RAS blockade inhibit ethanol-induced T cell ROS generation

To confirm a causal relationship between VDR/RAS and ethanol-induced T cell ROS generation, Jurkat cells pretreated with VD and losartan were incubated in media containing either buffer or ethanol. Subsequently, cells were co-labeled with Red CC1 and Mitotracker green. Representative microphotographs are shown in Fig. 4A. Ethanol treated Jurkat cells displayed mitochondrial generation of ROS. However, both VD, and losartan attenuated ethanol-induced ROS generation

Effect of VDR activation, RAS blockade, and antioxidants on ethanol-induced T cell double strand breaks and DNA repair

To determine the effect of VD, RAS blockade and free radical scavengers on ethanol-induced DNA damage, Jurkat cells were pretreated either with buffer, or VD (EB1089, 10 nM), losartan (10^{-7} M), catalase (500 U) or tempol (1mM, a membrane permeable superoxide scavenger) for 4 hours followed by ethanol treatment for 12 hours. Subsequently, control and experimental cells were co-labeled for H2AX (double strand breaks) and KU80 (NHEJ, DNA repair protein) expression. Ethanol enhanced double strand breaks in Jurkat cells and displayed only moderate expression of KU80 (Fig. 4B). On the other hand, VD, losartan, catalase, and tempol enhanced expression of KU80 in ethanol treated cells. These findings indicated that down regulation VDR is associated with inadequate DNA repair response, whereas the activation of VDR by VD is associated with diminished double strand breaks and enhanced DNA repair response. Since both catalase and tempol not only prevented ethanol-induced DNA damage but also enhanced DNA repair, it appears that ethanol induced DNA damage was mediated through ROS generation.

Ethanol enhances Jurkat cell apoptosis

Jurkat cells were incubated in serum free media containing either buffer or variable concentrations of ethanol (25, 50, and 100 mM) for 24 hours. Subsequently, cells were prepared for TUNEL assay. Ethanol treated T cells displayed greater number of TUNEL +ve cells (Fig. 5 A)

Role of VDR activation and RAS blockade on ethanol-induced T cell apoptosis

To establish a causal relationship between VDR/Ang II and induction of ethanol-induced T cell apoptosis, Jurkat cells were pretreated with either VD or losartan, followed by treatment with either buffer or ethanol. Subsequently cells were assayed for apoptosis by TUNEL assay. Representative microphotographs and cumulative data of control, ethanol, ethanol +VD-, and ethanol + Losartan- treated Jurkat cells are shown in Fig 5B. Ethanol promoted ($P<0.01$) T cell apoptosis, however this effect of ethanol was inhibited by VD and losartan (Fig. 5B).

Effect of ethanol on human T cells—Since primary T cells are more sensitive to ethanol effects, we evaluated the effect of ethanol on freshly isolated T cells. T cells were incubated with either buffer or variable concentrations of ethanol (25, 50, and 100 mM) for 24 hours and assayed for apoptosis by flow cytometry. Ethanol stimulated apoptosis in 30 to 50% of cells (Fig. 6A). Representative flow scans are shown as insets. Cells treated under similar conditions were also assayed by TUNEL assay. Thirty to 48% percent of cells were TUNEL +ve after ethanol treatment (Fig. 6B). To determine the effect of VD and losartan on ethanol-induced T cell apoptosis, freshly isolated T cells were pretreated with either VD or losartan, followed by treatment with either buffer or ethanol. Subsequently cells were assayed for apoptosis by TUNEL assay. Ethanol stimulated apoptosis in T cells, however this effect of ethanol was inhibited both by VD and losartan (Fig. 6C).

