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Alcohol consumption modulates host defense in rhesus macaques by altering gene expression in circulating leukocytes

Tasha Barr^{*}, Thomas Girke[†], Suhas Sureshchandra^{*}, Christina Nguyen^{*}, Kathleen Grant[‡], and Ilhem Messaoudi^{*.§}

^{*}Division of Biomedical Sciences, School of Medicine, University of California-Riverside, Riverside, CA 92521, USA

[†]Institute of Integrative Genome Biology, University of California-Riverside, Riverside, CA 92521, USA

[‡]Division of Neurosciences, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR 97006, USA

Abstract

Several lines of evidence indicate that chronic alcohol use disorder leads to increased susceptibility to several viral and bacterial infections whereas moderate alcohol consumption decreases incidence of colds and improves immune responses to some pathogens. In line with these observations, we recently showed that heavy ethanol intake (average blood ethanol concentrations (BECs) >80 mg/dl) suppressed, whereas moderate alcohol consumption (BEC <50 mg/dl) enhanced T and B-cell responses to Modified Vaccinia Ankara (MVA) vaccination in a nonhuman primate model of voluntary ethanol consumption. To uncover the molecular basis for impaired immunity with heavy alcohol consumption and enhanced immune response with moderate alcohol consumption, we performed a transcriptome analysis using PBMCs isolated on day 7 post-MVA vaccination, the earliest time point at which we detected differences in T-cell and antibody responses. Overall, chronic heavy alcohol consumption reduced expression of immune genes involved in response to infection and wound healing, and increased expression of genes associated with the development of lung inflammatory disease and cancer. In contrast, chronic moderate alcohol consumption upregulated expression of genes involved in immune response and reduced expression of genes involved in cancer. In order to uncover mechanisms underlying the alterations in PBMC transcriptomes, we profiled the expression of microRNAs within the same samples. Chronic heavy ethanol consumption altered the levels of several microRNAs involved in cancer and immunity and known to regulate expression of mRNAs differentially expressed in our dataset.

INTRODUCTION

Alcohol use disorder (AUD) results in a significant increase in both incidence and severity of infections such as bacterial pneumonia, tuberculosis, hepatitis C virus, and HIV (1–3).

[§]To whom correspondence should be addressed: Ilhem Messaoudi, PhD, University of California Riverside, 900 University Avenue, Riverside, CA 92521, Tel.: 951-827-7774, Fax: 951-579-4118, ilhem.messaoudi@ucr.edu.

Similarly, chronic ethanol consumption in rodents results in increased pathogen burden and impaired ability to clear *Listeria monocytogenes* (4), *Mycobacterium tuberculosis* (5), and influenza virus (6). Likewise, rhesus macaques given ethanol via intragastric cannula show increased simian immunodeficiency virus replication compared to controls (7). Increased vulnerability to infection in individuals with AUD is due to changes in barrier function as well as innate and adaptive immunity (8). Dysregulation of tight junction proteins in the lungs and gut increases permeability, leading to bacterial translocation into the alveolar space and circulation, respectively (9, 10). In addition, AUD results in the inhibition of phagocytic functions, reduction of chemotaxis and aberrant cytokine production, and diminished lymphocyte numbers and antigen-specific responses (11).

In contrast, data from several studies support a beneficial role for moderate alcohol consumption on immunity. Moderate alcohol consumption is associated with decreased incidence of the common cold in humans (12–14) as well as improved bacterial clearance and increased delayed cutaneous hypersensitivity response following infection with *Mycobacteria bovis* in rats (15). Recently, we showed using a macaque model of ethanol self-administration (16) that moderate consumption resulted in a more robust T-cell and antibody vaccine response to Modified Vaccinia Ankara (MVA), while heavy drinkers generated blunted T-cell and antibody response compared to controls (17). Moreover, we showed that the dose-dependent effects of ethanol on the immune response to the MVA vaccine were independent of changes in frequency of major immune cell subsets. Specifically, numbers of circulating lymphocyte, monocyte, and neutrophil as well as the frequency of CD4 T cell, CD8 T cell, and CD20 B cells (and their naïve and memory subsets) did not differ between control and ethanol consuming animals (17). Instead, we detected changes in the expression of several microRNAs (miRNAs) associated with development and function of the immune system, suggesting that ethanol dose-dependent modulation of immunity is mediated by changes in gene expression. Therefore, in this study, we compared the transcriptomes of PBMCs isolated from controls, moderate, and heavy drinkers on day 7 post-MVA vaccination.

Our results revealed that chronic heavy ethanol consumption was associated with significant downregulation of genes involved in immune response to infection and wound healing as well as upregulation of genes associated with development of obstructive lung disease and cancer. In contrast, chronic moderate alcohol consumption was associated with reduced expression of genes involved in neoplasia and the upregulation of genes involved in host defense. In order to uncover mechanisms underlying the alterations in PBMC transcriptomes, we also examined changes in miRNA expression. Our analysis showed that chronic heavy ethanol consumption altered the expression of several miRNAs whose targets were differentially expressed in our data set and are involved in cancer progression and immune function. Overall, data presented in this manuscript provide novel insight into the mechanisms by which excessive alcohol consumption interferes with immune responses, and exacerbates co-morbidities such as poor wound healing, lung disease, and cancer, while moderate consumption improves immunity.

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).

Animal studies and sample description

The animal model and vaccination strategy were previously described (17). Briefly, we used schedule-induced polydipsia to establish reliable self-administration of 4% (w/v) ethanol in 8 male rhesus macaques (16). Four animals served as controls for a total of 12 animals. Following a 4-month induction period, animals were allowed a choice of 4% ethanol or water for 22hr/day every day for 12 months. In this nonhuman primate model of voluntary self-administration, animals segregate naturally into heavy and moderate drinkers within 2–3 months, and these patterns remain stable for at least 12 months (18). In this specific cohort, ethanol-drinking animals segregated into two cohorts, n=4 each based on average blood ethanol (BEC) values: moderate drinkers with average BEC of 22.3–48.8 mg/dl and heavy drinkers with average BEC of 90–126 mg/dl (17). All 12 animals were vaccinated with MVA prior to induction of ethanol and again after 7 months of open access to ethanol. We used PBMCs isolated 7 days after booster vaccination for RNA and microRNA expression analysis. Only 3 animals from each group had sufficient numbers of PBMCs for RNA sequencing.

