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Chronic Ethanol consumption modulates growth factor release, mucosal cytokine production and microRNA expression in nonhuman primates

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Abstract

BACKGROUND—Chronic alcohol consumption has been associated with enhanced susceptibility to both systemic and mucosal infections. However, the exact mechanisms underlying this enhanced susceptibility remain incompletely understood.

METHODS—Using a nonhuman primate model of ethanol self-administration, we examined the impact of chronic alcohol exposure on immune homeostasis, cytokine and growth factor production in peripheral blood, lung and intestinal mucosa following twelve months of chronic ethanol exposure.

RESULTS—Ethanol exposure inhibited activation-induced production of growth factors HGF, G-CSF and VEGF by peripheral blood mononuclear cells (PBMC). Moreover, ethanol significantly reduced the frequency of colonic Th1 and Th17 cells in a dose-dependent manner. In contrast, we did not observe differences in lymphocyte frequency or soluble factor production in the lung of ethanol-consuming animals. To uncover mechanisms underlying reduced growth factor and Th1/Th17 cytokine production, we compared expression levels of microRNAs in PBMC and intestinal mucosa. Our analysis revealed ethanol-dependent upregulation of distinct microRNAs in affected tissues (miR-181a and miR-221 in PBMC; miR-155 in colon). Moreover, we were able to detect reduced expression of the transcription factors STAT3 and ARNT, which regulate expression of VEGF, G-CSF and HGF and contain targets for these microRNAs. To confirm and extend these observations, PBMC were transfected with either mimics or antagomirs of miR181 and 221 and protein levels of the transcription factors and growth factors were determined.

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Transfection of microRNA mimics led to a reduction in both STAT-3/ARNT as well as VEGF/HGF/G-CSF levels. The opposite outcome was observed when microRNA antagomirs were transfected

CONCLUSION—Chronic ethanol consumption significantly disrupts both peripheral and mucosal immune homeostasis, and this dysregulation may be mediated by changes in microRNA expression.

Keywords

Ethanol; Nonhuman Primate; Self-Administration; Immunity; microRNA

INTRODUCTION

In the US alone, 18 million persons meet the criteria for alcohol abuse, which remains the third leading cause of preventable death in with ~ 25,000 alcohol-related deaths/year (Kochanek et al., 2011). One of the most striking alcohol-related morbidities is increased susceptibility to infection, most likely due to ethanol-mediated dysregulation of the immune system (reviewed by (Szabo and Mandrekar, 2009)). Studies in humans, non human primates (NHPs) and rodents indicate ethanol may act to either promote or inhibit Th1/Th17 CD4 T cell and CD8 cytotoxic T cell responses, with varying results dependent on ethanol exposure duration and dosage, as well as tissue examined (Szabo and Mandrekar, 2009, Molina et al., 2010, von Haefen et al., 2010, Gurung et al., 2009). Tissue specific effects of alcohol consumption are likely to be critical to its impact on host fitness. For instance, impaired immune function in the lung is associated with increased susceptibility to pulmonary infection in alcoholics, while increased epithelial permeability and systemic translocation of intestinal endotoxin are proposed to potentiate hepatic inflammation and eventual cirrhosis. However, alcohol's effect on tissue resident immune cells, particularly in mucosal sites such as the lung and gut remains incompletely understood due to the difficulties in: 1) obtaining mucosal tissues from patients; 2) determining accurate alcohol dose/exposure history and 3) presence of additional alcoholism associated co-morbidities. Therefore, animal models of alcohol abuse can significantly help to address these concerns.

We have developed a NHP model of chronic alcohol consumption, in which rhesus macaques are trained to self-administer alcohol following schedule-induced polydipsia and then allowed voluntary access to ethanol and water for 22hr/day. This model enables the immune effects of alcohol exposure to be examined at known and biologically relevant doses over an extended time period. Using this animal model, we have shown that chronic alcohol consumption significantly modulates serum levels of several bioactive mediators including cytokines, chemokines, growth factors, stress hormones and vasoactive peptides (Helms et al., 2012). However, the impact of ethanol on both peripheral and mucosal immune cells in this model is unknown.

In this study, we examined the effect of chronic ethanol consumption on cytokine, chemokine and growth factor production by peripheral blood mononuclear cells (PBMC). Moreover, we determined the impact of chronic alcohol consumption on cytokine production by lymph node-, lung- and gut-resident immune cells. We found that PBMC and colonic T cells exhibited a marked ethanol-dependent disruption of growth factor and inflammatory cytokine production respectively. We also detected differences in microRNA expression levels in these affected tissues. These studies contribute to a growing area of research into the interaction between ethanol exposure, microRNA expression and immune homeostasis.

MATERIALS AND METHODS

Ethics Statement

This study was performed in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the United States Department of Agriculture. All animal work was approved by the ONPRC Institutional Animal Care and Use Committee (IACUC). All efforts were made to minimize animal suffering and all procedures performed with qualified veterinary staff present.

Ethanol self-administration model

We used a schedule-induced polydipsia approach described previously (Grant et al., 2008a, Grant et al., 2008b) to establish self-administration of 4% (w/v) ethanol in rhesus macaques. One male and one female cohort (n = 9 females, aged 5–6; n=12 males, aged 5–6) were used in this study. The average daily alcohol dosage (avg g/kg) for each animal ranged from 3.8–5.6 for females (n=6, with 3 controls) and 1.9–3.3 for males (n=8, with 4 controls), as outlined in Table 1.

Tissue collection and processing

All tissues were collected following terminal anesthesia (8 mg/kg ketamine followed by i.v. pentobarbital). Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over histopaque (Sigma, St Louis, MO) as per manufacturer's protocol. Mesenteric and tracheobronchial lymph nodes, jejunum, duodenum, ileum and descending colon were collected in RPMI supplemented with FBS, streptomycin/ penicillin, and L-glutamine. RPMI was supplemented with 10% FBS for the collection of lymphoid tissues and 3% FBS for the collection of intestinal tissues. Lymph node lymphocytes were isolated by mechanical disruption and filtration of lymph nodes with a 70µm cell strainer (BD Pharmingen, San Diego, CA). Intestinal lymphocytes were isolated using DTT/EDTA digest (intra-epithelial cells) and collagenase IX/DNAase digest (lamina propria lymphocytes) and subsequently purified over Percoll (GE Healthcare Biosciences, Pittsburgh, PA) gradient as described previously (Veazey et al., 1997). Lavage of the right lung was with PBS was also performed at necropsy, with bronchial alveolar lavage (BAL) cells filtered, pelleted and resuspended in RPMI supplemented with 10% FBS. Cells were cryopreserved in Fetalplex™ Animal Serum Complex (Gemini Bio-Products, West Sacramento, CA)/DMSO.

