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The Impact of Ethanol on Increasing HIV-1 Replications in U1 Cells

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Keywords

- HIV; Ethanol; Macrophage; Latency; Transcription factors

Abstract

Alcohol consumption leads to injury in various tissues/organs, and impairs immune functions. The effects of alcohol consumption on HIV-1 infection and acceleration of disease progression remain controversial. It is also not well-known if alcohol abuse leads to reactivation of HIV-1 replication from latency and causes virologic failure. Using susceptible U1 cells chronically infected with HIV-1, we examined the effects of alcohol on latency in macrophages. We found that low levels (0.05~0.2 g/ml) of ethanol treatments increased HIV-1 replication with upregulating expression of AP-1 and NFAT and activation of NF-κBp65 through inducing TCR-related pathways, P-TEFb pathway and MAPK-signaling pathways, regulating expression of epigenetic factors with enhanced expression of histone acetylation, inhibition of HDAC and controlling histone methylation. Ethanol treatments increased $p47^{phox}$ expression and decreased cell viabilities in a dose-dependent manner with inducing both apoptotic pathways and autophagy pathways. High levels (0.4 g/ml) of ethanol treatment caused significantly lower cell viability with no detectable changes in reactivation of HIV-1 replication through inhibited expression/activation of CD3, CD28, PKCθ and ZAP-70 in TCR-related pathways. These results indicate that low levels of ethanol enhance HIV-1 replication without significant loss of cells while ethanol at high levels reduces cell counts with no detectable changes of HIV-1 replication.

INTRODUCTION

HIV-1/AIDS remains a major problem despite recent advances in antiretroviral therapy (ART). However, HIV-1 infection remains incurable because of latent infection, leading to some infected cells persisting as a latent reservoir, and the virus persists in latently infected cells or viral reservoirs which can be reactivated upon discontinuation of cART [1]. One of the factors that has been associated with increased risk of the acquisition of HIV-1 infection and accelerated disease progression is the use of alcohol or drugs of abuse [2].

Blood alcohol concentration is usually expressed as a percentage of ethanol in the blood in units of mass of alcohol per volume of blood or mass of alcohol per mass of blood in North America. From US Federal Aviation Regulation (CFR) 91. 17, person with 0.001~0.029g in one ml of blood (0.001~0.029 g/ml) appears normal, 0.03~0.059 g/ml shows mild euphoria. 0.08 g/ml or higher is considered legally impaired, and a range of 0.35 g/ml to 0.40 g/ml usually represents potentially fatal alcohol poisoning. It has been estimated that over 50% of the general population are at least occasional users of alcohol and estimates of the proportion of the general population who are heavy drinkers are as high as 8% [2]. Alcohol consumption leads to injury in various tissues and organs including liver, pancreas, heart, brain and muscle, and impairs immune functions [3].

The use of alcohol has been reported to be associated with increased risk of the acquisition of HIV-1 infection and accelerated disease progression as measured primarily by decreased CD4+ cell counts in patients receiving ART [4]. Despite antiretroviral therapy, HIV positive individuals experienced increased mortality and physiologic injury at lower levels of alcohol use compared with uninfected individuals [5], suggesting that patients with HIV infection who ingest alcohol are at greater risk for alcohol associated adverse events and toxicities and underscores the need for simultaneous treatment of alcohol use disorders and HIV in patients with co-occurring conditions [5].

The most studied strategy in HIV-1 infection is the so called "shock and kill" therapy [6]. This strategy is based on reactivation of dormant viruses from the latently-infected reservoirs (the shock) followed by the eradication of the reservoirs (the kill). Several drugs have been developed to target some of cellular factors involved in HIV-1 latency, called Latency Reversing Agents (LRAs), which include: i) can activate host transcription factors that are required for HIV-1 transcript, such as NF-B, NFAT, and AP1 through activation of TCR-related signaling pathways and PKC signaling pathway; ii) are able to activate P-TEFb (positive transcription elongation factor b) signaling pathway involved in HIV-1 Tat molecule, and iii) can control epigenetic factors to open the nucleosomes to facilitate HIV transcription [7]. However, it is not well known if alcohol abuse plays any roles in reactivation of HIV-1 replication from its latent infection.

Macrophages contribute to HIV-1 pathogenesis by forming

a viral reservoir that serve as a viral source for the infection of CD4 T cells. Cells of the monocyte/macrophage lineage are one of the major cellular targets of HIV-1 and are relatively resistant to the cytopathic effect of HIV as these cells can survive infection with HIV for extended periods of time [8]. A recent study using a humanized mouse model shows that HIV-1 can persist in macrophages during antiretroviral therapy, suggesting that macrophages may represent an obstacle to efforts to cure HIV-1 infection [9]. Here, using HIV-1-infected cell line, U1 cells, a subclone of U937 chronically infected with HIV-1, which can be used as an in vitro model for HIV-1 latent study [8], treated with ethanol, we found that ethanol treatments could reactivate HIV-1 replication from its latent infection in macrophages with inducing cell activation, and apoptotic/autophagy signaling pathways.