RNA isolation and mRNA library preparation

Total RNA was isolated from PBMCs using the miRNeasy kit (Qiagen, Valencia, CA). One microgram of RNA was used to generate libraries using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). Poly(A)-enriched mRNA was fragmented followed by cDNA synthesis with random hexamers. This product underwent end-repair, adapter ligation, and size selection using AMPure XP beads (Beckman Coulter Inc., Brea, CA) to isolate cDNA templates of 320 nucleotides that were amplified by PCR. Each library was prepared with unique index primers for multiplexing and subjected to single-end 100 bp sequencing on the HiSeq2500 platform (Illumina, San Diego, CA).

Small RNA library preparation

One microgram of total RNA extracted as described above underwent adapter ligation and primer hybridization prior to cDNA synthesis and PCR amplification using the NEBNext Small RNA Library Prep Set for Illumina Kit (New England Biolabs, Ipswich, MA). Size selection was performed with AMPure XP beads (Beckman Coulter Inc., Brea, CA) to isolate cDNA templates of 140 nucleotides. Each library was prepared with unique index primers for multiplexing and subjected to single-end 50 bp sequencing on the HiSeq2500 platform (Illumina, San Diego, CA). We were unsuccessful in generating one library from one of the heavy drinkers.

RNA-Seq analysis

Data analysis was performed with the RNA-Seq workflow module of the *systemPiperR* package available on Bioconductor (19, 20). Quality reports were generated with the *seeFastq* function. RNA-Seq reads were mapped with the splice junction aware short read alignment suite Bowtie2/TopHat2 (21, 22) against the *Macaca mulatta* genome sequence from Ensembl (23). For the alignments, we used default parameters of TopHat2 optimized for mammalian genomes. Raw expression values in form of gene-level read counts were generated with the *summarizeOverlaps* function (24). Here, we counted only reads overlapping exonic regions of genes, discarding reads mapping to ambiguous regions of exons from overlapping genes. Given the non-stranded nature of RNA-Seq libraries, the read counting was performed in a non-strand-specific manner. The RNA sequencing data have been deposited in NCBI's Sequence Read Archive (SRA) under the accession number SRP064253 (<http://www.ncbi.nlm.nih.gov/sra>). Analysis of differentially expressed genes (DEGs) was performed with the GLM method from the *edgeR* package (25, 26). DEGs were defined as those with a fold change of ≥ 2 and a false discovery rate (FDR) of ≤ 0.05 . Enrichment analysis of functional annotations was performed to identify significant biological pathways including gene ontology (GO) terms and disease biomarkers using MetaCore™ software (GeneGo, Philadelphia, PA).

Small RNA-Seq analysis

Adaptor contaminations were removed (trimmed) from the reads using the *preprocessReads* function from the *systemPipeR* package. The preprocessed reads were aligned with Bowtie2 (21, 22) against the *Macaca mulatta* genome sequence with settings optimized for miRNA alignments, including tolerance of multiple mappings. Reads overlapping with miRNA gene ranges were counted with the *summarizeOverlaps* function as described above, but in a strand-specific manner. The miRNA gene coordinates, required for this step, were downloaded from miRBase (Release version 19). The small RNA sequencing data have been deposited in NCBI's SRA under the accession number SRP064540 (<http://www.ncbi.nlm.nih.gov/sra>). Differentially expressed miRNA genes were identified with *edgeR* as described above. TargetScan was used to predict genes for each differentially expressed microRNA with a high context ratio of 0.95. These targets were then compared with our list of differentially expressed genes among the three groups of rhesus macaques. These combinations of differentially expressed mRNA and miRNA were then segregated based on the directions of fold changes.

Gene validation via qRT-PCR

cDNA was synthesized from RNA isolated as above, using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). mRNA expression was determined by quantitative reverse transcription PCR using Taqman primer and probe kits specific for *Macaca mulatta* cDNA sequences and a StepOnePlus instrument (Life Technologies, Grand Island, NY). mRNA expression levels of LYZ (Rh02902590), PTGS2 (Rh02787804), THBS1 (Rh00962902), KLF4 (Rh02847953), TLR4 (Rh01060206), CD14 (Rh03648680), CD163 (Hs00174705), and FN1 (Rh02621780) for each sample were calculated relative to control RPL32 mRNA expression using $\Delta\Delta C_t$ calculations.

RESULTS

Heavy alcohol consumption leads to significant changes in gene expression

There were 514 DEGs between controls and heavy drinkers (C7-H7), 479 of which were annotated with 356 downregulated and 123 upregulated genes with heavy drinking (Supplementary Table I). We identified 368 DEGs between moderate and heavy drinkers (M7-H7), 347 of which were annotated with 290 downregulated and 57 upregulated genes with heavy drinking (Supplementary Table II). Finally, of the 60 DEGs between controls and moderate drinkers (C7-M7), 47 were annotated with 29 downregulated and 18 upregulated with moderate ethanol consumption (Supplementary Table III, Fig. 1A).

Heavy ethanol consumption was associated with the largest changes in gene expression compared to both controls and moderate consumption (Fig. 1A, 1B), with 171 downregulated (Fig. 1C), and 26 upregulated DEGs compared to controls and moderate drinkers (Fig. 1D). The overwhelming majority of the downregulated (Fig. 1E) and upregulated (Fig. 1F) DEGs showed a 2–4 fold-change in expression. To confirm the RNA-Seq results, 8 genes differentially expressed with chronic heavy ethanol consumption (LYZ, PTGS2, THBS1, KLF4, TLR4, CD14, CD163, and FN1) were selected for confirmation using qRT-PCR. Changes in expression level of all 8 DEGs were confirmed (Supplementary Fig. 1). In order to better understand the biological relevance of these gene expression changes, we conducted functional enrichment analysis using the MetaCore™ pathway-mapping tool.