Flow cytometry

For flow cytometric analysis of cytokine production by mucosal and lymph node lymphocytes, $1-2 \times 10^6$ cells were stimulated for 6hr at 37 °C in supplemented RPMI (3% FBS) only or in the presence of phorbol myristate acetate/ionomycin. Brefeldin A was added for the last 5hr of the incubation to all wells. Following incubation, cells were surface stained for CD3, CD4 (eBioscience, San Diego, CA) and CD8 (Beckman Coulter, Brea, CA) and with AQUA Live/Dead stain (Invitrogen, San Diego, CA). Cells were subsequently fixed, permeabilized and stained intracellularly as described (Asquith et al., 2011) for TNFα, IFNγ (eBioscience) IL-2, and IL-17A (BioLegend). All flow cytometry samples were acquired with LSRII instrument (Beckton Dickinson) and analyzed using FlowJo software (TreeStar, Ashland, OR). Frequencies of cytokine-producing cells represent the stimulated sample value minus that of its unstimulated (media alone) control.

Cytokine, chemokine and growth factor analysis

PBMC culture supernatant samples were analyzed using Invitrogen Cytokine Monkey Magnetic 28-plex panel or G-CSF singleplex (Life Technologies, Grand Island, NY) per the

manufacturer's instructions. Samples were run in triplicates. Values below the limit of detection were designated as ND, or not detected.

RNA isolation, cDNA synthesis and analysis of microRNA and transcription factor expression

Total RNA was isolated from 2×10^6 PBMC cells using Trizol method (Invitrogen, Grand Island, NY) as described previously (Asquith et al., 2012). Both PBMC and colon RNA was precipitated in isopropanol overnight at -20°C prior to final extraction. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in conjunction with Taqman® microRNA 5x RT primer kits specific for each microRNA. MicroRNA expression was determined by quantitative Real Time PCR using Taqman® microRNA assays (Applied Biosystems) and a StepOnePlus instrument (Life Technologies, Grand Island, NY). MicroRNA expression levels were normalized to control U6 miRNA expression for each sample using ΔCt calculations (Pfaffl, 2001).

To measure transcription factor expression, cDNA was synthesized from RNA isolated as above, using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene expression was determined using custom Taqman® primer/probe kits specific for *Macaca mulatta* cDNA sequences. Transcription factor expression levels were calculated relative to the housekeeping gene *Macaca mulatta* glutathione synthetase. Samples with low cDNA yield (MGSS cycle number <35 cycles) were excluded from analysis.

Identification of miRNA targets

MicroRNA targets were first analyzed using the TargetScan algorithm (release 6.2, June 2012), using *Macaca mulatta* reference sequences. Positive hits were then verified using a second algorithm to improve specificity and avoid false positives as has been described previously (Asirvatham et al., 2008). The second algorithm used was miRanda software (August 2010 release) available at www.microRNA.org. For each putative target gene analyzed by miRanda for miRNA target sites, the homo sapiens sequence was analyzed using a mirSVR threshold ≤ -0.1 . Some transcription factor target genes selected for this approach were selected bioinformatically. In this instance, transcription factors predicted to bind to the promoters for VEGF, G-CSF, EGF and MIF were identified using the Champion ChIP Transcription Factor Portal (Qiagen), which uses SABiosciences' Text Mining Application and the UCSC Genome Browser (available at www.sabiosciences.com).

Transfection of miRNA mimics and antagomirs into PBMC

PBMC were cultured at $1-2 \times 10^6$ cells per well in a 96-well plate with RPMI-1640 supplemented with 10% FBS and transfected with 60 nM of either mimics (miR-181b, miR-221, miRNA- neg ctrl) or antagomirs (miR- 181b, miR- 221 or miRNA- neg ctrl) (Thermo-scientific) using nucleofection technology (human T cell Nucleofector kit, program F1-115) in accordance with manufacturer's recommendations. After a 24 h incubation, cells were stimulated overnight with 100 ng/ml PMA and 500 ng/ml ionomycin and harvested 14 h later for western blot analysis. Each transfection experiment was done in triplicate.

Western Blot Analysis

Total protein extracts were prepared in RIPA lysis buffer. The protein concentrations were determined by Bradford assay (BioRad). Approximately 30–40 μg of lysate was separated on a 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were incubated in 5% nonfat milk powder diluted in TBST for 30 min at room temperature, and then probed with a human monoclonal anti-STAT3 (1:2000), anti-ARNT antibody (1:1000), anti-HGF (1:5000), anti-VEGF (1:5000) (cell signalling) in 1%

milk diluted in 1xTBST overnight at 4°C. The membrane was washed three times with 1xTBST and incubated with horseradish peroxidase conjugated secondary antibody (goat antirabbit, 1:5000) for 2 h at room temperature. The Membrane was then washed three times with 1xTBST and 1x TBS respectively. Immuno-complexes were detected with an enhanced chemiluminescence method using DURA kit (GE Healthcare). The same membranes were stripped and re-probed with anti- β -actin monoclonal antibody (1:2000, cell signaling). Images of autoradiography were acquired using a scanner EPSON Perfection 2580 Photo (EPSON) and quantified by Image J 1.34 Software (<http://rsb.info.nih.gov/ij>).

Statistics

Statistical analysis and graphing was conducted with GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA). Correlation analyses were performed with Spearman rank correlation test. Analyses that compared drinkers vs controls were performed using Student's t test or nonparametric Mann-Whitney U test appropriate to normality distribution.

RESULTS

The impact of chronic alcohol consumption on cytokine, chemokine and growth factor production by PBMC

To initially examine the impact of chronic alcohol consumption on immune function, we compared soluble factor production by polyclonally-stimulated PBMC from drinkers versus those of control animals. Phorbol myristate acetate/ionomycin stimulation was chosen for these initial experiments given their broad range of action and their robust effect on cytokine production by T cells. We used a 28-plex array to measure protein levels of 13 cytokines (IFN γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-12, IL-15, IL-17, TNF α , IL-1ra, IL-10 and MIF), 6 growth factors (EGF, FGF, G-CSF, GM-CSF, HGF and VEGF) and 9 chemokines (MCP-1, MIP1 α , MIP1 β , RANTES, EOTAXIN, MDC, IL-8, MIG and I-TAC). There were no differences in cytokine (Fig 1A) or chemokine (Fig 1B) production by PBMC obtained from control versus ethanol-consuming animals. By contrast, hepatocyte growth factor (HGF), granulocyte-colony stimulating factor (G-CSF) and vascular-endothelial growth factor (VEGF) were produced at significantly lower levels in drinkers versus controls (Fig 1). Epithelial growth factor (EGF) also showed a trend towards decreased expression in drinkers ($p = 0.076$). Since females had higher BECs than males, we also compared cytokines, chemokines and growth factor production after PMA/ionomycin stimulation between these two groups. We noted higher expression IL-1b, IL-15, ITAC, EGF, and IL-8 expression in females compared to males (data not shown). However, it is unclear whether these differences are attributed to ethanol, gender or both.