MATERIALS AND METHODS

Chemicals and Reagents

Antibodies against Atg4D, AP-1, Bax, Bechlin-1, Brd4, CD4, CD28, CDK9, ERK1/2, FADD, Grb2, HDAC1, HDAC2, HEXIM1, IL-2, JNK, Lat, MBD2, NFAT, NF-kB p65, Nod-1, p38, $p47^{phox}$, PCAF, PKCθ, PLCγ-1, PARP-1, RAS, TCRβ, and ZAP-70 were purchased from Santa Cruz Biotechnology]Santa Cruz, CA). Acetyl-H3, Caspase-3, Caspase-8, Cyclin T1, H3K4, H3K27, phosphor-FADD, IFI16, LC3, mTor, phosphor-NF-kB p65, phospho-p38, p-Rpb-CTD, Ulk1, phosphor-ZAP-70 and GAPDH were bought from Cell Signaling Technology, Inc (Danvers, MA). Pure ethyl alcohol (≥99.99%) and all other chemicals were from Sigma (St. Louis, MO).

Cell Culture And Treatment with Ethanol

U1 (promonocytic) cells, a subclone of U937 chronically infected with HIV-1, were obtained from the National Institutes of Health AIDS Research Reference and Reagent Program (Germantown, MD) and cultured at 37 ° C in 5% CO2 in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. For ethanol (EtOH) treatments, U1 cells were seeded at 2×10^5 cells/ml and cultured in the medium containing 0, 0.05, 0.1, 0.2, or 0.4 g per ml of medium (weight (g)/volume (ml, g/ml) for 3 days.

Real-time PCR

Quantitative real-time reverse-transcriptase (RT) PCR was used for quantitation of viral RNA. Viral RNA was isolated from 140 μl of culture supernatant by using the QIAamp Viral RNA Mini Kit (Valencia, CA 91355) according to the manufacturer's protocol. The primers and TaqMan probe were designed in the *gag* capsid (p24) region, which is the variable region among most of the HIV-1 subtype B isolate sequences according to GenBank database. The forward primer was 5`-GACATCAAGCAGCCATGCAA-3`, corresponding to nucleotides 1367-1386, and the reverse primer was 5`- CTATCCCATTCTGCAGCTTCCT -3`, corresponding to nucleotides 1430-1409. The TaqMan probe was oligonucleotide 5`-ATTGATGGT CTCTTTTAACA-3`, corresponding to nucleotides 1488-1507, coupled with a reporter dye [6-carboxy fluorescein] (FAM) at the 5` end and a non-fluorescent quencher and a minor groove binder [MGB], which is a Tm enhancer, at the 3` end. The nucleic acids were amplified and detected in an automated TaqMan 7500 Analyzer by using QuantiTect™ Probe RT-PCR kit (Qiagen Inc., Valencia, CA). The 25-μl PCR mixture consisted of 100 nM primers and 100 nM probe. Following three thermal steps at 55 °C for 5 min, at 50 °C for 30 min and at 95 °C for 10 min, 45 cycles of two-step PCR at 95 °C for 15 s and at 60 °C for 1 min were performed.

The data are expressed as copy numbers/ml. Known concentrations of HIV-1 (MN) viral RNA (serially diluted: 10^8 to 100 copies) were used as templates and quantitative RT-PCR performed to generate a standard curve. Each value represents the average concentration of six reactions in triple isolated repeats based on the standard curve.

Western blot analysis

Proteins were isolated from U1 cells with RIPA buffer (1×PBS, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.1 mg/ml PMSF, 30 μl/ml aprotinin, 1 mM

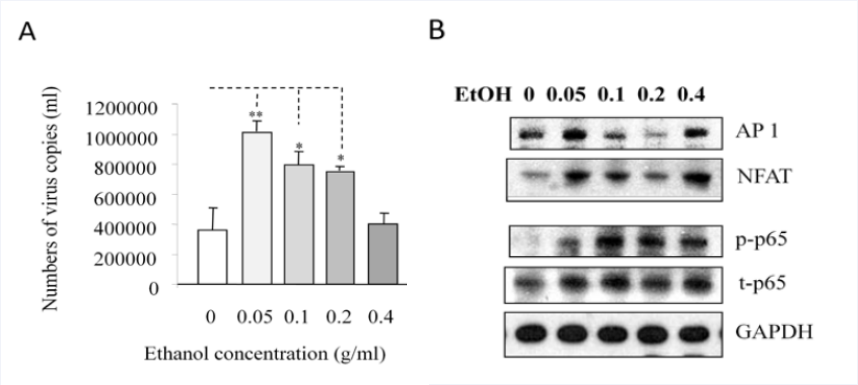

Figure 1 Ethanol treatment increased the activation of HIV-1 replication. U1 cells were cultured in medium containing 0, 0.05, 0.1, 0.2, and 0.4 g/ml of ethanol for 3 days. (A) 140 μl of culture supernatants containing HIV-1 particles were used to isolate viral RNA. 10 μl in 50 μl of the RNA was used as template to perform real-time PCR. Known concentrations of HIV-1 (MN) viral RNA (serially diluted: 10^8 to 100 copies) were used as templates and quantitative RT-PCR performed to generate a standard curve. Each value represents the average concentration of six reactions in triple isolated repeats based on the standard curve. The value of $p < 0.05$ (*) was considered significant, $p < 0.01$ (**) very significant. (B) The cell pellets were lysed with RIPA buffer and the lysates were subjected to Western blot analysis to detect to AP-1, NFAT, the phosphorylation of NF-κB p65 and total NF-κB p65.