Discussion

In the present study, ethanol induced down regulation of T cell VDR expression; the latter led to the enhanced T cell activation of the RAS in the form of increased T cell expression of renin and increased T cell Ang II production. Since T cells silenced for VDR also displayed an increase in their Ang II content, it appeared that ethanol-induced RAS activation was mediated through down regulation of VDR. Ethanol also enhanced T cell ROS generation in a dose dependent manner. Jurkat cells silenced for VDR displayed enhanced ROS generation both under basal and ethanol-stimulated states. Since both VD

and losartan inhibited ethanol-induced ROS generation, it confirmed that ethanol-induced ROS production was mediated through down regulation of VDR and up regulation of the RAS. Since anti-oxidants inhibited ethanol-induced DNA damage, it suggested that ethanol-induced DNA damage was mediated through ROS generation. Both VD and LOS not only attenuated ethanol-induced DNA damage but also inhibited ethanol-induced T cell apoptosis; these findings indicated that ethanol-induced T cell apoptosis was mediated through ROS generation in response to ethanol-induced down regulation of VDR and associated activation of the RAS.

Clinical and experimental reports on alcohol-induced ROS generation in T cells are scanty (34). Nonetheless, the effect of alcohol on liver cell ROS generation has been studied extensively (35–42). Acute intragastric infusion of alcohol in animals resulted in enhanced lipid peroxidation, formation of the 1-hydroxyethyl radical and lipid radicals, and decrease in the hepatic antioxidant defense, particularly GSH levels (35–39). Moreover, severity of liver cell injury further worsened after addition of increased amount of iron (enhanced hydroxyl radical formation) in their diets (40). On the other hand, the addition of antioxidants prevented the development of liver cell injury. In *in vitro* studies in liver cells, revealed enhanced ROS generation in response to alcohol and associated cellular injury; on the other hand, alcohol-induced liver cell injury could be blocked by anti-oxidants (41, 42). In the present study, anti-oxidants also inhibited alcohol-induced T cell DNA damage. Thus, our findings are consistent with these investigators.

The electron transport chain of mitochondria is the major source of intracellular ROS in the cell (43,44). We previously reported that ethanol translocated T cell mitochondrial cytochrome C to the cytosol (23). In these studies ethanol not only decreased the T cell expression of Bcl-2 but also promoted the expression of Bax. Moreover, an inhibitor of caspase-9 attenuated the ethanol-induced T cell apoptosis. In the present study, ethanol-induced T cell ROS generation was also predominantly localized to mitochondria. Chiramonte et al., evaluated direct effect of H₂O₂ on the induction of Jurkat cells apoptosis (45). These investigators demonstrated that H₂O₂ induced Jurkat cell apoptosis through down regulation of Bcl₂. In *in vivo* studies, alcohol binge drinking also promoted T cell apoptosis through attenuation of T cell Bcl₂ expression and cleavage of caspase-3 as reported earlier (23).

Oxidative pathway of alcohol is well characterized in liver cells (35–42). Alcohol dehydrogenase, cytochrome P450 2E1 (CYP2E1) and catalase contribute to oxidative metabolism of alcohol. Usually cytosolic alcohol dehydrogenase converts ethanol to acetaldehyde by involving nicotinamide adenine dinucleotide (NAD⁺), which is reduced by two electrons to form NADH; however, at higher ethanol concentrations, microsomal CYP2E1 converts ethanol to acetaldehyde. Peroxisomal catalase also oxidizes alcohol to acetaldehyde in the presence of H₂O₂. Acetaldehyde is metabolized to acetate and NADH in mitochondria by aldehyde dehydrogenase2. However, alcoholic metabolism is not so well studied in T cells. Nonetheless, we have previously reported involvement of mitochondrial dysfunction in alcohol-induced T cell apoptosis (23). Moreover, in the present study, we observed that mitochondria was the major site for ROS generation in ethanol milieu. However, in the present study we implicated Ang II for mitochondrial ROS generation in response to ethanol. Nonetheless, it did not exclude the direct effect of ethanol in mitochondrial ROS generation. Thus, it will be important to evaluate the direct effect of ethanol on T cell ROS generation in future studies.

Ang II has been reported to induce mitochondrial dysregulation in heart, kidney, and vascular smooth muscle cells (46–49). Dysregulation of mitochondria led to enhanced production of ROS (50, 51). Direct effect of Ang II on isolated mitochondria and intact

endothelial cells revealed that Ang II increased mitochondrial H₂O₂ production (52); this increase was blocked both protein kinase C inhibitor and NADPH oxidase inhibitor, mitochondrial ATP-sensitive potassium channel inhibitor. These investigators suggested that Ang II induced mitochondrial dysfunction via protein kinase C dependent pathway by activating endothelial cell NADPH oxidase (52). It will be interesting to evaluate these effects of Ang II on T cell mitochondrial functions in future studies.