Heavy alcohol consumption downregulates genes that promote host defense compared to both moderate drinkers and controls

Of the 171 genes repressed in H7 compared to C7 and M7, 114 mapped to the following GO terms: response to stress, response to wounding, inflammatory response, response to lipid, and positive regulation of response to external stimulus (Fig. 2A). Several DEGs mapping to “response to stress” encode microbial sensors, notably formyl peptide receptor 2 (FPR2, FC=128.2), TLR4 (FC=5.6), CD14 (FC=3.8), pyrin domain-containing-3 (NLRP3, FC=3.3), TLR8 (FC=2.9), and nucleotide-binding oligomerization domain-containing-2 (NOD2, FC=2.4). Other DEGs encode immune receptors such as cadherin EGF LAG seven-pass G-type receptor-1 (CELSR1, FC=30.6), syndecan-2 (SDC2, FC=7.8), plasminogen activator receptor (PLAUR, FC=6.5), macrophage scavenger receptor-1 (MSR1, FC=5.7), IL-1 receptor type-1 (IL1R1, FC=5.7), IL-13 receptor alpha-1 (IL13RA1, FC=4.8), CCR1 (FC=4.5), and neuropilin-1 (NRP1, FC=3.4). Additional DEGs encode chemokines, cytokines, growth factors, and antimicrobial peptides, including matrix metalloproteinase-1 (MMP1, FC=8.6), CXCL8 (FC=7.5), oncostatin-M (OSM, FC=4.4), IL-1β (FC=3.7), vascular endothelial growth factor-A (VEGFA, FC=5.2), heparin-binding EGF-like growth factor (HBEGF, FC=3.4), and S100 calcium binding protein-8/9 (S100A8/9, FC =3.6/2.9).

Several genes listed above also mapped to “response to wounding” and “inflammatory process” and play a role in wound healing (Fig. 2B). For instance, CELSR1, SDC2, VEGFA, HBEGF, and NRP1 promote wound closure (27, 28), while NOD2, NLRP3, CD14, coagulation factor plasminogen activator inhibitor-2 (SERPINB2, FC=17.6), complement

component 5a receptor-1 (C5AR1, FC=4.0) and 3a receptor-1 (C3AR1, FC=2.9), and IL-1 β (which activates CXCL8 FC=7.5) promote chemotaxis and leukocyte extravasation into injury sites (29–32).

Additional analysis showed that 89 genes map to these disease categories: obstructive lung diseases, pathologic processes, hypersensitivity, bacterial infection and mycoses, and inflammation (Fig. 2C). Of the 52 genes mapping to “obstructive lung diseases” (Fig. 2D), 24 interact with each other (Fig. 2E), and are important for lung homeostasis, notably MMP1 (FC=8.6, involved in lung alveolar epithelial cell migration (33)), VEGFA (important for alveolar structure (34)), cathelicidin antimicrobial peptide (CAMP/LL37, FC=2.8), and aryl hydrocarbon receptor (AHR, FC=2.7, regulates apoptosis of lung epithelial cells (35)). DEGs mapping to “pathological processes” include Annexin-2 receptor (ANXA2R, FC=17.1), cysteine-rich secretory protein LCCL domain-containing-2 (CRISPLD2, FC=9.7), epiregulin (EREG, FC=9.2), triggering receptor expressed on myeloid cells-1 (TREM1, FC=4.7), and HBEGF, which are involved in protection against cancer, sepsis, endotoxin shock, and necrotizing enterocolitis (36–40).

Heavy drinking downregulates genes that promote wound healing and protect against chronic disease compared only to controls

Of the 185 DEGs repressed in H7 compared to C7, 128 mapped to these GO terms: response to wounding, regulation of response to stimulus, positive regulation of response to stimulus, response to stimulus, and system development (Fig. 3A). The 37 genes mapping to “response to wounding” play an important role in wound healing (Fig. 3B) including diacylglycerol kinase (DGKH, FC=5.8, regulates fibroblast migration (41)), catenin alpha-1 (CTNNA1, FC=3.4, promotes wound repair in bronchial epithelial cells (42)), metalloproteinase inhibitor-2 (TIMP2, FC=3.4, involved in wound closure (43)), solute carrier family-11 (SLC11A1, FC=2.2, regulates macrophage activation in cutaneous wounds (44)), CSF1 (FC=2.1, involved in neoangiogenesis (45)), and hepatocyte growth factor (HGF, FC=2.0, accelerates wound re-epithelialization (28)). One highly downregulated gene mapping to “response to stimulus” is eosinophil peroxidase (EPX, FC=22.6), a potent toxin for bacteria and parasites (46).

Further analysis showed 106 genes mapped to these disease categories: obstructive lung diseases, pathological processes, immune system diseases, bronchial diseases, and immediate hypersensitivity (Fig. 3C). Genes in “obstructive lung diseases” play an important role in lung function (Fig. 3D). For instance, IL-1 receptor-like-1 (IL1RL1, FC=9.2), TLR5 (FC=6.0), arachidonate 15-lipoxygenase (ALOX15, FC=5.5), TREM2 (FC=2.6), and TNF-receptor superfamily-1A (TNFRSF1A, FC=2.0) promote host defense against bacterial infection in the lungs and regulate lung inflammation, whereas HGF (FC=2.0) promotes lung regeneration after injury (28). Polymorphisms in myeloperoxidase (MPO, FC=7.4) and A Disintegrin and Metalloproteinase domain-12 (ADAM12, FC=4.5) modulate the development of lung cancer (47, 48).