sodium orthovanadate). Equal amounts of protein were boiled in the loading buffer (100 mM Tris–HCl, 200 mM DTT, 4% SDS, 0.2% bromphenol blue, 20% glycerol), separated on SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The data represented are from three independent experiments. The relative quantitation of protein expression was determined with Image J (Image Processing and Analysis in Java) from NIH website (https://imagej.nih.gov/ij/).

Cell viability assay

Cell viability was determined by Trypan blue exclusion analysis (Life Technologies).

Statistical analysis

The unpaired Student's *t* test was used for data analyses as indicated, and a value of $p < 0.05$ [*]was considered significant, $p < 0.01$ [**] very significant.

RESULTS

Ethanol Treatment Increased the Activation of HIV-1 replication

Alcohol abuse is an important public health problem, frequently unrecognized among people living with HIV/AIDS [2]. It has been reported that alcohol abuse in people with HIV/AIDS is positively associated with a lower CD4 cell count [4], although it is controversial if alcohol abuse has relationship with higher HIV-1 viral load values. To investigate if ethanol treatment can increase HIV-1 replication in macrophages, U1 cells were cultured in medium containing different concentration of ethanol for 3 days; the supernatants from cell culture were used as RT-PCR and proteins from cell pellets were subjected to Western blotting analysis. As shown in Fig. 1A, ethanol treatment significantly increased HIV-1 replication in a dose of 0.05~0.2g/ml, lower concentration of ethanol showed increased HIV-1 RNA production and treatments of 0.4 g/ml of ethanol had no effect on HIV-1 replication in U1 cells, suggesting that ethanol is able to reactivate HIV-1 replication from its latent state at certain levels of ethanol treatment.

It is well known that the HIV-1 LTR has been shown to contain recognition sequences for many cellular transcription factors, including AP1, NFAT, and NF-κB, which play very important roles in HIV-1 replication [10]. In order to examine whether ethanol treatments in increased activation of HIV-1 replication affect these host transcription factors, proteins from the cell pellets were subjected to Western blotting to detect Ap1, NFAT, total NF-κB p65, and phosphorylation of NF-κB p65. As shown in Fig. 1B, ethanol treatment dramatically upregulated expression of Ap-1, NFAT and phosphorylation of NF-κB p65.

These data indicate that ethanol can activate HIV-1 replication from HIV-1 latent infection via increasing expression of some host transcription factors.

Ethanol treatment could activate T-cell receptor (TCR)/CD3-related signaling pathways

Latent HIV proviruses are thought to be primarily reactivated *in vivo* through stimulation of the T-cell receptor (TCR)/CD3 [11]. TCR/CD3 activation induces multiple signal transduction pathways leading to the ordered nuclear migration of the HIV transcription initiation factors NF-κB and NFAT as well as potential effects on HIV transcriptional elongation [11]. As shown in Fig. 2, U1 cells treated with lower concentration [0.05~0.20 g/ml] of ethanol displayed increased expression of TCRβ, and 0.05~0.1 g/ml of ethanol upregulated CD3 expression, suggesting that ethanol can increase/activate the TCR/CD3 complex, which is required for HIV-1 replication.

CD28 is one of the proteins expressed on T cells that provide co-stimulatory signals required for T cell activation and survival [12]. Ethanol treatments with lower concentrations (0.05~0.10 g/ml) were able to increase expression of protein CD28 [Fig. 2A].

The transmembrane adapter LAT (linker for activation of T cells) plays a central role in signaling by ITAM bearing receptors and leads to the phosphorylation of tyrosine residues present in the intracellular domain of LAT and formation of a multiprotein complex with other adapter molecules and enzymes including Grb2 and PLCγ isoforms [13], which is recognized to play a central role in linking TCR-mediated activation, such as ZAP-70 and PKCΘ, at the membrane to the stimulation of the vast majority of downstream events [13]. U1 cells treated with lower concentration (0.05~0.20 g/ml) of ethanol displayed increased expression of LAT, 0.05~0.1 g/ml of ethanol up regulated the expression of Grb2 [Fig. B], PLCγ-1 and PKCθ, activated ZAP-70 by its phosphorylation [Fig. 2C].

High level (0.4 g/ml) of ethanol did not increase HIV-1 production [Fig. 1A], which might be because 0.4 g/ml of ethanol treatment down regulated of expression of CD3, CD28 [Fig. 2A], PLCγ-1 and PKCθ [Fig. 2C], and inhibited activation of ZAP-70 [Fig. 2C].

These results indicate that the important role of T cell activation pathways and others triggered by TCR stimulation is to activate NFAT and NF-κB through TCR-related pathways. Ethanol may increase T cell activation through TCR/CD3 signaling pathways, leading to activation of NFAT and NF-κB pathways, and increased HIV-1 replication within a certain level of ethanol treatments.