The impact of chronic alcohol consumption on mucosal cytokine production

In light of the increased susceptibility of alcoholics to lung infection, we examined cytokine, growth factor and chemokine production by lung resident immune cells. However, by contrast to PBMC, bronchoalveolar lavage (BAL) cells collected from drinkers and controls exhibited equivalent production of all proteins examined upon polyclonal stimulation (Fig S1). Similarly, the frequency of IL-2+, TNF α +, IFN γ + and IL-17+ CD4 and CD8 T cells in BAL and tracheobronchial lymph node were comparable between the two groups (Fig 2A and B). Moreover, drinkers and controls had a similar frequency of both central and effector memory CD4 and CD8 T cells amongst BAL cells (Fig 2C and D) as well as total CD4 and CD8 T cells illustrated by comparable CD4/CD8 T cell ratio in the BAL (Fig 2E).

We next examined the frequency of CD4 and CD8 T cells producing IL-2, TNF α , IFN γ and IL-17 in jejunum, duodenum, ileum and colon, as well as the intestinal draining mesenteric lymph node by intracellular cytokine staining (Fig 3, S2, and S3). Both intra-epithelial and

lamina propria lymphocytes were analyzed since they represent distinct subpopulations of the intestinal immune axis. Cytokine responses were typically more variable among drinkers, with more 'low responders' detected in this group. We detected a lower frequency of TNF-expressing CD8 T cells within the duodenal lamina propria of ethanol consuming animals (Fig 3A). In light of several recent reports that IL-17 and IFN γ are often co-expressed by mucosal T cells with both protective and pathogenic effector functions, we also enumerated the frequency of IFN γ /IL-17 co-expressing T cells. Interestingly, chronic alcohol exposure significantly reduced the frequency of these cells within CD4+ intraepithelial T cells of the jejunum (Fig 3C) and CD8+ T cells of the ileal lamina propria (Fig 3D). No differences in IL-2, IFN γ or IL-17 production by CD4 or CD8 T cells was observed between ethanol-consuming and control animals (Fig S3).

Since cytokine responses were particularly variable amongst drinkers, we next performed correlation analyses to establish dose-dependent effects of ethanol consumption on tissue cytokine production. We observed a negative association in the frequency of colonic CD4 T cells producing multiple cytokines as a function of increasing ethanol dose (Fig 4). This trend was significant for IL-2+, TNF α +, IFN γ + and IFN γ /IL-17+ cells. We also observed a trend towards negative association with IL-17+ cells, albeit non-significant. CD8+ T cells did not show a significant association between increasing alcohol exposure and cytokine production. Thus, CD4+ T cells of the colon appeared exquisitely susceptible to immune modulation by ethanol. Moreover, the ratio of CD4:CD8 T cells was equivalent between controls and drinkers in all gut tissues examined (Fig S4), further indicating that this reduction was at the functional level.

Chronic alcohol consumption modulates microRNA expression

MicroRNAs are ~ 22 nucleotide long endogenous RNAs that target mRNAs for translational repression or degradation (Tomankova et al., 2011). MicroRNAs are increasingly appreciated as potent regulators of immune function, and reports indicate that alcohol may modulate microRNA expression (Miranda et al., 2010). We therefore compared the expression of a panel of microRNAs highly expressed in lymphoid cells (Felli et al., 2005, Xiao et al., 2008, Li et al., 2007, Lu et al., 2010, Rouas et al., 2009, Ma et al., 2011, Lu et al., 2009, Rodriguez et al., 2007) by quantitative real-time PCR in both PBMC and colon. This panel included microRNAs miR-181a-5p, miR-146a-5p, miR-155-5p, miR-221-3p, miR-17-3p, miR-17-5p, miR-21-5p and miR29a.

Strikingly, both PBMC and colon exhibited differential expression of specific microRNAs between drinkers and control animals (Fig 5). Within PBMC, the expression of both miR-181a and miR-221 was significantly upregulated in animals that chronically consumed ethanol with the rest showing a trend of equivalent or higher expression than controls (Fig 5A). Further analysis revealed increased expression of miRNA 221, miRNA 17-5p, and miRNA 21 in females compared to males following ethanol consumption (data not shown). However, as noted earlier, it is unclear at this point whether this difference could be attributed to increase in ethanol intake, gender or both. Notably, none of the microRNAs examined was downregulated in PBMC from drinkers. By contrast to PBMC, miR-181 and miR-221 had equivalent expression in colon sections from ethanol-consuming and controls (Fig 5B). Instead, microRNA miR-155 was significantly upregulated in colon from ethanol-exposed animals. Therefore, reduced growth factor and cytokine production following chronic alcohol consumption is associated with tissue-specific upregulation of microRNAs with potential immune modulatory activity.

Expression of transcription factors containing predicted miRNA target sites in drinkers vs controls

Since we observed an alcohol-dependent upregulation of microRNAs miR-181a and miR-221 in PBMC, we next examined whether cytokines found to be differentially expressed were potential microRNA targets. Using miRanda software, we used a bioinformatics approach to search target sequences for either miR181a or miR-221 in the sequence of growth factors downregulated in PBMC from ethanol-consuming animals (VEGF, G-CSF, HGF). Using a high threshold to identify potential binding sites (mirSVR threshold ≤ -0.1) to identify potential binding sites, we found that none of the microRNAs examined were predicted to bind the aforementioned cytokine target sequences. We therefore tested the hypothesis that these microRNAs may bind transcription factors known or predicted to regulate expression of these genes. Interestingly, multiple transcription factors (summarized in Fig S5) were found to contain 3'-UTR sequences with target sites for miR221 and miR181a.