Several DEGs that mapped to “immune system diseases” also mapped to “obstructive lung diseases” and “pathological processes” including C-type lectin domain family-10A (CLEC10A, FC=4.8) and ATP-binding cassette subfamily-C-2/3 (ABCC2/3, FC=2.9/3.2),

which play a role in antigen recognition and presentation (49, 50); as well as EGF-like module receptor-1 (EMRI, FC=3.7) and interleukin-12 receptor-beta-2 (IL12R β 2, FC=2.8), which are critical for host defense (51). Genes unique to this category (Fig. 3E) regulate inflammation such as leucine-rich repeat-containing-18 (LRRC18, FC=104.5) and hemoglobin subunit gamma-2 (HBG2, FC=8.9 (52, 53)); as well as lymphocyte proliferation and differentiation including SH2B adaptor-3 (SH2B3, FC=2.0 (54)) and killer cell lectin-like receptor subfamily-G1 (KLRG1, FC=2.4 (55)).

Heavy alcohol consumption reduces expression of genes that regulate the immune system compared to moderate consumption only

Of the 119 genes downregulated in H7 compared to M7, 66 mapped to the following GO terms: immune system processes, response to stress, immune response, defense response, and regulation of immune system processes (Fig. 4A). In total, 47 mapped to immune system-related GO terms, 20 of which interact with each other (Fig. 4B). DEGs mapping to these GO terms are involved in: 1) lymphocyte activation and recruitment (CXCL10, FC=17.3; SLC16A1, FC=3.2 (56); sterile alpha-motif-domain Src homology-domain nuclear localization signals-1, SAMS1, FC=3.7 (57); ICAM1, FC=5.5; CD83, FC=3.0 (58)); 2) antimicrobial response (TNF α , FC=7.9; ficolin-2, FCN2, FC=6.5 (59); MD2, FC=2.8); and 3) regulation of gene expression (v-ets avian erythroblastosis virus oncogene homolog-2, ETS2, FC=2.5; B-cell lymphoma 2-related protein-A1, BCL2A1, FC=5.4 (60); B-cell CLL/lymphoma-3/6, BCL3/6, FC=2.5/2.0 (61, 62); Kruppel-like factor-10, KLF10, FC=3.0 (63); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha, NFKBIA, FC=2.9 (64)).

Several genes mapping to “immune system process” (Fig. 4C) also map to “response to stress”, notably hypoxia-inducible factor-1-alpha (HIF1A, FC=3.0) that regulates expression of genes that counter oxidative stress and ETS2, which is induced by shear stress to preserve integrity of microvascular walls (65). Additional notable DEGs that only mapped to “response to stress” included Gadd45-gamma (GADD45G, FC=8.4, important in anti-tumor immune responses (66)) and BMX non-receptor tyrosine kinase (BMX, FC=11.5, promotes tight junction formation in epithelial cells during chronic hypoxia (67)).

Heavy drinking increases expression of genes associated with impaired wound healing, cardiovascular disease and cancer

Within the 26 genes upregulated in H7 compared to both C7 and M7 (Fig. 5A), several encode transcription factors associated with skin, colorectal, breast, and lymphoma cancers, notably IFN alpha-inducible protein 27-like-1 (IFI27L1, FC=68.0 (68)), AXIN2 (FC=2.2 (69)), lymphoid enhancer-binding factor-1 (LEF1, FC=2.0 (70)), Meis homeobox-1 (MEIS1, FC=2.4 (71)), and four-and-a-half LIM domains-1 (FHL1, FC=2.1 (72)). Interestingly, increased expression of retinoid X receptor-gamma (RXRG, FC=5.2), which is associated with sensation seeking (73), a behavioral trait common among alcoholics, was detected. Another overexpressed gene was serum deprivation protein response (SDPR, FC=2.8), which induces deformation of plasma membrane invaginations impairing endocytosis and potentially antigen presentation (74). Finally, expression of regulator of G-protein signaling-18 (RGS18, FC=3.3), growth factor-independent 1B transcription repressor

(GFI1B, FC=2.3), and rho guanine nucleotide exchange factor-4 (ARHGEF4, FC=6.1), which play a role in megakaryocyte differentiation (75, 76), were also increased.

Of the 97 genes upregulated in H7 compared to only C7, 26 mapped to the following GO terms: response to wounding, regulation of body fluids, platelet activation, wound healing, and platelet degranulation. Genes in “response to wounding” (Fig. 5B) include connexin-43 (GJA1, FC=11.0), a gap junction associated with impaired wound healing (77); IL-17F (FC=6.7), which can delay wound closure (78); and P-selectin (SELP, FC=2.3), a glycoprotein highly expressed in wounds (79). Genes with roles in cardiovascular disease mapped to the disease category “infarction” including carbonic anhydrase III (CA3, FC=5.2), glycoprotein VI (GP6, FC=2.9), phosphodiesterase-6H (PDE6H, FC=2.6), and integrin-alpha-2b (ITGA2B, FC=2.6 (80–83)). Furthermore, heavy ethanol consumption upregulated genes associated with cancer, notably transient receptor potential cation channel-M1 (TRPM1, FC=14.9), tripartite motif containing-31 (TRIM31, FC=9.8), RGS6 (FC=7.6), and CLDN5 (FC=2.2 (84–86)).

Twenty-one of the 31 genes upregulated in H7 compared to only M7 mapped to “neuroectodermal tumors” (Fig. 5C). These genes are either expressed at high levels in cancer including delta-like-1 (DLK1, FC=7.3 (87)) and insulin receptor substrate-1 (IRS1, FC=4.8 (88)); or involved in progression of cancer such as phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor-2 (PREX2, FC=11.7 (89)), latent transforming growth factor-beta-binding protein-2/3 (LTBP2/3, FC=3.6/2.6 (90, 91)), and stromal antigen-3 (STAG3, FC=2.3 (92)).

Moderate drinking activates genes associated with immunity and represses genes associated with cancer compared to controls

Of the 29 annotated genes upregulated in M7 compared to C7 (Fig. 6A), four play a role in chemotaxis: CXCL3 (FC=50.3, critical for leukocyte chemotaxis), IL-1 α (FC=23.4, recruitment of neutrophils), CCL3 (FC=17.1, recruits T-cells (93)), and CCL4L1/2 (FC=8.1/5.5, trafficking of NK cells (94)). Other genes significantly upregulated in M7 include acute phase protein pentraxin-3 (PTX3, FC=15.3 (95)); ceruloplasmin (CP, FC=3.7) and granzyme A (GZMA, FC=2.2), expressed primarily by NK cells and play a role in host defense (96, 97); and GJA1 (FC=9.6), important for barrier function (98).