Ethanol treatment could activate signaling pathways of positive transcription elongation factor b (P-TEFb) and MAPK signaling pathways

The positive transcription elongation factor, P-TEFb, a cellular kinase composed of Cyclin T1 and CDK9, is a cyclin dependent kinase that can phosphorylate the DRB (5,6-dichloro-1-β-d-ribofuranosylbenzimidazole) sensitivity inducing factor (DSIF) and negative elongation factor (NELF), which is essential for processive HIV-1 transcription [14]. P-TEFb complex is expressed at low levels in resting cells. P-TEFb can be activated by HIV-1 protein Tat. RNA polymerase II pauses after transcription of TAR in the absence of Tat. Tat recruits P-TEFb via CycT1 to TAR, allowing CDK9 to phosphorylate the C-terminal domain (CTD) of RNAP polymerase II. As shown in Fig. 3A, ethanol could increase CDK9 expression, and 0.05~0.1 g/ml of ethanol treatments showed increased expression of Cyclin T1, and CTD, suggesting that ethanol can increase/activate P-TEFb signaling pathway in HIV-1-infected macrophages.

It has been reported that protein HEXIM1 or 2 associated

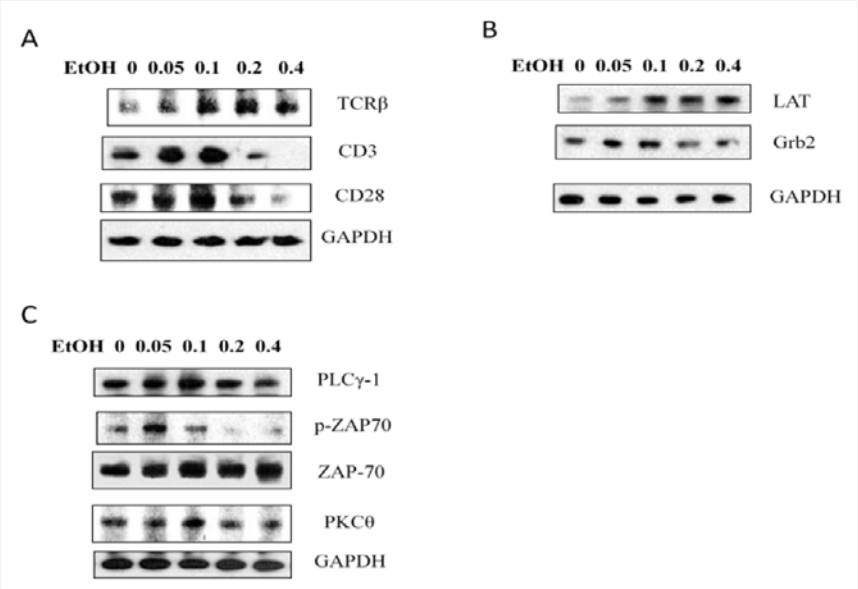

Figure 2 Ethanol treatment could activate T-cell receptor (TCR)/CD3-related signaling pathways. U1 cells were cultured in medium containing 0, 0.05, 0.1, 0.2, and 0.4 g/ml of ethanol for 3 days. Proteins isolated from the cell pallets with RIPA buffer were subjected to Western blot analysis to detect to (A) TCRβ, CD3 and CD28; (B) LAT and Grb2; and (C) PLCγ-1, phosphorylation of ZAP-70, ZAP-70 and PKCθ.