Enhanced intracellular ROS generation carry a number of direct and indirect outcomes in the form of apoptosis or necrosis (53). ROS-induced protein modifications can alter either signaling pathways or cellular function or both (54). ROS can directly modify signaling proteins through nitrosylation, carbonylation, disulphide bond formation and glutathionylation (55). These modifications alter a protein's activity in general and events involved in cell death particular (56). In the present study, ROS not only directly induced DNA damage but also altered cellular signaling, which compromised DNA repair. The net outcome of these events was displayed in the form of T cell apoptosis.

Apoptosis and autophagy constitute the two processes through which unwanted cells or organelles are eliminated (57–61). Autophagy is a highly regulated process that is involved in the turnover of damaged proteins, whole organelles or distinct organelles (59, 60); whereas, apoptosis removes damaged or unwanted cells. It appears that the same stimuli can induce either autophagy or apoptosis depending on the threshold of the stimuli (57). For an example hydrogen peroxide at lower concentrations will stimulate autophagy and at higher concentrations will induce apoptosis (62). Interestingly, von Haefen et al demonstrated that ethanol promoted apoptosis by inhibiting autophagy in Jurkat cells (63). Theoretically, ethanol-induced ROS generation expected to stimulate autophagy so that oxidized proteins can be degraded. Nonetheless, it appears that T cells in ethanol milieu are in double jeopardy-overwhelming oxidative stress and compromised ability to degrade oxidized proteins and damage organelles, which seemed to have promoted apoptosis. Recently, VD has been reported to enhance autophagy in macrophages (64), we speculate that VD induced inhibition of ethanol-induced apoptosis might have also contributed by its pro-autophagic effects. It will be worthwhile to carry out these studies in future.

In conclusion, the present study not only delineates the involved molecular mechanism in ethanol-induced T cell loss but also provides opportunities to develop therapeutic strategies to provide protection against T cell loss in ethanol milieu.

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References

1. Adams HG, Jordan C. Infections in the alcoholic. *Med Clin North Am.* 1984; 68:179–200. [PubMed: 6361412]
2. MacGregor RR. Alcohol and immune defense. *JAMA.* 1986; 256:1474–1479. [PubMed: 3747066]
3. Young CL, MacGregor RR. Alcohol and host defenses: infectious consequences. *Infect Med.* 1980; 6:163–75.
4. Capps JA, Coleman GH. Influence of alcohol on prognosis of pneumonia in Cook County Hospital: a statistical report. *JAMA.* 1923; 80:750–2.
5. Young GP, Van der Weyden MB, Ros IS, Dudley FJ. Lymphopenia and lymphocyte transformation in alcoholics. *Experientia.* 1979; 35:268–269. [PubMed: 421856]
6. Roselle GA, Mendenhall CL, Grossman CJ, Weesner RE. Lymphocyte subset alterations in patients with alcoholic hepatitis. *J Clin Lab Immunol.* 1988; 26:169–173. [PubMed: 3199427]