Several of the 18 genes downregulated in M7 compared to C7 (Fig. 6B) are involved in cancer progression including transmembrane protein-98 (TMEM98, FC=286.9), serine-protease temperature requirement-A1 (HTRA1, FC=24.1), DNA nucleotidylexotransferase (DNMT, FC=5.7), MMP9 (FC=3.5), and ten-eleven translocation-1 (TET1, FC=3.0 (99–103)). Interestingly, ABCA9 (FC=6.9), retinoic acid-binding receptor-related orphan receptor-C (RORC, FC=3.6), 2'-5'-oligoadenylate synthetase-2 (OAS2, FC=2.6), CD84 (FC=2.2), and myxovirus resistance protein-1 (MX1, FC=2.2), which play a role in innate immunity, were downregulated in M7.

Heavy ethanol consumption alters the expression of microRNAs involved in cancer and immune function

To begin uncovering the mechanisms underlying changes in gene expression regulation with moderate and heavy ethanol consumption, we compared the miRNA expression profiles of the same PBMCs isolated from controls, moderate, and heavy drinkers on day 7 post-MVA vaccination. MiRNAs are ~22 nucleotide long endogenous RNAs that target mRNAs for translational repression or degradation (104), and several reports indicate that ethanol can modulate miRNA expression (105). As described for mRNA expression, the largest differences in miRNA expression were observed between controls and heavy drinkers, and only a few miRNAs were differentially expressed between controls and moderate drinkers. Interestingly, no differentially expressed miRNAs were detected between heavy and moderate drinkers. There were 79 differentially expressed miRNAs between controls and heavy drinkers, 37 of which were upregulated (Fig. 7A) and 42 were downregulated (Fig. 7B).

Importantly, 53 of these miRNAs have mRNA targets within our dataset. Heavy drinking led to the upregulation of 29 microRNAs known to regulate 25 target mRNAs that were downregulated in our dataset. A subset of these mRNA-miRNA pairs are shown in Fig. 7C; for a complete list, please refer to Table 1. Some of the downregulated mRNAs were targeted by several differentially expressed miRNAs. For instance, miR-16, miR-15b, miR-195, and miR-374b target VEGFA, which was downregulated >5-fold. Similarly, miR-30b and miR-30c target SH2B3, downregulated 2 fold, while miR-125a and miR-125b both target SCARB1, which was downregulated >3-fold. Of the 24 microRNAs downregulated with heavy drinking, 7 were associated with an increase in their mRNA targets (Fig. 7C, Table 1). For example, the downregulated miR-101 targets GJA1, which was upregulated 11-fold with heavy drinking. MiR-144 targets AXIN2, which was upregulated 2-fold. MiR-183 and miR-202 both target ROBO2, which was upregulated more than 3-fold. Finally, miR-29a, miR-29b, and miR-29c all target SH3PXD2A, which was upregulated 4-fold in our dataset.

DISCUSSION

Using a macaque model of ethanol self-administration, we recently showed that heavy ethanol consumption suppresses, whereas moderate ethanol consumption enhances, T and B-cell responses to MVA (16). The goal of this study was to uncover the molecular mechanisms underlying this dose-dependent effect. We used RNA-Seq to identify changes in gene expression on day 7 following vaccination with MVA, the earliest time point at which we detected differences in antibody and T-cell responses (16). We also sequenced small RNA molecules to gain insight into mechanisms underlying the changes in gene regulation. Overall, our study revealed robust changes in gene and miRNA expression between control, moderate, and heavy drinkers, with fewer changes between moderate drinkers and controls compared to either group versus heavy drinking status.

The changes in gene expression reported herein provide novel insight into the reduced immune response to vaccination and the increased vulnerability to infection seen in humans with AUD. Specifically, we detected large decreases in the expression of microbial sensors

that are critical in the detection of bacterial peptides (FPR2/3 (106)), LPS (MD2, CD14, TLR4), bacterial flagellin (TLR5), and certain helminths and filoviruses (CLEC10A (49)). There was also decreased expression of genes important for antigen presentation (ABCC2/3, CLEC10A (49, 50)); recruitment of immune cells (C3AR1, C5AR1, CCR2, CXCL3, CXCL8, CXCL10, CCL3, CCL4L1/2, ICAM1 (31, 93, 107–109)); and soluble mediators that play a role in response to infection (TNF α , TNFRSF1A, IFN γ , IFNGR1, IL-1R, IL-1R1, IL-1RL1, IL-1 β , S100A8/9, LL37, EPX, GRZMA (46, 110)). As previously described (111), expression of interferon signaling interferon receptor-2 (IFNAR2) was downregulated in heavy drinkers, which would contribute to deficits in both innate and adaptive immunity.

We also found decreased expression of lymphocyte activation markers including CD83 and KLRG1 (55). In our previous study, excessive ethanol consumption suppressed whereas moderate ethanol consumption enhanced MVA-specific IgG responses (17). These defects in antibody production could be partially explained by the decreased expression of transcription factors BCL3 and BCL6, which are important in germinal center formation, isotype class switching, and hypermutation (61, 62), SH2B3, which regulates B-cell development (54), and SAMSN1, an adapter protein involved in immune cell signaling (57). Reduced expression of KLF10, which suppresses regulatory T-cells by upregulating TGF β (63), could further explain the reduction in T and B-cell responses.

Our study also revealed insight into the mechanisms by which moderate alcohol consumption stimulates immunity. Animals that drank moderate amounts of ethanol showed increased expression of chemokines CCL3 and CCL4L1, which signal through CCR5 to recruit memory T-cells (93), and IL-1 α and CXCL3, which recruit neutrophils. Although a vigorous innate immune response is critical, it is equally important for the host to minimize damaging inflammation. PTX3, which plays a role in the resolution of inflammation (95), and the NF κ B inhibitor, NFKBIA, were significantly upregulated with moderate drinking. These observations are in line with previous studies that showed that moderate alcohol consumption in humans significantly alters genes involved in B-cell, T-cell, and IL-15 signaling pathways, and attenuates NF κ B signaling pathways in leukocytes (112).