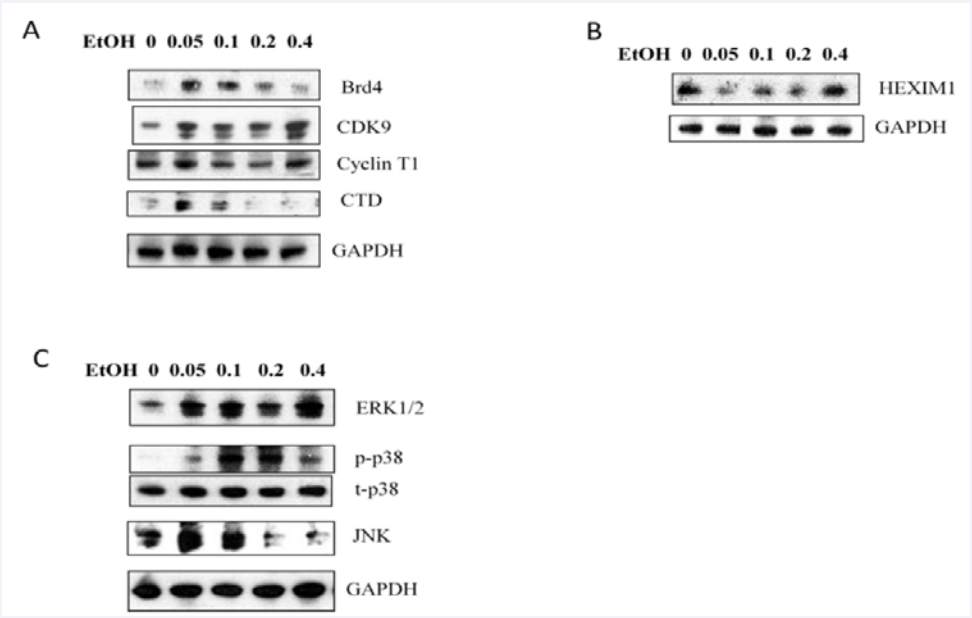

Figure 3 Ethanol treatment could activate signaling pathways of positive transcription elongation factor b (P-TEFb) and MAPK signaling pathways. U1 cells were cultured in medium containing 0, 0.05, 0.1, 0.2, and 0.4 g/ml of ethanol for 3 days. Proteins isolated from the cell pallets with RIPA buffer were subjected to Western blot analysis to detect to (A) Brd 4, CDK9, Cyclin T1, and CTD; (B) HEMIM1; and (C) ERK1/2, phosphorylation of p38, p38 and JNK.

with a noncoding RNA, 7SK, to bind to P-TEFb can inactivate the elongation of transcription by RNA polymerase II [14]. 0.05~0.2 g/ml of ethanol treatment inhibited the expression of HEXIM1 [Fig. 3B]. These data indicate that ethanol can activate P-TEFb signaling pathway to increase HIV-1 transcription.

Previously, we reported that MAPKs (mitogen-activated protein kinase), extracellular signal-regulated kinases (ERK) ½, p38, and c-jun-N-terminal kinases ½ (JNK), were upregulated in HIV-1 primary infection [15]. ERK1/2 and JNK were reported to reactivate HIV-1 replication from its latent infection through AP-1 and NF-κB, and p38 can reactivate replication from HIV-1 latency via C/EBPβ-mediated induction of HIV-1 gene expression in U1 cells [8]. Here, we found that U1 cells treated with ethanol displayed increased expression of ERK1/2, p38, phosphorylation of p38, and U1 cells treated with 0.05~0.1 g/ml of ethanol

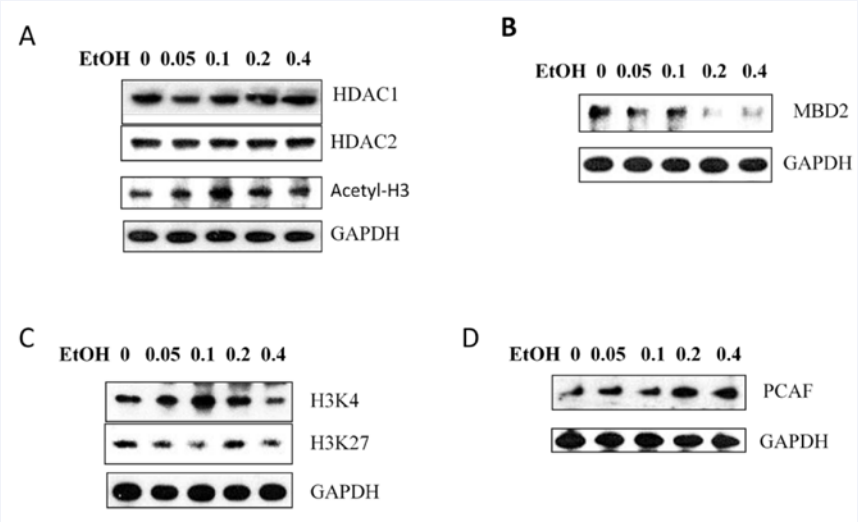

Figure 4 Ethanol treatment could affect expression of epigenetic factors. U1 cells were cultured in medium containing 0, 0.05, 0.1, 0.2, and 0.4 g/ml of ethanol for 3 days. Proteins isolated from the cell pallets with RIPA buffer were subjected to Western blot analysis to detect (A) HDAC1, HDAC2 and Acetyl-H3; (B) MBD2; (C) H3K4 and H3K27; and (D) PCAF.