7. Pol S, Artru P, Thepot V, Berthelot P, Nalpas B. Improvement of the CD4⁺ cell count after alcohol withdrawal in HIV-positive alcoholic patients. *AIDS*. 1996; 10:1293–1294. [PubMed: 8883595]
8. Laso FJ, Madruga JI, Lopez A, Ciudad J, Alvarez-Mon M, San Miguel J. Distribution of peripheral blood lymphoid subsets in alcoholic liver cirrhosis: influence of ethanol intake. *Alcohol Clin Exp Res*. 1996; 20:1564–1568. [PubMed: 8986204]
9. Gheorghiu M, Bara C, Pasarica D, Brasoveanu L, Bleotu C, Toparceanu F. Ethanol-induced dysfunction of hepatocytes and leukocytes in patients without liver failure. *Roum Arch Microbiol Immunol*. 2004; 63:5–33. [PubMed: 16295318]
10. Kazbariene B, Krikstaponiene A, Monceviute-Eringiene E. Disturbance of human immunohomeostasis by environmental pollution and alcohol consumption. *Acta Microbiol Immunol Hung*. 2006; 53:209–218. [PubMed: 16956130]
11. Helm RM, Wheeler G, Burks AW, Hakkak R, Badger TM. Flow cytometric analysis of lymphocytes from rats following chronic ethanol treatment. *Alcohol*. 1996; 13:467–471. [PubMed: 8888943]
12. Chadha KC, Stadler I, Albin B, Nakeeb SM, Thacore HR. Effect of alcohol on spleen cells and their functions in C57BL/6 mice. *Alcohol*. 1991; 8:481–485. [PubMed: 1723602]
13. Shellito JE, Olariu R. Alcohol decreases T-lymphocyte migration into lung tissue in response to *Pneumocystis carinii* and depletes T-lymphocyte numbers in the spleens of mice. *Alcohol Clin Exp Res*. 1998; 22:658–663. [PubMed: 9622447]
14. Peterson JD, Herzenberg LA, Vasquez K, Waltenbaugh C. Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc Natl Acad Sci U S A*. 1998; 17:3071–3076. [PubMed: 9501217]
15. Saad AJ, Jerrells TR. Flow cytometric and immunohistochemical evaluation of ethanol-induced changes in splenic and thymic lymphoid cell populations. *Alcohol Clin Exp Res*. 1991; 15:796–803. [PubMed: 1755511]
16. Jerrells TR, Saad AJ, Domiati-Saad R. Effects of ethanol on parameters of cellular immunity and host defense mechanisms to infectious agents. *Alcohol*. 1992; 9:459–463. [PubMed: 1472300]
17. Padgett EL, Sibley DA, Jerrells TR. Effect of adrenalectomy on ethanol-associated changes in lymphocyte cell numbers and subpopulations in thymus, spleen, and gut-associated lymphoid tissues. *Int J Immunopharmacol*. 2000; 22:285–298. [PubMed: 10689102]
18. Louria DB. Susceptibility to infection during experimental alcohol intoxication. *Trans Assoc Am Physicians*. 1963; 76:102–10.
19. Liu YK. Leukopenia in alcoholics. *Am J Med*. 1973; 54:605–10. [PubMed: 4701945]
20. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol*. 2011; 30:16–34. [PubMed: 21235323]
21. Lindenbaum J, Hargrove L. Thrombocytopenia in alcoholics. *Ann Intern Med*. 1968; 68:526–32. [PubMed: 5643676]
22. McFarland W, Libre EP. Abnormal leukocyte response in alcoholism. *Ann Intern Med*. 1963; 59:865–77. [PubMed: 14082738]
23. Kapasi AA, Patel G, Goenka A, Nahar N, Modi N, Bhaskaran M, Reddy K, Franki N, Patel J, Singhal PC. Ethanol promotes T cell apoptosis through the mitochondrial pathway. *Immunology*. 2003; 108:313–20. [PubMed: 12603597]
24. von Essen MR, Kongsbak M, Schjerling P, Olgaard K, Odum N, Geisler C. Vitamin D controls T cell antigen receptor signaling and activation of human T cells. *Nat Immunol*. 2010; 11:344–9. [PubMed: 20208539]
25. Irvin BJ, Williams BL, Nilson AE, Maynor HO, Abraham RT. Pleiotropic contributions of phospholipase C- γ 1 (PLC- γ 1) to T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC- γ 1-deficient Jurkat T-cell line. *Mol Cell Biol*. 2000; 20:9149–9161. [PubMed: 11094067]
26. Christakos S, Dhawan P, Benn B, Porta A, Hediger M, Oh GT, Jeung EB, Zhong Y, Ajibade D, Dhawan K, Joshi S. Vitamin D: molecular mechanism of action. *Ann N Y Acad Sci*. 2007; 1116:340–8. [PubMed: 18083936]
27. White JH. Vitamin D metabolism and signaling in the immune system. *Rev Endocr Metab Disord*. 2012; 13:21–9. [PubMed: 21845364]