Several of the genes that were differentially expressed with ethanol consumption have previously been described as being important for mounting immune responses to vaccination. For instance, studies that investigated yellow fever vaccine (YF-17D)-induced signatures in blood of healthy adults have reported significant increases in the expression of proinflammatory mediators CXCL10 and IL-1 α and complement gene C3AR1, which were found to be predictive of robust vaccine responses (113). Another study identified ETS2 as an additional key regulator of the early innate immune response to YF-17D (114). In our study, we observed a 17-fold downregulation of CXCL10, a 3-fold downregulation of C3AR1, and a 2.5-fold downregulation of ETS2 with excessive ethanol consumption, which could explain the suppression of vaccine responses in this cohort. In contrast, we detected a 23-fold upregulation of IL-1 α in moderate drinkers compared to controls, re-enforcing the association of this marker with successful immune responses.

Our gene expression analysis is also in line with clinical observations linking alcohol abuse with impaired wound healing (115), increased susceptibility of wound infections (116), and delay of wound closure (117). This defect has significant clinical ramifications since half the emergency room trauma cases involve alcohol exposure (118). Previous studies showed that ethanol exposure at the time of traumatic injury impairs wound closure via decreased pro-inflammatory cytokine release, neutrophil recruitment, and phagocytic function (115). Our gene expression data support and extend these earlier observations. We detected significantly decreased expression of multiple components of the innate immune system that play a critical role in the prevention of wound infections including pattern recognition receptors (FPR2/3, NALP3, NOD2, MD2, CD14, TLR4, TLR5, TLR8, CLEC10A, SCARB1), proinflammatory cytokines and their receptors (TNFRSF1A, IFN γ , IFN γ 1, IL-1R, IL-1R1, IL-1RL1, IL-1 β , IL13RA1), as well as chemokines and their receptors (CXCL2, CXCL8, CXCL10, CCR1, CCR2, OSM, CSF3R, CSF1).

Earlier studies suggested the disruption of VEGF signaling and reduced expression of HIF-1 α in endothelial cells with chronic alcohol consumption interferes with wound closure (117). Our gene expression data also show a significant decrease in the expression of both of those genes with excessive ethanol consumption. Moreover, we detected fewer transcripts of NRP1, which is expressed by endothelial cells and associates with the VEGF receptor to promote angiogenesis and wound repair (119). We also report decreased expression of CELSR1 and SDC2 that have been shown to promote effective wound repair (27, 120) as well as secreted proteins such as fibronectin and MMP-1 which promote platelet aggregation, angiogenesis, and tissue remodeling (121, 122). The expression of additional growth factors, HGF and HB-EGF, which promote angiogenesis and tissue regeneration, was also significantly decreased (28). Furthermore, heavy alcohol abuse increased expression of additional genes known to interfere with wound healing (PSEL, VWF, CX43, IL-17 (78, 79, 123)).

Additionally, our data is in line with clinical observations that heavy alcohol consumption is associated with increased incidence of chronic obstructive pulmonary disease (COPD (124, 125)), lung injury in response to inflammatory insults (126), acute respiratory distress syndrome (ARDS (127)), and risk of mortality in acute lung injury patients (128). Impaired immunity and increased oxidative stress, both consequences of AUD, are considered risk factors for COPD and ARDS (129). Our transcriptome analysis provides new insight into the mechanisms that underlie increased susceptibility and severity of lung injury and chronic lung inflammatory diseases. Heavy ethanol consumption was associated with downregulation of several genes important for maintaining lung homeostasis that can be categorized into: transcription factors (AHR, P21, ROR γ , ATF/CREB); receptors (TREM1, FC γ R, NOR1); immune signaling molecules (IL-1R, IL-1 β , CXCL8, CD14, CD163, G-CSF, TNFR1, TLR5, ALOX15, ADAM12); transporters (SLC11A1/16A1); and growth factors (VEGF, HBEGF, HGF). AHR and ROR γ play a role in suppressing lung inflammation (35, 130) while decreased p21 expression is associated with hypoxia-induced lung disease (131). In addition, transcripts associated with immune genes that promote host defense against pulmonary infection (ALOX15, CD14, G-CSF, TREM1, TLR5, NRAMP1, TNFR1) were reduced with heavy drinking (132–135). Critical growth factors important for repairing lung injury were also downregulated. Decreased levels of VEGF correlate with loss of alveolar

structure in emphysema patients (34) and a compromised integrity of the alveolar-capillary barrier (136). Finally, HGF is important for lung development and also promotes regeneration after lung injury in animals (28).

Chronic alcohol consumption is associated with an increased risk of cardiovascular disease (137) and stroke (138). Our analysis has revealed increased expression of genes implicated in heart disease including CLDN5, VWF, PDE6H, ITGA2B, and CA3 (80, 82, 83, 139, 140), as well as megakaryocyte differentiation (RGS18, GFI1B, and ARHGEF4 (75, 76)). ITGA2B is used as a biomarker for myocardial infarction risk and therapeutic modulation of phosphodiesterases is one strategy for treating cardiovascular disease. Interestingly, claudin-5 levels are reduced in human and mice models of cardiomyopathy; therefore, its increased expression here might be a compensatory mechanism.

Finally, heavy alcohol use is a major risk factor for liver, head and neck, and colorectal cancers (141–143). Our gene expression analysis showed increased expression of several genes that promote cancer progression with chronic heavy alcohol consumption (LTBP2/3, IRS-1, SFRP5, LEF1, DLK1, STAG3, and H2B). Higher levels of IRS1 are found in hepatocellular carcinoma, breast, ovarian, and colorectal cancers (144–147). Increased expression of LEF1 is associated with human endometrial tumors, prostate, and colon cancer (148–150). DLK1 is also expressed at higher levels in colon adenocarcinomas, pancreatic islet carcinomas, and small cell lung carcinomas (87). In contrast, moderate alcohol consumption is associated with a reduced risk of developing kidney cancer (151), Hodgkin's lymphoma (152), and thyroid cancer (153). Our study revealed moderate drinking repressed genes associated with reduced cancer incidence including TMEM98 (102), HTRA1 (101), DNTT (99), TET1 (100), and MMP9 (103).