We therefore analyzed expression of some of the transcription factors with predicted miRNA target sites (cFos – miR181a and miR221; ARNT – miR221 and miR181a; STAT3 – miR181a; RUNX1 – miR221 and AhR – miR221) in PBMC from both drinkers and control animals (Fig 6A). Notably, chronic alcohol consumption led to significant downregulation of both ARNT and STAT3 mRNA expression. By contrast, expression of transcription factors cFos, RUNX1 and AhR mRNA in PBMC was comparable between both groups. We then compared the protein expression levels of STAT-3 and ARNT in PBMC isolated from drinking or control animals (Fig 6B). In line with the reduction in gene expression levels, protein levels of both STAT-3 and ARNT were reduced in ethanol-consuming animals (Fig 6B and C). These data suggest that chronic ethanol consumption led to a significant downregulation of transcription factors that regulate cytokine expression and are predicted targets for ethanol sensitive microRNAs.

miR-181 and miR-221 modulate STAT3, ARNT, HGF, VEGF and G-CSF protein expression

We next determined whether STAT3 and ARNT expression is modulated by these two microRNAs. To that end, we analyzed STAT3 and ARNT expression in PBMC following transient transfection of chemically-stabilized dsRNA oligomers that mimic the function of endogenous mature miRNA (mimics), or chemically-modified single-stranded antisense oligomers that inhibit miRNA function (antagomirs) (Fig 7). In PBMC transfected with miRNA mimics specific for miR-181, miR-221, miR-ctrl and then stimulated them with PMA and ionomycin we observed down-regulation of both STAT3 and ARNT protein levels (Fig 7A and C). As a corollary, transfection of the polyclonally stimulated PBMC with antagomirs induced up regulation of STAT-3 and ARNT protein levels (Fig 7B and C).

Since STAT3 and ARNT modulate HGF, VEGF and G-CSF expression (Albasanz-Puig et al., 2012, Lin et al., 2012, Tacchini et al., 2003, Nguyen-Khac et al., 2006, Zhang et al., 2012, Zimmers et al., 2003), we analyzed HGF and VEGF levels by western blot in lysates of PBMC transfected with miRNA 181/221 and mimics or antagomirs and then stimulated with PMA/ionomycin (Fig 7D–F). Secretion of G-CSF into culture supernatant was carried using singleplex assay because we were not able to detect protein expression using the commercially available antibody (Fig 7G). Immunoblot analysis revealed that levels of VEGF and HGF were reduced in PBMC transfected with mimics (Fig 7D and F) while the opposite effect was observed with antagomirs (Fig 7E and F). Our analysis also shows that increasing levels of miR-181a and 221 through transfection of mimics led to decreased levels of G-CSF. Conversely, microRNA antagomirs results in increased secretion of G-CSF (Fig 7G).

DISCUSSION

In this study we used a nonhuman primate model of voluntary ethanol intake to investigate the impact of alcohol on immune homeostasis. Rhesus macaques share close physiological, behavioral and genetic relatedness to humans and have a strong propensity for alcohol consumption (Grant et al., 2008a). In our study, the majority of animals (9/14) consumed on average >3g ethanol/kg/day. Our findings indicate this level of alcohol consumption over the period of 12 months significantly perturbed immune function in mucosal tissues and the periphery. Intriguingly, reduced growth factor and canonical cytokine production by PBMC and intestinal immune cells respectively was associated with increased expression of distinct microRNAs in these tissues. We propose that the dysregulation of these microRNAs may contribute to the immune suppressive effects of ethanol in our study, acting at the level of transcriptional regulation of cytokine expression.

Our initial finding that PBMC production of most cytokines was not altered in ethanol-consuming animals was somewhat unexpected since peripheral blood dendritic cells from alcoholics without liver disease (AWLD) patients exhibit higher spontaneous production of IL-1 β , TNF α , IL-6 and IL-12 than healthy controls (Laso et al., 2007). Likewise, increased IL-2 and IFN γ production has been reported in peripheral blood T cells from alcoholics (Laso et al., 1999). In contrast to these findings, we observed reduced production of growth factors VEGF, HGF and G-CSF in drinkers. This could reflect the fact that we studied total PBMC stimulated with PMA/ionomycin rather than purified subset of immune cells.

VEGF is a growth factor produced by vascular endothelium, but is also produced by monocytes and T cells (Ferrara, 2004). The association between alcohol consumption and both cardiovascular disease and impaired wound healing prompted several studies on ethanol exposure and VEGF expression. Ethanol reduces VEGF expression in aortic tissue, smooth muscle and fetal lung tissue (Husain et al., 2011, Lazic et al., 2011). It has also been demonstrated that NHPs chronically exposed to ethanol have reduced vasculogenesis *in vitro*, consistent with disrupted VEGF expression (Williams et al., 2008) and experienced a decrease in circulating VEGF and EGF levels (Helms et al., 2012). Nonetheless, other studies indicate ethanol can also upregulate VEGF expression. For example, rats exposed to ethanol for 2–52 weeks rapidly increase VEGF expression in brain, heart and skeletal muscle (Louboutin et al., 2012). These opposing effects may reflect differences in ethanol metabolism of NHPs and rodents, highlighting the distinct effects of ethanol on VEGF biology according to species, tissue type or dosage.

G-CSF is also produced by a variety of cells including fibroblasts, stromal cells, monocytes and macrophages (reviewed by (Panopoulos and Watowich, 2008)). Given its pivotal role in leukocyte mobilization, it is plausible that ethanol-mediated reduction in peripheral G-CSF response could adversely affect host immunity. Consistent with this hypothesis, acute ethanol exposure in mice has been shown to impair G-CSF synthesis and neutrophil mobilization following systemic bacterial infection (Bagby et al., 1998). Moreover, studies of alcohol abuse in humans have demonstrated reduced granulopoiesis and enhanced susceptibility to bacterial infection (Happel and Nelson, 2005b).

The final growth factor that was significantly suppressed in PBMC by ethanol in our study was HGF. First identified as a potent hepatocyte mitogen, HGF acts upon epithelial, endothelial and mesenchymal cells (reviewed by (Matsumoto and Nakamura, 1997)). Interestingly, HGF administration can delay or prevent ethanol-mediated liver injury in rodent models and hepatocyte cell lines (reviewed by (Dhanda et al., 2012)). Thus, the inhibition of HGF production by ethanol in our own study implicates dysregulation of

growth factor as a potentially contributory mechanism preceding ethanol-induced liver damage.

In contrast to ethanol's limited impact on canonical cytokine production in the periphery, we observed significantly reduced production of IL-2, TNF α , IFN γ and IL-17 by mucosal T cells, particularly those of the colonic lamina propria. These findings are mirrored by another NHP study, in which rhesus macaques administered ethanol intragastrically for 8 weeks exhibited a reduced frequency of intestinal effector memory T cells in the jejunum, but not observed in lymph nodes or periphery (Poonia et al., 2006). The reduced frequency of CD4⁺ Th1 and Th17 cells observed in our study may be acutely relevant, given the central role both subsets play in mucosal immunity (Maloy and Powrie, 2011). Consistent with impaired intestinal immunity, EtOH-exposed mice have enhanced susceptibility to oral *Salmonella typhii* infection (Sibley and Jerrells, 2000), due to diminished Th1 responses in the upper GI tract. Together our findings demonstrate that ethanol may significantly inhibit the immune effector functions of intestinal T cells, rendering the host more susceptible to infection of the intestinal mucosa.