28. Costa EM, Feldman D. Measurement of 1,25-dihydroxyvitamin D₃ receptor turnover by dense amino acid labeling: changes during receptor up-regulation by vitamin D metabolites. *Endocrinology*. 1987; 120:1173–1178. [PubMed: 3026787]
29. Li YC, Kong J, Wei M, Chen ZF, Liu SQ, Cao LP. 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. *J Clin Invest*. 2002; 110:229–38. [PubMed: 12122115]
30. Li YC, Qiao G, Uskokovic M, Xiang W, Zheng W, Kong J. Vitamin D: a negative endocrine regulator of the renin-angiotensin system and blood pressure. *J Steroid Biochem Mol Biol*. 2004; 89–90:387–92.
31. Qi X, Pramanik R, Wang J, Schultz RM, Maitra RK, Han J, DeLuca HF, Chen G. The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D₃-induced growth inhibition. *J Biol Chem*. 2002; 277:25884–25892. [PubMed: 11983707]
32. Husain M, Meggs LG, Vashistha H, Simoes S, Griffiths KO, Kumar D, Mikulak J, Mathieson PW, Saleem MA, Del Valle L, Pina-Oviedo S, Wang JY, Seshan SV, Malhotra A, Reiss K, Singhal PC. Inhibition of p66ShcA longevity gene rescues podocytes from HIV-1-induced oxidative stress and apoptosis. *J Biol Chem*. 2009; 284:16648–58. [PubMed: 19383602]
33. Salhan D, Pathak S, Husain M, Tandon P, Kumar D, Malhotra A, Meggs LG, Singhal PC. HIV gene expression deactivates redox-sensitive stress response program in mouse tubular cells both in vitro and in vivo. *Am J Physiol Renal Physiol*. 2012; 302(1):F129–40. [PubMed: 21993884]
34. Hoch NE, Guzik TJ, Chen W, Deans T, Maalouf SA, Gratz P, Weyand C, Harrison DG. Regulation of T-cell function by endogenously produced angiotensin II. *Am J Physiol Regul Integr Comp Physiol*. 2009; 296:R208–R216. [PubMed: 19073907]
35. Knecht KT, Adachi Y, Bradford BU, Iimuro Y, Kadiiska M, Xuang QH, Thurman RG. Free radical adducts in the bile of rats treated chronically with intragastric alcohol. *Molecular Pharmacology*. 1995; 47:1028–1034. [PubMed: 7746269]
36. Tsukamoto H, Lu SC. Current concepts in the pathogenesis of alcoholic liver injury. *FASEB Journal*. 2001; 15:1335–1349. [PubMed: 11387231]
37. Iimuro Y, Bradford BU, Yamashina S, Rusyn I, Nakagami M, Enomoto N, Kono H, Frey W, Forman D, Brenner D, Thurman RG. The glutathione precursor L-2-oxothiazolidine-4-carboxylic acid protects against liver injury due to chronic enteral ethanol exposure in the rat. *Hepatology*. 2000; 31:391–398. [PubMed: 10655262]
38. Nanji AA, Zhao S, Sadrzadeh SM, Dannenberg AJ, Tahan SR, Waxman DJ. Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-treated ethanol-fed rats. *Alcoholism: Clinical and Experimental Research*. 1994; 18:1280–1285.
39. Morimoto M, Zern MA, Hagbjörk AL, Ingelman-Sundberg M, French SW. Fish oil, alcohol, and liver pathology: Role of cytochrome P450 2E1. *Proceedings of the Society for Experimental Biology and Medicine*. 1994; 207:197–205. [PubMed: 7938050]
40. Tsukamoto H, Horne W, Kamimura S, Niemelä O, Parkkila S, Ylä-Herttuala S, Brittenham GM. Experimental liver cirrhosis induced by alcohol and iron. *J Clin Invest*. 1995; 96:620–630. [PubMed: 7615836]
41. Adachi M, Ishii H. Role of mitochondria in alcoholic liver injury. *Free Radical Biology & Medicine*. 2002; 32:487–491. [PubMed: 11958949]
42. Bailey SM, Cunningham CC. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radical Biology & Medicine*. 2002; 32:11–16. [PubMed: 11755312]
43. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J*. 1973; 134:707–716. [PubMed: 4749271]
44. Boveris A, Cadenas E. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Lett*. 1975; 54:311–314. [PubMed: 236930]
45. Chiamonte R, Bartolini E, Riso P, Calzavara E, Erba D, Testolin G, Comi P, Sherbet GV. Oxidative Stress Signalling in the Apoptosis of Jurkat T-Lymphocytes. *Journal of Cellular Biochemistry*. 2001; 82:437–444. [PubMed: 11500920]