We also investigated differences in miRNA expression levels between the three experimental groups. MiRNAs can modulate gene expression through translational repression or degradation of target mRNAs and play a critical role in regulating immune function (154). We identified several differentially expressed miRNAs with validated mRNA targets present in our RNA-Seq dataset. Interestingly, and as described for mRNA, several of these upregulated miRNAs have also been implicated in the development and progression of cancer. For instance, miR-494 has been shown to be upregulated in hepatocellular carcinomas and promote proliferation in tumor cells (155), while miR-106a is upregulated in gastric, colorectal, and pancreatic cancers in humans (156). As described previously in human hepatocytes and cholangiocytes treated with ethanol (157), miR-34a was upregulated greater than 5-fold in our dataset. Furthermore, ethanol-induced hypomethylation of the miR-34a promoter, which results in increased expression of this miRNA, plays a role in the development of alcoholic liver disease (157).

Moreover, several upregulated miRNAs in our dataset are involved in modulating immune responses. For example, and as we recently reported (17), miR-221 was upregulated in PBMCs of heavy drinkers. We previously showed that increased levels of miR-221 resulted in decreased expression of transcription factors STAT3 and ARNT, which in turn regulate expression of VEGF, G-CSF, and HGF (17, 158). Indeed, VEGFA and HGF were downregulated in this study by 5- and 2-fold, respectively. Another target of miR-221,

RGS6, was also downregulated in this study. In addition, upregulation of miR-125b interferes with the innate immune response following LPS stimulation or microbial infection (159). Finally, miR-223, up-regulated 19-fold in our dataset, inhibits NF κ B activation, angiogenesis, and endothelial cell proliferation, thereby impairing wound healing (160) and inflammation (161).

Many of the downregulated miRNAs are also involved in cancer. For instance, miR-203 has been identified as a tumor suppressor and inhibits proliferation in colorectal cancer cell lines (162). Similarly, expression of miR-144 is significantly decreased in human lung cancer and inhibits proliferation in lung cancer cell lines (163). Finally, chronic ethanol feeding in a mouse model of alcoholic steatohepatitis has also led to downregulation of miR-183 (164). Additionally, most of the downregulated miRNAs modulate immunity. MiR-183 levels have been shown to be positively associated with phagocytosis by macrophages (165). As we reported previously (17, 158), miR-29a expression was modulated by heavy drinking. Specifically, miR29a was downregulated and its target SH3PXD2A was upregulated. Finally, in addition to its role as a tumor suppressor, increased expression of miR-203 has also been shown to be important for anti-inflammatory responses (166).

In summary, our studies revealed that heavy ethanol consumption results in the downregulation of genes that promote resolution of infection, wound healing, and protect against obstructive lung diseases and cancer whereas, moderate drinkers showed increased expression of genes associated with enhancing immune responses. Heavy drinking status also resulted in upregulation of genes involved in impaired wound healing and cancer progression compared to controls and moderate drinkers, whereas moderate ethanol consumption lowered expression of genes associated with cancer. Moreover, heavy ethanol consumption altered the expression of several miRNAs whose targets were differentially expressed in our data set and are involved in cancer progression and immune function. One of the strengths of this study is that we used an outbred animal model of voluntary self-administration that faithfully recapitulates human behavior and physiology. However, a caveat of the current study is that only 3 animals per group were analyzed. Future studies are needed to extend these observations using a larger cohort of animals to other infectious agents such as influenza. Future studies will also investigate the mechanisms underlying dose-dependent changes in gene expression by uncovering factors regulating gene expression such as epigenetic changes within specific immune cells that influence expression of both mRNA and miRNA. Using the nonhuman primate model of alcohol self-administration, longitudinal gene regulation, and epigenetic changes in immune cells and target organs, offer the promise for understanding the complicated and dose-dependent impact of alcohol on immunity and health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

MVA	Modified Vaccinia Ankara
BEC	blood ethanol concentration
DEG	differentially expressed gene
miRNA	microRNA

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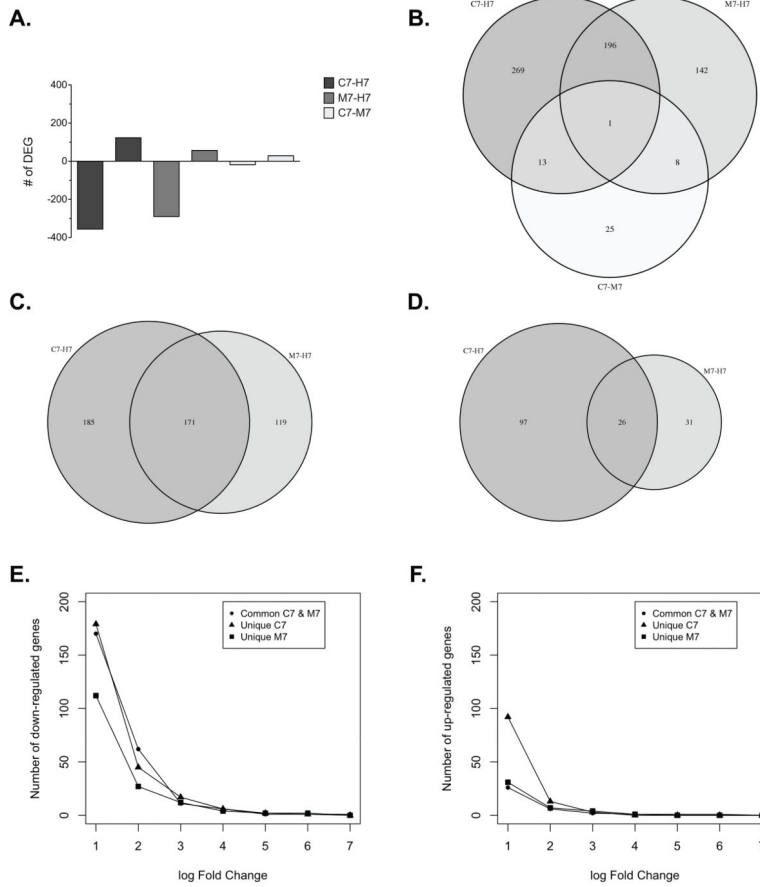