Previous studies have shown alcohol exposure reduces Th1 and Th17 responses as well as chemokine production in the lung (reviewed by (Happel and Nelson, 2005a)). In our study however, global cytokine production and the frequency of CD4 and CD8 BAL-resident T cells were not dysregulated. The reason for this discrepancy is unclear but we propose that 12-month alcohol exposure in our model causes fewer physiological disruptions than acute/binge ethanol intake and accumulated years of alcohol abuse observed in clinical studies of alcoholics and pulmonary immune function.

Our findings that chronic alcohol consumption upregulates multiple microRNAs contributes to a growing body of data identifying ethanol-sensitive microRNAs (Miranda et al., 2010). However, while changes in expression levels of several microRNAs have been identified in neuronal, hippocampal and hepatic tissue, there are relatively few reports of ethanol-sensitive microRNA in immune cells or intestinal. To our knowledge, we report for the first time that chronic ethanol self-administration leads to overexpression of miR-181a and miR-221 in PBMC. Functionally, miR-181a expression is reported to augment the sensitivity of T cells to antigenic stimulation (Li et al., 2007). By contrast, miR221 is reported to be specifically upregulated upon T cell activation, providing regulatory feedback (Grigoryev et al., 2011). Our findings however indicate that upregulation of miR-181a and miR-221 by ethanol in our model were associated with reduced growth factor production rather than increased cytokine production.

Consistent with the notion that ethanol differentially modulates microRNA expression in distinct tissues, we found miR-155 was upregulated in colon but not in PBMC, corresponding with reduced colonic cytokine expression. As mentioned, miR-155 has previously been reported as an ethanol sensitive microRNA in both RAW macrophages *in vitro*, and more recently in murine cerebellum (Lippai et al., 2013) and liver cells *in vivo* (Bala et al., 2011). However, in those studies, increased miR-155 expression was associated with enhanced inflammation potentially by targeting suppressor of cytokine signaling 1 (SOCS1) (Dudda et al., 2013). Therefore, our finding that higher levels of miR-155 in colonic biopsies were associated with lower Th1 (IL-2, IFN γ and TNF α) and IL-17 cytokine production by resident CD4 T cells was surprising. One possible explanation for this discrepancy is that while the reduction of cytokine production was specifically observed within CD4 T cells, miR-155 expression levels were measured in the entire tissue. Another potential explanation is that mi-155 has also been reported to be expressed at 10–20 fold higher levels in FoxP3⁺ regulatory T cells than conventional T cells (Lu et al., 2009). Of note, upregulation of microRNA miR-155 has been observed in colon cancer (Zhang et al.),

which is particularly relevant given the association that has been made between alcoholism and colon cancer (Cho et al., 2004).

We finally propose that upregulated microRNA expression by ethanol may lead to reduced cytokine responses either directly, or indirectly as in the case of miR181a and 221 via down-regulation of transcription factors that promote their expression. Cytokine expression however undergoes complex transcriptional regulation. Testament to this is the fact that while transcription factors ARNT and STAT3 were downregulated in PBMC, correlating with reduced expression of cytokines G-CSF, VEGF and HGF, other cytokines that may also be regulated by these transcription factors were not downregulated with chronic ethanol exposure. For example, STAT3 also promotes transcription of IL-17, which was induced to comparable levels in a cytokine with equivalent expression between drinker and control PBMC. Thus, we propose this a putative but by no means sole contributory mechanism for the impact increased microRNA expression may have upon cytokine and growth factor expression. Nevertheless, further study of the interaction between ethanol exposure, microRNA expression and immune homeostasis offers a wealth of future translational research

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
cFos-FBJ	murine osteosarcoma viral oncogene homolog
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
MCP-1	Monocyte chemoattractant protein-1
MDC	Macrophage-derived chemokine
MIP1	Macrophage inhibitory factor
PBMC	peripheral blood mononuclear cells
RANTES	regulated and normal T cell expressed and secreted
RUNX1	Runt-related transcription factor 1
STAT3	signal transducer and activator of transcription 3
VEGF	Vascular endothelial growth factor