46. de Cavanagh EM, Piotrkowski B, Basso N, Stella I, Inserra F, Ferder L, Fraga CG. Enalapril and losartan attenuate mitochondrial dysfunction in aged rats. *FASEB J*. 2003; 17: 1096–1098. [PubMed: 12709417]
47. de Cavanagh EM, Toblli JE, Ferder L, Piotrkowski B, Stella I, Inserra F. renal mitochondrial dysfunction in spontaneously hypertensive rats is attenuated by losartan but not by amlodipine. *Am J Physiol Regul Integr Comp Physiol*. 2006; 290:R1616–R1625. [PubMed: 16410402]
48. Monteiro P, Duarte AI, Goncalves LM, Providencia LA. Valsartan improves mitochondrial function in hearts submitted to acute ischemia. *Eur J Pharmacol*. 2005; 518:158–164. [PubMed: 16055115]
49. Kimura S, Zhang GX, Nishiyama A, Shokoji T, Yao L, Fan YY, Rahman M, Abe Y. Mitochondria-derived reactive oxygen species and vascular MAP kinases: comparison of angiotensin II and diazoxide. *Hypertension*. 2005; 45:438–444. [PubMed: 15699441]
50. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, Utsumi H, Hamasaki N, Takeshita A. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res*. 2001; 88:529–535. [PubMed: 11249877]
51. Iuchi T, Akaike M, Mitsui T, Ohshima Y, Shintani Y, Azuma H, Matsumoto T. Glucocorticoid excess induces superoxide production in vascular endothelial cells and elicits vascular endothelial dysfunction. *Circ Res*. 2003; 92:81–87. [PubMed: 12522124]
52. Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ Res*. 2008 Feb 29; 102(4):488–96. [PubMed: 18096818]
53. Fuertes MA, Castillab J, Alonso C, Perez JM. Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. *Curr Med Chem*. 2003; 10:257–266. [PubMed: 12570712]
54. Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signaling. *Biochem J*. 2012; 441:523–540. [PubMed: 22187934]
55. Brigelius-Flohé RFlohé L. Basic Principles and Emerging Concepts in the Redox Control of Transcription Factors. *Antioxid Redox Signal*. 2011; 15:2335–2381. [PubMed: 21194351]
56. Suen D-F, Norris KL, Youle RJ. Mitochondrial dynamics and apoptosis. *Genes Dev*. 2008; 22:1577–1590. [PubMed: 18559474]
57. Galluzzi L, Morselli E, Vicencio JM, Kepp O, Joza N, Tajeddine N, Kroemer G. Life, death and burial: multifaceted impact of autophagy. *Biochem Soc Trans*. 2008; 36:786–90. [PubMed: 18793137]
58. Gozuacik D, Kimchi A. Autophagy and cell death. *Curr Top Dev Biol*. 2007; 78:217–245. [PubMed: 17338918]
59. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. *Int J Biochem Cell Biol*. 2004; 36:2445–2462. [PubMed: 15325584]
60. Mizushima N. Autophagy: process and function. *Genes Dev*. 2007; 21:2861–2873. [PubMed: 18006683]
61. Baehrecke EH. Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol*. 2005; 6:505–510. [PubMed: 15928714]
62. Yadav A, Vallabu S, Arora S, Tandon P, Slahan D, Teichberg S, Singhal PC. Ang II promotes autophagy in podocytes. *Am J Physiol Cell Physiol*. 2010; 299:C488–96. [PubMed: 20484657]
63. von Haefen C, Siffringer M, Menk M, Spies CD. Ethanol enhances susceptibility to apoptotic cell death via down-regulation of autophagy-related proteins. *Alcohol Clin Exp Res*. 2011; 35:1381–91. [PubMed: 21410490]
64. Yuk JM, Shin DM, Lee HM, Yang CS, Jin HS, Kim KK, Lee ZW, Lee SH, Kim JM, Jo EK. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe*. 2009; 6:231–43. [PubMed: 19748465]