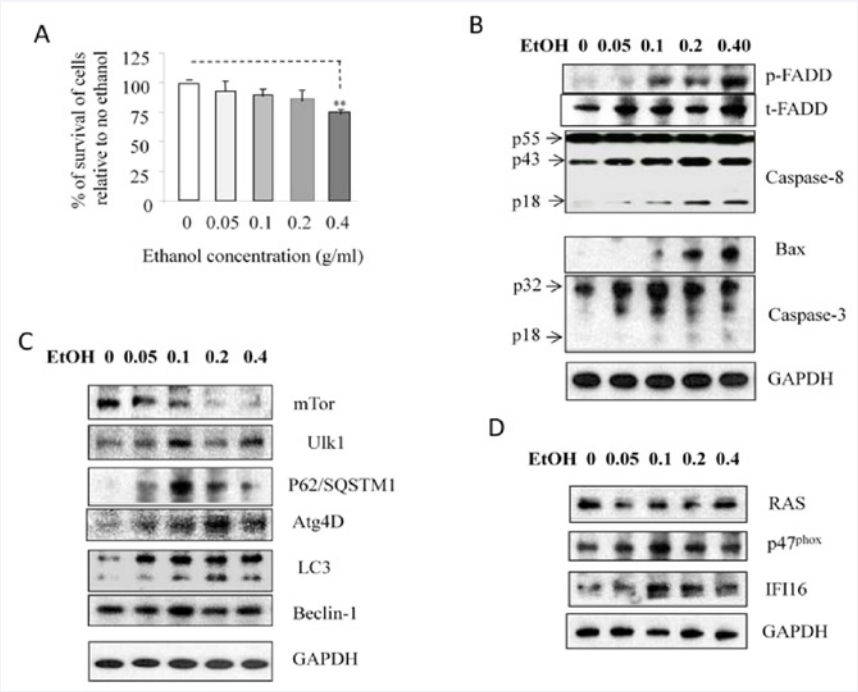

Figure 5 Ethanol treatment could activate both apoptotic and autophagy signaling pathways. U1 cells were cultured in medium containing 0, 0.05, 0.1, 0.2, and 0.4 g/ml of ethanol for 3 days. (A) Cell viability (% of survival of cells treated with ethanol vs. non-treatment) was determined by Trypan blue exclusion analysis. Proteins isolated from the cell pallets with RIPA buffer were subjected to Western blot analysis to detect (B) phosphorylation of FADD, FADD, Caspase-8, Bax and Caspase-3; (C) mTor, Ulk1, p62/SQSTM1, Atg4D, LC3 and Beclin-1; (D) RAS, p47^{phox}, and IFI16.

increased JNK expression [Fig. 3C], suggesting that ethanol treatments can activate MAPK signaling pathways.

Ethanol treatment could affect expression of epigenetic factors

In resting CD4$^+$ T cells, chromatin structure at the site of provirus integration plays critical role in repressing provirus transcription. Sequence-specific transcription factors can recruit histone deacetylases (HDACs) and other chromatin-modifying enzymes to the provirus promoter, resulting in transcriptional repression and virus latency; while chromatin acetylation by histone acetyltransferases (HATs) promotes chromatin opening and is associated with active euchromatin [16]. U1 cells treated with 0.05 g/ml of ethanol showed a decreased expression of

HDAC1 while HDAC2 had no changes [Fig. 4A]. Further, ethanol could upregulated Acetyl-Histone H3 protein expression [Fig. 4A].

Methyl-CpG binding domain protein 2 (MBD2), which binds methylated HIV DNA, was identified as a regulator of HIV-1 latency. Ethanol treatments downregulated MBD2 expression in U1 cells [Fig. 4B].

Histone methylation can be either linked to transcriptional repression or activation, depending on the site of modification. Methylation of H3 at lysine residue 4(H3K4) is associated with transcriptional activation while methylation of H3K27 is associated with transcriptional repression [16]. 0.05~0.2 g/ml of ethanol upregulated H3K4 expression and 0.05~0.1 g/ml of ethanol down regulated H3K27 expression in U1 cells [Fig. 4C].

The HIV protein, Tat, stimulates transcription from the viral long-terminal repeats [LTR] through an RNA hairpin element, transactivation responsive region (TAR) [17]. It has been reported that Tat-associated histone acetyltransferases (TAHs), p300 and p300/CBP-associating factor (PCAF), assists functionally in the activation of chromosomally integrated HIV-1 LTR [16]. Ethanol-treated U1 cells displayed increased expression of PCAF [Fig. 4D].

These data indicate that ethanol treatment can change the structure of nucleosome by modulating proteins, HDAC, DNA methylation and histone acetylation, which open nucleosomes to facilitate HIV transcription.

Ethanol treatment could activate both apoptotic and autophagy signaling pathways

It was reported that alcohol activated T cells that normally underwent apoptosis [18]. To test if ethanol inhibits cell growth and induce apoptosis and autophagy, U1 cells were treated with different concentration of ethanol for 3 days. High levels (0.4 g/ml) of ethanol very significantly inhibited the growth of U1 cells relative to control (0 g/ml of ethanol) [Fig. 5A].

Apoptosis is a process of programmed cell death that occurs in multicellular organisms, which are biochemical events leading to characteristic cell changes (morphology) and death. We found that ethanol treatment could activate both external and internal apoptotic pathways in U1 cells with increased expression of protein FADD and Bax, and activation of FADD, caspase-8 and caspase-3 [Fig. 5B].

Autophagy is a genetically programmed, evolutionarily conserved intracellular degradation pathway involved in the trafficking of long-lived proteins and cellular organelles to the lysosome for degradation to maintain cellular homeostasis. Emerging evidence suggests that autophagy is involved in alcohol-induced tissue injury [3]. Autophagy pathways were activated by ethanol treatments in U1 cells with inhibition of mTOR expression and increased the expression of p62/SQSTM1, Atg4D, Ulk1, LC3 and Beclin-1 [Fig. 5C], suggesting that ethanol treatments may activate autophagy pathways.