REFERENCES

- Albasanz-Puig A, Murray J, Namekata M, Wijelath ES. Opposing Roles Of Stat-1 And Stat-3 In Regulating Vascular Endothelial Growth Factor Expression In Vascular Smooth Muscle Cells. *Biochem Biophys Res Commun*. 2012; 428:179–184. [PubMed: 23068100]
- Asirvatham AJ, Gregorie CJ, Hu Z, Magner WJ, Tomasi TB. MicroRNA Targets In Immune Genes And The Dicer/Argonaute And Are Machinery Components. *Mol Immunol*. 2008; 45:1995–2006. [PubMed: 18061676]
- Asquith M, Haberthur K, Brown M, Engelmann F, Murphy A, Al-Mahdi Z, Messaoudi I. Age-Dependent Changes In Innate Immune Phenotype And Function In Rhesus Macaques (Macaca Mulatta). *Pathobiol Aging Age Relat Dis*. 2012; 2
- Bagby G, Zhang P, Stoltz D, Nelson S. Suppression Of The Granulocyte Colony-Stimulating Factor Response To Escherichia Coli Challenge By Alcohol Intoxication. *Alcoholism, Clinical And Experimental Research*. 1998; 22:1740–1745.
- Bala S, Marcos M, Kodys K, Csak T, Catalano D, Mandrekar P, Szabo G. Up-Regulation Of MicroRNA-155 In Macrophages Contributes To Increased Tumor Necrosis Factor {Alpha} (Tnf{Alpha}) Production Via Increased Mrna Half-Life In Alcoholic Liver Disease. *J Biol Chem*. 2011; 286:1436–1444. [PubMed: 21062749]
- Cho E, Smith-Warner S, Ritz J, Van Den Brandt P, Colditz G, Folsom A, Freudenheim J, Giovannucci E, Goldbohm R, Graham S, Holmberg L, Kim D-H, Malila N, Miller A, Pietinen P, Rohan T, Sellers T, Speizer F, Willett W, Wolk A, Hunter D. Alcohol Intake And Colorectal Cancer: A Pooled Analysis Of 8 Cohort Studies. *Annals Of Internal Medicine*. 2004; 140:603–613. [PubMed: 15096331]
- Dhanda AD, Lee RW, Collins PL, Mccune CA. Molecular Targets In The Treatment Of Alcoholic Hepatitis. *World J Gastroenterol*. 2012; 18:5504–5513. [PubMed: 23112542]
- Dudda JC, Salaun B, Ji Y, Palmer DC, Monnot GC, Merck E, Boudousquie C, Utzschneider DT, Escobar TM, Perret R, Muljo SA, Hebeisen M, Rufer N, Zehn D, Donda A, Restifo NP, Held W, Gattinoni L, Romero P. MicroRNA-155 Is Required For Effector Cd8+ T Cell Responses To Virus Infection And Cancer. *Immunity*. 2013; 38:742–753. [PubMed: 23601686]
- Felli N, Fontana L, Pelosi E, Botta R, Bonci DE, Facchiano F, Liuzzi F, Lulli V, Morsilli O, Santoro S, Valtieri M, Calin G, Liu C-G, Sorrentino A, Croce C, Peschle C. MicroRNAs 221 And 222 Inhibit Normal Erythropoiesis And Erythroleukemic Cell Growth Via Kit Receptor Down-Modulation. *Proc Natl Acad Sci U S A*. 2005; 102:18081–18086. [PubMed: 16330772]
- Ferrara N. Vascular Endothelial Growth Factor: Basic Science And Clinical Progress. *Endocrine Reviews*. 2004; 25:581–611. [PubMed: 15294883]
- Grant KA, Leng X, Green HL, Szeliga KT, Rogers LS, Gonzales SW. Drinking Typography Established By Scheduled Induction Predicts Chronic Heavy Drinking In A Monkey Model Of Ethanol Self-Administration. *Alcohol Clin Exp Res*. 2008a; 32:1824–1838. [PubMed: 18702645]
- Grant KA, Stafford J, Thiede A, Kiley C, Odagiri M, Ferguson B. Who Is At Risk?. Population Characterization Of Alcohol Self-Administration In Nonhuman Primates Helps Identify Pathways To Dependence. *Alcohol Health & Research World*. 2008b; 31:289.
- Grigoryev Y, Kurian S, Hart T, Nakorchevsky A, Chen C, Campbell D, Head S, Yates J, Salomon D. MicroRNA Regulation Of Molecular Networks Mapped By Global MicroRNA, Mrna, And Protein Expression In Activated T Lymphocytes. *Journal Of Immunology (Baltimore, Md: 1950)*. 2011; 187:2233–2276.
- Gurung P, Young B, Coleman R, Wiechert S, Turner L, Ray N, Waldschmidt T, Legge K, Cook R. Chronic Ethanol Induces Inhibition Of Antigen-Specific Cd8+ But Not Cd4+ Immunodominant T Cell Responses Following Listeria Monocytogenes Inoculation. *Journal Of Leukocyte Biology*. 2009; 85:34–77. [PubMed: 18820175]
- Happel K, Nelson S. Alcohol, Immunosuppression, And The Lung. *Proceedings Of The American Thoracic Society*. 2005a; 2:428–460. [PubMed: 16322595]
- Happel KI, Nelson S. Alcohol, Immunosuppression, And The Lung. *Proc Am Thorac Soc*. 2005b; 2:428–432. [PubMed: 16322595]

- Helms C, Messaoudi I, Jeng S, Freeman W, Vrana K, Grant K. A Longitudinal Analysis Of Circulating Stress-Related Proteins And Chronic Ethanol Self-Administration In Cynomolgus Macaques. *Alcoholism, Clinical And Experimental Research*. 2012; 36:995–1998.
- Husain K, Ferder L, Ansari R, Lalla J. Chronic Ethanol Ingestion Induces Aortic Inflammation/ Oxidative Endothelial Injury And Hypertension In Rats. *Human & Experimental Toxicology*. 2011; 30:930–939. [PubMed: 20921064]
- Kochanek KD, Xu J, Murphy SL, Miniño AM, Kung HC. National Vital Statistics Reports. *National Vital Statistics Reports*. 2011; 59:1. [PubMed: 22808755]
- Laso F, Iglesias-Osma C, Ciudad J, López A, Pastor I, Orfao A. Chronic Alcoholism Is Associated With An Imbalanced Production Of Th-1/Th-2 Cytokines By Peripheral Blood T Cells. *Alcoholism, Clinical And Experimental Research*. 1999; 23:1306–1311.
- Laso F, Vaquero J, Almeida J, Marcos M, Orfao A. Chronic Alcohol Consumption Is Associated With Changes In The Distribution, Immunophenotype, And The Inflammatory Cytokine Secretion Profile Of Circulating Dendritic Cells. *Alcoholism, Clinical And Experimental Research*. 2007; 31:846–900.
- Lazic T, Sow F, Van Geelen A, Meyerholz D, Gallup J, Ackermann M. Exposure To Ethanol During The Last Trimester Of Pregnancy Alters The Maturation And Immunity Of The Fetal Lung. *Alcohol (Fayetteville, N.Y.)*. 2011; 45:673–680.
- Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P, Klein LO, Davis MM, Chen CZ. Mir-181a Is An Intrinsic Modulator Of T Cell Sensitivity And Selection. *Cell*. 2007; 129:147–161. [PubMed: 17382377]
- Lin J, Qin X, Zhu Z, Mu J, Zhu L, Wu K, Jiao H, Xu X, Ye Q. Fhl Family Members Suppress Vascular Endothelial Growth Factor Expression Through Blockade Of Dimerization Of Hif1alpha And Hif1beta. *Iubmb Life*. 2012; 64:921–930. [PubMed: 23086815]
- Lippai D, Bala S, Csak T, Kurt-Jones EA, Szabo G. Chronic Alcohol-Induced MicroRNA-155 Contributes To Neuroinflammation In A Tlr4-Dependent Manner In Mice. *Plos One*. 2013; 8:E70945. [PubMed: 23951048]
- Louboutin J-P, Marusich E, Gao E, Agrawal L, Koch W, Strayer D. Ethanol Protects From Injury Due To Ischemia And Reperfusion By Increasing Vascularity Via Vascular Endothelial Growth Factor. *Alcohol (Fayetteville, N.Y.)*. 2012; 46:441–454.
- Lu L-F, Boldin M, Chaudhry A, Lin L-L, Taganov K, Hanada T, Yoshimura A, Baltimore D, Rudensky A. Function Of Mir-146a In Controlling Treg Cell-Mediated Regulation Of Th1 Responses. *Cell*. 2010; 142:914–929. [PubMed: 20850013]
- Lu L-F, Thai T-H, Calado D, Chaudhry A, Kubo M, Tanaka K, Loeb G, Lee H, Yoshimura A, Rajewsky K, Rudensky A. Foxp3-Dependent MicroRNA155 Confers Competitive Fitness To Regulatory T Cells By Targeting Socs1 Protein. *Immunity*. 2009; 30:80–91. [PubMed: 19144316]
- Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, Hua M, Li N, Yao H, Cao X. The MicroRNA Mir-29 Controls Innate And Adaptive Immune Responses To Intracellular Bacterial Infection By Targeting Interferon- Γ . *Nature Immunology*. 2011; 12:861–869. [PubMed: 21785411]
- Maloy KJ, Powrie F. Intestinal Homeostasis And Its Breakdown In Inflammatory Bowel Disease. *Nature*. 2011; 474:298–306. [PubMed: 21677746]
- Matsumoto K, Nakamura T. Hepatocyte Growth Factor (Hgf) As A Tissue Organizer For Organogenesis And Regeneration. *Biochem Biophys Res Commun*. 1997; 239:639–644. [PubMed: 9367820]
- Miranda R, Pietrzykowski A, Tang Y, Sathyan P, Mayfield D, Keshavarzian A, Sampson W, Hereld D. MicroRNAs: Master Regulators Of Ethanol Abuse And Toxicity? *Alcoholism, Clinical And Experimental Research*. 2010; 34:575–662.
- Molina PE, Happel KI, Zhang P, Kolls JK, Nelson S. Focus On: Alcohol And The Immune System. *Alcohol Health & Research World*. 2010; 33:97.
- Nguyen-Khac F, Della Valle V, Lopez RG, Ravet E, Mauchauffe M, Friedman AD, Huang LE, Fichelson S, Ghysdael J, Bernard OA. Functional Analyses Of The Tel-Arnt Fusion Protein Underscores A Role For Oxygen Tension In Hematopoietic Cellular Differentiation. *Oncogene*. 2006; 25:4840–4847. [PubMed: 16547490]