Figure 1. Ethanol down regulates T cell VDR

A. Primed T cells were incubated in media containing variable concentrations of ethanol (0, 10, 25, and 50 mM) for 24 hours (n=3). Immunoblots were probed for VDR. The same blots were restriped and probed for actin. A representative gel is shown. The upper lane shows VDR expression by control and ethanol treated cells. The lower lane displays cellular actin content under similar conditions. Cumulative data from three different sets of experiments are shown in bar graphs. *P<0.01 compared to ethanol 0 and 10 mM.

B. Jurkat cells were incubated in media containing either buffer or ethanol (25 mM) for 24 hours. Cellular lysates were electrophoresed and probed for VDR and actin. Representative gels from three different sets of experiments are shown. Cumulative data (n=3) are shown in a bar diagram. *P<0.01 compared to control.

C. Jurkat cells were incubated either in buffer (control, C1 and C2) or ethanol + variable concentrations of EB20189 (0, 10, or 50 nM; VD1/0 and VD2/0; VD1/10 and VD2/10; VD1/50 and VD2/50) for 24 hours. Immunoblots were probed for VDR and actin. Representative gels of two sets of experiments are shown. The upper lane shows VDR expression by control and experimental cells. The lower lane displays cellular actin content under similar conditions.

Figure 2. Ethanol up regulates renin angiotensin system (RAS) through down regulation of VDR

A. T cells were incubated in media containing either buffer or variable concentrations of ethanol (0, 10, 25, and 50 mM) for 24 hours. Cellular lysates were electrophoresed and probed for renin and actin. Representative gels are shown. The upper lane shows renin expression by control and experimental cells. The lower lane displays cellular actin content under similar conditions.

B. Jurkat cells were incubated in serum-free media containing either buffer or ethanol (25 and 100 mM) for 24 hours. Subsequently, cellular lysates were assayed for their Ang II levels by ELISA. Results (means $\pm$ SD) representing 3 sets of experiments are shown.

* $P < 0.01$ compared to control.

C. Jurkat cells (control), Jurkat cells transfected with siRNA-VDR, and Jurkat cells transfected with scrambled siRNA (SCR-siRNA) were assayed for their Ang II content by ELISA ($n=4$). * $P < 0.001$ compared to control and SCR/siRNA.

Figure 3. VD modulates ethanol-induced ROS generation

A. To determine the effect of ethanol on T cell ROS generation, Jurkat cells were loaded with DCFDA, followed by treatment with ethanol (0, 25, 50, 100 mM; control, ETH25, ETH50, ETH100) and measurement of ROS generation at the indicated time periods (every 10 minutes) by a fluorometer. * $P < 0.00$ Compared to control; ** $P < 0.05$ compared ETH, 100 mM; *** $P < 0.1$ compared to control and ETH, 100 mM.

B. Jurkat cells transfected with siRNA-VDR and Jurkat cells transfected with scrambled siRNA (SCR-siRNA) and then loaded with DCFDA for 30 min. After washing, cells were treated with either buffer or ethanol (25 mM) and then ROS generation was measured by a fluorometer (at every 10 minutes). * $P < 0.05$ compared to control; ** $P < 0.01$ compared to control; *** $P < 0.05$ compared to ethanol.

C. Jurkat cells were incubated in media containing either buffer or EB1089 (10 nM, VD) for 4 hours and then loaded with DCFDA. Thirty minutes later, cells were treated with either buffer or ethanol and then followed by measurement of ROS generation at the indicated time periods. * $P < 0.001$ compared to control and VD alone. ** $P < 0.01$ compared to ETH, control and VD alone.