One mechanism contributing to ethanol-induced apoptosis in T cells could involve downregulation of a signaling molecule called renin angiotensin (RAS) [19]. Lowered RAS levels in turn induce dysregulation of mitochondria and enhance production of reactive oxygen species (ROS) that can damage various molecules in the cells [19]. Ethanol treatment could decrease RAS expression in U1 cells [Fig. 5D]. ROS acts as primary or secondary messengers to promote cell growth or death [15]. $p47^{phox}$ is one of the oxidase activators required for NADPH oxidase to produce ROS [15,19]. As shown in Fig. 5D, $p47^{phox}$ protein levels were increased during ethanol treatments in U1 cells.

The DNA sensors interferon-inducible protein 16 (IFI16) has been reported to be increased in treatment-naive patients, and IFI16 expression is correlated with high viral load and low CD4 cell count [20]. Ethanol treatment increased IFI16 expression in U1 cells [Fig.5D].

These results indicate that ethanol can produce ROS and induce cell death through apoptotic and autophagy pathways with an increased reactivation of HIV-1 replication.

Ethanol treatments could upregulate expression of PARP-1, IL-2 and Nod-1.

Poly (ADP-ribose) polymerase 1 (PARP-1) is an abundant nuclear enzyme that catalyzes the successive transfer of the ADP-ribose moiety of NAD^+ to a variety of nuclear proteins, including itself [21]. PARP-1 is required for the activation of NF-κB-dependent target genes, including HIV-1 LTR in reporter constructs; PARP-1 may also play a role in HIV-1 integration [21].

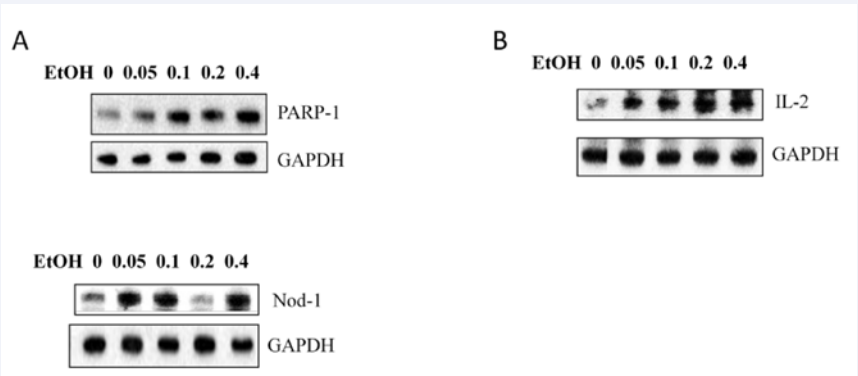

Figure 6 Ethanol treatments could upregulate expression of PARP-1, IL-2 and Nod-1. U1 cells were cultured in medium containing 0, 0.05, 0.1, 0.2, and 0.4 g/ml of ethanol for 3 days. Proteins isolated from the cell pallets with RIPA buffer were subjected to Western blot analysis to detect (A) PARP-1; (B) IL-2; (C) Nod-1.

Ethanol treatment increased PARP-1 expression [Fig. 6A].

IL-2, an autocrine T-cell growth factor, is produced predominantly by CD4+ T cells. IL-2 stimulates the proliferation of CD4+ and CD8+ T cells and NK cells, and enhances cytolytic activity against a variety of target cells. It has been demonstrated that the intermittent administration of rIL-2 with cART results in substantial and significantly higher CD4+ T-cell increases than those achieved with either dual nucleoside reverse transcriptase inhibitor therapy or more potent cART alone [22]. Ethanol treatment significantly enhanced IL-12 expression [Fig. 6B], suggesting that ethanol can activate monocyte cells.

During HIV-1 infection macrophages can be activated, and NOD-1 (nucleotide oligomerization domain-1) can synergize IL-32 that stimulates IL-1β and IL-6 release [23]. U1 cells treated with ethanol displayed increased Nod-1 expression [Fig. 6C].

DISCUSSION

The direct effect of alcohol of immune toxicity induces T-cell apoptosis and diminishes important functionality of natural killer cells and macrophages [18]. HIV-infected individuals have higher prevalence of alcohol use disorders than do uninfected individuals [24]. The studies with rhesus macaques have showed that chronic alcohol intake prior to and during HIV/SIV infection results in a higher viral set point, more rapid progression to end-stage disease, and AIDS wasting [25]. Alcohol consumption is associated with lower CD4 counts [4]. A cohort study in US Military was demonstrated to be a statistically significant relationship between virologic failure and at-risk drinking [26]. However, the mechanism in which alcohol leads to virologic failure is unknown.

Several mechanisms to silence HIV gene expression and replication in HIV-1 latency have been reported, such as mutations in viral genome, blocking accession of RNA polymerase into nucleosome, inactivation/absence of transcription factors, and problems with RNA processing and transportation [7]. Since 1987, it has been clear that activation of infected cells is required for HIV to replicate, which follows the translocation of transcription factors, such as NF-κB, into the nucleus [10, 11]. Lower levels of alcohol can activate U1 cells and increase the expression of some host transcription factors [Fig.1 & 2].