- Panopoulos A, Watowich S. Granulocyte Colony-Stimulating Factor: Molecular Mechanisms Of Action During Steady State And 'Emergency' Hematopoiesis. *Cytokine*. 2008; 42:277–365. [PubMed: 18400509]
- Pfaffl M. A New Mathematical Model For Relative Quantification In Real-Time Rt-Pcr. *Nucleic Acids Research*. 2001; 29
- Poonia B, Nelson S, Bagby G, Veazey R. Intestinal Lymphocyte Subsets And Turnover Are Affected By Chronic Alcohol Consumption: Implications For Siv/Hiv Infection. *Journal Of Acquired Immune Deficiency Syndromes* (1999). 2006; 41:537–584. [PubMed: 16652027]
- Rodriguez A, Vigorito E, Clare S, Warren M, Couttet P, Soond D, Van Dongen S, Grocock R, Das P, Miska E, Vetrie D, Okkenhaug K, Enright A, Dougan G, Turner M, Bradley A. Requirement Of Bic/Microrna-155 For Normal Immune Function. *Science (New York, N.Y.)*. 2007; 316:608–611.
- Rouas R, Fayyad-Kazan H, El Zein N, Lewalle P, Rothe F, Simion A, Akl H, Mourtada M, El Rifai M, Burny AN, Romero P, Martiat P, Badran B. Human Natural Treg Microrna Signature: Role Of Microrna-31 And Microrna-21 In Foxp3 Expression. *European Journal Of Immunology*. 2009; 39:1608–1626. [PubMed: 19408243]
- Sibley D, Jerrells T. Alcohol Consumption By C57bl/6 Mice Is Associated With Depletion Of Lymphoid Cells From The Gut-Associated Lymphoid Tissues And Altered Resistance To Oral Infections With Salmonella Typhimurium. *J Infect Dis*. 2000; 182:482–491. [PubMed: 10915079]
- Szabo G, Mandrekar P. A Recent Perspective On Alcohol, Immunity, And Host Defense. *Alcohol Clin Exp Res*. 2009; 33:220–232. [PubMed: 19053973]
- Tacchini L, Matteucci E, De Ponti C, Desiderio MA. Hepatocyte Growth Factor Signaling Regulates Transactivation Of Genes Belonging To The Plasminogen Activation System Via Hypoxia Inducible Factor-1. *Exp Cell Res*. 2003; 290:391–401. [PubMed: 14567996]
- Tomankova T, Petrek M, Gallo J, Kriegova E. Micrornas: Emerging Regulators Of Immune-Mediated Diseases. *Scandinavian Journal Of Immunology*. 2011
- Veazey RS, Rosenzweig M, Shvets DE, Pauley DR, Demaria M, Chalifoux LV, Johnson RP, Lackner AA. Characterization Of Gut-Associated Lymphoid Tissue (GALT) Of Normal Rhesus Macaques. *Clin Immunol Immunopathol*. 1997; 82:230–242. [PubMed: 9073546]
- Von Haefen C, Mei W, Menk M, Klemz R, Jones A, Wernecke KD, Spies CD. Ethanol Changes Gene Expression Of Transcription Factors And Cytokine Production Of Cd4+ T-Cell Subsets In Pbmcs Stimulated With Lps. *Alcohol Clin Exp Res*. 2010; 35:621–631. [PubMed: 21143255]
- Williams J, Baptista P, Daunais J, Szeliga K, Friedman D, Soker S. The Effects Of Ethanol Consumption On Vasculogenesis Potential In Nonhuman Primates. *Alcoholism, Clinical And Experimental Research*. 2008; 32:155–161.
- Xiao C, Srinivasan L, Calado D, Patterson H, Zhang B, Wang J, Henderson J, Kutok J, Rajewsky K. Lymphoproliferative Disease And Autoimmunity In Mice With Increased Mir-17-92 Expression In Lymphocytes. *Nature Immunology*. 2008; 9:405–414. [PubMed: 18327259]
- Zhang H, Li W, Nan F, Ren F, Wang H, Xu Y, Zhang F. Microrna Expression Profile Of Colon Cancer Stem-Like Cells In Ht29 Adenocarcinoma Cell Line. *Biochemical And Biophysical Research Communications*. 2011; 404:273–278. [PubMed: 21130073]
- Zhang X, Yue P, Page BD, Li T, Zhao W, Namanja AT, Paladino D, Zhao J, Chen Y, Gunning PT, Turkson J. Orally Bioavailable Small-Molecule Inhibitor Of Transcription Factor Stat3 Regresses Human Breast And Lung Cancer Xenografts. *Proc Natl Acad Sci U S A*. 2012; 109:9623–9628. [PubMed: 22623533]
- Zimmers TA, McKillop IH, Pierce RH, Yoo JY, Koniaris LG. Massive Liver Growth In Mice Induced By Systemic Interleukin 6 Administration. *Hepatology*. 2003; 38:326–334. [PubMed: 12883476]

Figure 1. Chronic ethanol self-administration reduces PBMC growth factor production
 PBMC were collected from controls (closed circles, $n = 13$) or drinkers (open circles, $n = 14$) and stimulated o/n in the presence of PMA/ionomycin. Cytokine (A), chemokine (B) and growth factor (C) production were determined in supernatants by multiplex bead array. Horizontal lines represent geometric mean, each symbol represents an individual animal. * = $p < 0.05$, *** = $p < 0.001$.