D. Jurkat cells were incubated in media containing either buffer or losartan (10^{-7} M) for 4 hours and then loaded with DCFDA. Subsequently, cells were treated with either buffer or ethanol (25 mM) followed by measurement of ROS generation at the indicated time periods. * $P < 0.001$ compared to control and LOS alone. ** $P < 0.01$ compared to ETH, control and LOS alone.

Figure 4. VDR activation and RAS blockade inhibit ethanol-induced mitochondrial T cell ROS generation and DNA damage/repair A

Jurkat cells pretreated with VD (EB 1089, 10 nM) and losartan (LOS, 10⁻⁷M) were incubated in media containing either buffer or ethanol (25 mM). Subsequently, cells were co-labeled with Red CC1 and Mitotracker green. Representative microphotographs are shown. Ethanol treated Jurkat cells displayed mitochondrial generation of ROS (indicated by bright red fluorescence in REDCC-1 panel and orange/yellow fluorescence in merged panel). Both VD and LOS diminished intensity of fluorescence in ethanol treated cells.

B. Jurkat cells were pre-incubated in serum free media containing either buffer, or VD (EB1089, 10 nM), losartan (LOS, 10⁻⁷M), catalase (CAT 500 U) or tempol (TEMP, 1mM) for 4 hours. Subsequently, aliquot of ethanol was added (final concentration 25 mM) and incubation was continued for another 12 hours. Subsequently, cells were co-labeled for anti-H2AX and KU80 antibodies. Representative microphotographs are shown. Control cells displayed minimal double strand breaks (green fluorescence) and DNA repair proteins (red fluorescence). Ethanol treated cells displayed enhanced numbers of double strand breaks and diminished numbers of DNA repair proteins. VD, LOS, CAT, and tempol attenuated these effects of ethanol.

Figure 5. Effect of Ethanol on Jurkat cell apoptosis

A. Jurkat cells were incubated in serum free media containing either buffer or variable concentrations of ethanol (25, 50, and 100 mM) for 24 hours. Subsequently, cells were prepared for TUNEL assay. Representative microphotographs are shown as inserts. TUNEL +ve cells are indicated by dark blue color. Cumulative data (n=3) are shown in a bar diagram. *P<0.05 compared with control; **P<0.01 compared to control.

B. Jurkat cells were pre-incubated in media containing either buffer, VD (10 nM), or losartan (LOS, 10^{-7} M), followed by addition of aliquot of ethanol (final concentration, 25mM) and continued incubation for 24 hours. Subsequently, cells were assayed for apoptosis by TUNEL assay. Representative microphotographs are shown as inserts. TUNEL +ve cells are indicated by dark blue color. Cumulative data (n=3) are shown in bar graphs. *P<0.01 with all other variables.

Figure 6. Effect of Ethanol on primary T cells

A. Freshly isolated T cells were treated with either buffer or variable concentrations of ethanol (25, 50, and 100 mM) for 24 hours. Cells were labeled with Annexin PE and 7AAD for flow cytometry. Representative flow scans are displayed. Cumulative data (n=3) are shown in a bar diagram. *P<0.01 compared to control, **P<0.001 compared to control;***P<0.05 compared to ETH, 25 mM.

B. Freshly isolated T cells were treated with either buffer or variable concentrations of ethanol (25, 50, and 100 mM) for 24 hours. Subsequently, cells were prepared for TUNEL assay. Cumulative data (n=3) are shown in bar graphs. *P<0.01 compared to control, **P<0.001 compared to control;***P<0.05 compared to ETH, 25 mM.

C. Freshly isolated T cells were pre-incubated in media containing either buffer, VD (10 nM), or losartan (LOS, 10^{-7} M), followed by treatment with ethanol (final concentration, 25mM) for 24 hours. Cells were prepared for TUNEL assay. Results $\pm$ SD (n=3) are shown in a bar diagram. *P<0.001 compared to all other variables.