Studies show that chronic alcohol abuse in humans and animal models result in reduced numbers of peripheral T cells, lymphopenia, which can increase homeostatic proliferation, and increase T cell differentiation and activation [18]. Chronic alcohol consumption can induce apoptosis with caspase-3 activation and exposure to different concentration of ethanol causes decreased cell viability in a dose-dependent manner [27] [Fig. 5A]. Furthermore, ethanol exposure promoted expression of the pro-apoptotic proteins, such as Bax and FADD, suggesting that ethanol treatments can sensitize T cells [28] and U1 cells [Fig. 5] to activation-induced cell death (AICD) [28]. Previously, we reported that pro-apoptotic proteins could activate the activation of T cell line, Jurkat cells and PBMC, and increase HIV-1 replication, while anti-apoptotic molecules could inhibit HIV-1 replication [29-31]. Here we found that ethanol treatment could activate U1 cells with inducing of TCR-related signaling pathways [Fig. 2], and inhibit cell viabilities by inducing both apoptotic signaling pathways [Fig. 5] and production of ROS leading to cellular damage.

Cumulative evidence has shown that virus replication is enhanced when host cells initiate the apoptotic program. This phenomenon is called an Alternative Replication Program (ARP), a process when the host cell is undergoing apoptosis during viral infection. Viruses adopt an emergency escape mechanism to produce a large amount of virus rapidly [32]. Expression/activation of some preapoptotic molecules from apoptotic pathways, such as Bax, FADD, caspase-3, and caspase-8 are sufficient to initiate the ARP in HIV-1 infection [29-31] and influenza A virus infection [33]. Treatment with cytotoxic drugs has been reported to produce and activate HIV-1 replication in pro-monocytic (U1) and lymphoid (ACH-2) cell lines persistently infected with HIV-1 [32]. However, further study is needed to determine if ethanol-induced apoptosis causes activation of U1 cells, or ethanol treatments lead to activation-induced cell death (AICD). These changes in turn compromise the cells' ability to respond to pathogens and contribute to increased susceptibility to infections, which may contribute to alcohol abuse increases in acquisition of HIV-1 infection and leads to virologic failure.

Autophagy is a dynamic multistep process that is tightly regulated by many signaling pathways involving nutrients, energy, and stress response [3]. mTORC1 is a negative regulator at the pre-initiation complex to regulate the initiation of autophagosome biogenesis [3]. It has been reported that HIV-1 infection induces autophagy in monocytes/macrophages and that knockdown of Beclin-1 decreases HIV-1 replication [34, 35]. However, it remains controversial whether HIV-1 infection induces or represses autophagy. Early in 2008, it was reported that in vitro HIV-1 infection could repress autophagy in T cells [36], but the conclusion resulted from the cell cultured under serum starvation. Previously we found that, in normal cell culture condition, HIV infections significantly increase transcription of ULK1, a member of the autophagy-initiated complex. Two ubiqutin-like conjugation systems, the Atg12 conjugation system and the microtubule-associated protein L chain 3 (LC3) conjugation system that control the elongation of the autophore to form the autophagosome [37]. We found here that ethanol could induce autophagy signaling pathways in HIV-1-infected microphage [Fig. 5C]. Further investigation is needed to determine what the functions of ethanol-induced autophagy are in HIV-1 replication and cell viability.

In humans, a direct effect of alcohol consumption has been more difficult to establish, and the effects of at-risk drinking on HIV infection remain controversial. Different studies on alcohol consumption in HIV-1 positive individuals reached different conclusions compared to HIV-1 negative drinkers, including: i) increased HIV-1 replication with no alterations in CD28-CD57+, naïve or memory T-cell phenotypes associated with immunosenescence [38]; ii) lower CD4 counts, but not progression to AIDS [39]; iii) lower CD4 counts with accelerated disease progression [4,40], and iv] decrease response to ART and virologic failure [28,41]. Besides differences in race, life styles and environment, lower level of alcohol consumption may be one of the reasons that there is an increase in HIV-1 replication with no significantly decreased cell counts, while high level of alcohol

drinking may significantly lower cell counts leading to HIV-1 replication with undetectable changes. Therefore, decreased HIV-1 replication may not progress fast enough to AIDS.

In conclusion, the monocytic U1 cell line, which shows minimal constitutive expression of HIV-1, when treated with different concentrations of ethanol, displayed increases in HIV-1 replication at 0.05~0.2 g/ml of ethanol and no undetectable changes at high level (0.4 g/ml) of ethanol. The ethanol-induced HIV-1 replication was related to upregulation/activation of host transcription factors, AP-1, NFAT and NF-κBp65 through increased expression/activation of TCR, CD3, CD28, PLCγ-1, ZAP-70 and PKCθ in TCR-related pathways, Brd4, CDK9, Cyclin T1 and CTD in P-TEFb pathway, and ERK1/2, p38 and JNK in MAPK signaling pathways. Ethanol treatment could modulate expression of epigenetic factors to easily open the nucleosomes to facilitate HIV transcription.

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Author Contributions

X.W. and I.H. conceived and designed the experiments; X.W., J.Z., and V.R. performed the experiments; and X.W. analyzed the data, and X.W. and I.H. wrote the paper. All authors have read and approved the final manuscript.

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