Figure 2. Lung cytokine responses and T cell phenotype are ethanol-independent

Bronchoalveolar lavage (BAL) and Tracheo bronchial lymph node (TBLN) cells were collected from controls (closed circles, $n = 7$) or drinkers that self-administered ethanol for 12 months (open circles, $n = 13-14$ /group). (A–D) The frequency of TNF α , IL-2, IFN γ or IL-17 producing CD4 (A) or CD8 (B) T cells was determined by flow cytometry following 6hr stimulation of BAL cells with PMA/ionomycin. (C–D) The frequency of central memory (CM) and effector memory (EM) CD4 and CD8 T cells was determined by flow cytometry. (E) The ratio of CD4/CD8 T cell frequency is shown. Horizontal lines represent group mean, each symbol represents an individual animal.

Figure 3. T cell cytokine production in intestinal tissues of rhesus macaques following chronic ethanol self-administration

Lymphocytes were isolated from MLN, duodenum, jejunum, ileum and colon from control animals (closed circles, n = 7) or animals self-administering ethanol for 12 months (open circles, n = 10–14/group). Cells were stimulated for 6hr with PMA/ionomycin. The frequency of TNFα+ and IFNγ+IL-17+ CD4 and CD8 T cells was determined by flow cytometry. Within intestinal tissue, cytokine production by intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) is shown, with the exception of colonic IEL. Horizontal bars represent group means, each symbol represents an individual animal. * = p < 0.05.

Figure 4. Increasing ethanol consumption is negatively associated with colonic CD4 T cell cytokine production

Lymphocytes were isolated from colonic lamina propria of controls ($n = 7$, closed circles) or drinkers ($n = 12$, open circles) and stimulated for 6hr with PMA/ionomycin. The frequency of IL-2⁺, TNF α ⁺, IFN γ ⁺, IL-17⁺ and IFN γ ⁺IL-17⁺ CD4 T cells was determined and plotted against each animal's mean daily ethanol intake (g/kg/day) during 12 month ethanol self-administration. Correlations between frequency of cytokine producing cells and ethanol intake were determined by Spearman's rank correlation. Two drinkers (ID# 26234 and 26235) were omitted due to insufficient cell yield for analysis.

Figure 5. Chronic ethanol consumption significantly upregulates expression of microRNAs miR-181a and miR-221 within PBMC and microRNA 155 within colon biopsy

The expression level of microRNAs 181a-5p, 146a-5p, 155-5p, 221-3p, 17-3p, 17-5p, 21-5p and 29a was determined in PBMC (A) and colon tissue (B) from controls (n = 14, closed circles) or drinkers (n = 14, open circles) by RT-qPCR. Expression is shown relative to housekeeping gene U6 (AU = arbitrary units). Horizontal lines represent group geometric means, each symbol an individual animal. * = p < 0.05.

Figure 6. Chronic ethanol consumption significantly downregulates expression of transcription factors ARNT and STAT3 in PBMC

(A) The expression level of transcription factors that regulate growth factor production and are predicted targets of microRNAs miR-181a and miR-221 was determined by RT-qPCR in PBMC from controls (n = 11, closed circles) or drinkers (n = 9, open circles). Expression is shown relative to housekeeping gene glutathione synthetase. Horizontal bars represent group geometric means, each symbol represents an individual animal. (B) The expression levels of STAT3 and ARNT protein levels was determined in PBMC from controls (n=4) or drinkers (n=4). (C) Quantification of the immunoblot intensity. * = $p < 0.05$.

Figure 7. miR-181 and miR-221 modulates transcription factors STAT3 and ARNT and growth factors VEGF, HGF and G-CSF protein expression
 (A–C) Western blot analysis of STAT3 and ARNT expression from PMA/ionomycin stimulated PBMC cells transfected with non-targeting control, miR-181 and miR-221 mimics (A), or with non-targeting control, miR-181 and miR-221 antagonists (B). Immunoblot intensity is summarized in panel (C). (D–F) Western blot analysis of VEGF and HGF expression from PMA/ionomycin stimulated PBMC cells transfected with non-targeting control, miR-181 and miR-221 mimics (D), or with non-targeting control, miR-181 and miR-221 antagonists (E). Immunoblot intensity is summarized in panel (F). (G)

Concentration of G-CSF in tissue culture supernatant. One of three independent experiments is illustrated.

Table 1

Summary of animals and drinking behavior in this study

Animal ID	Sex	Age at EtOH onset	Weight Pre-EtOH (kg)	Weight at Necropsy (kg)	Mean daily Ethanol intake (g/kg/day)	Group
26077	M	4 yrs 5 months	7.02	8.60	0	control
26082	M	4 yrs 5 months	6.54	8.75	0	control
26089	M	4 yrs 5 months	6.60	8.45	0	control
26104	M	4 yrs 5 months	5.70	6.90	0	control
25811	M	4 yrs 3 months	6.80	8.74	1.8	drinker
25790	M	4 yrs 8 months	6.46	7.65	1.9	drinker
26016	M	4 yrs 4 months	6.76	8.00	1.9	drinker
25742	M	4 yrs 4 months	6.70	7.40	2.3	drinker
25787	M	4 yrs 6 months	6.32	7.20	2.8	drinker
26148	M	4 yrs 4 months	6.38	8.60	3	drinker
25882	M	4 yrs 4 months	7.52	8.95	3.1	drinker
26168	M	4 yrs	5.52	8.75	3.3	drinker
25700	F	4 yrs 3 months	4.46	6.80	0	control
25893	F	4 yrs 3 months	4.63	5.80	0	control
26132	F	4 yrs 3 months	4.36	4.85	0	control
26234	F	4 yrs 2 months	4.64	6.30	3.8	drinker
26231	F	4 yrs 2 months	4.69	5.55	4.1	drinker
26235	F	4 yrs 2 months	4.34	4.60	4.2	drinker
25885	F	4 yrs 1 month	4.16	4.95	4.2	drinker
26158	F	3 yrs 10 months	4.56	5.20	5.2	drinker
25797	F	4 yrs 2 months	4.03	4.85	5.6	drinker

Mean daily ethanol intake, as calculated during the period of 12 month ethanol self-administration is shown.