Figure 1. Peripheral blood mononuclear cell (PBMC) gene expression
 (A) Bar graph showing the number of downregulated and upregulated genes for each comparison. (B) Venn diagram depicting the overlap of the annotated DEGs between controls (C), moderate drinkers (M), and heavy drinkers (H) on day 7 after booster vaccination (C7, M7 and H7 respectively). (C) Venn diagram depicting the overlap between genes that are downregulated with heavy alcohol consumption compared to controls and moderate drinkers. (D) Venn diagram depicting the overlap between genes that are upregulated with heavy alcohol consumption compared to controls and moderate drinkers. (E, F) Fold change of (E) downregulated and (F) upregulated genes with heavy drinking.

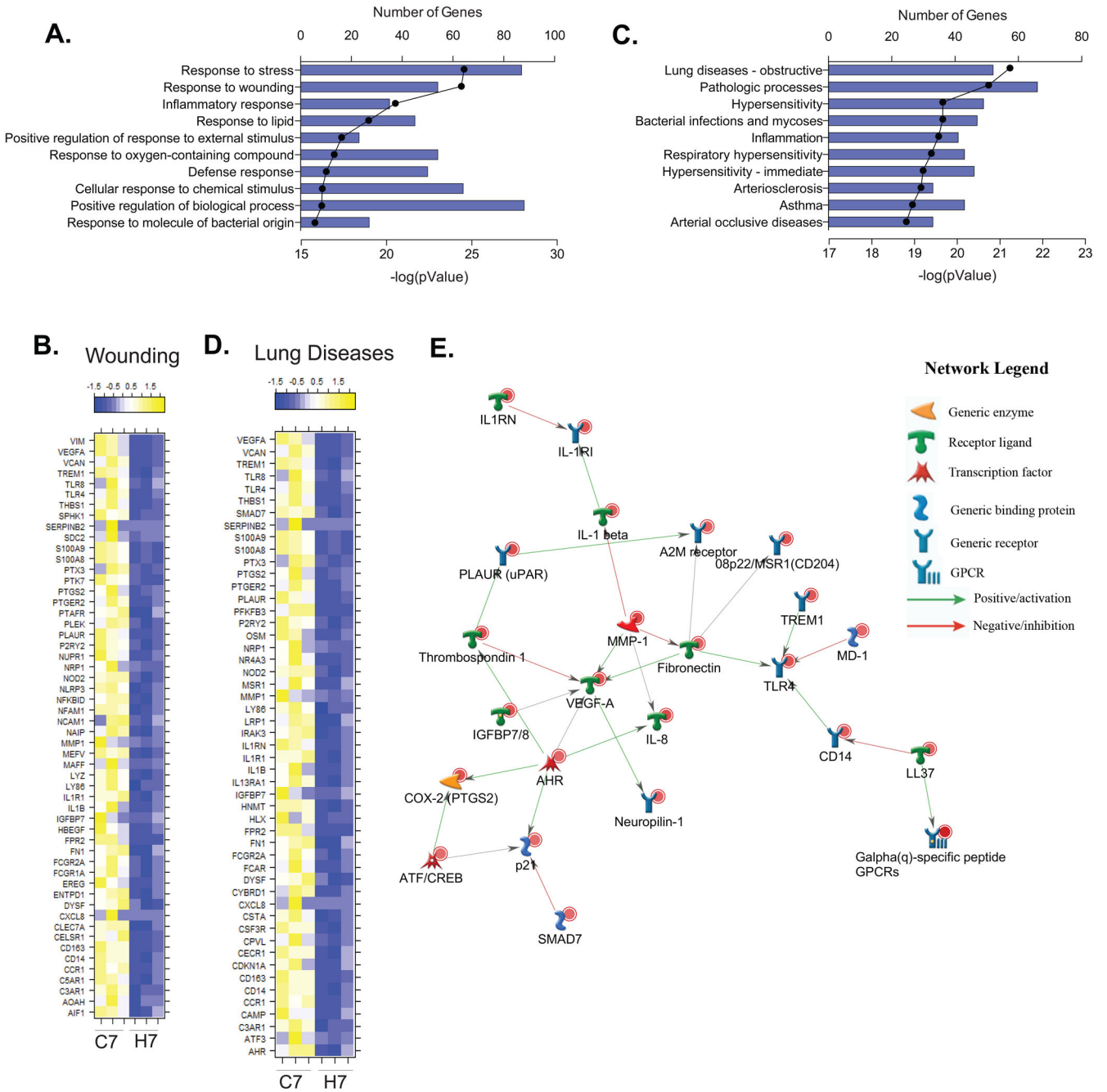


Figure 2. Chronic heavy alcohol consumption down-regulates genes that promote wound healing and contribute to obstructive lung diseases compared to controls and moderate drinkers (A) Bar graph displaying the 10 most significant GO terms associated with the 170 genes downregulated with heavy ethanol consumption (H7) compared to controls and moderate drinkers (C7 and M7). Line represents the $-\log(p)$ value associated with each GO term. (B) Heatmap of DEGs between H7 and C7 in the "response to wounding" GO term. (C) The 10 most significant diseases by biomarkers associated with the 170 genes downregulated with heavy ethanol consumption (H7) compared to controls and moderate drinkers (C7 and M7). (D) Heatmap of DEGs between H7 and C7 that mapped to "lung diseases-obstructive"

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category. (E) Network of DEGs that mapped to “obstructive lung diseases” and show direct interactions.

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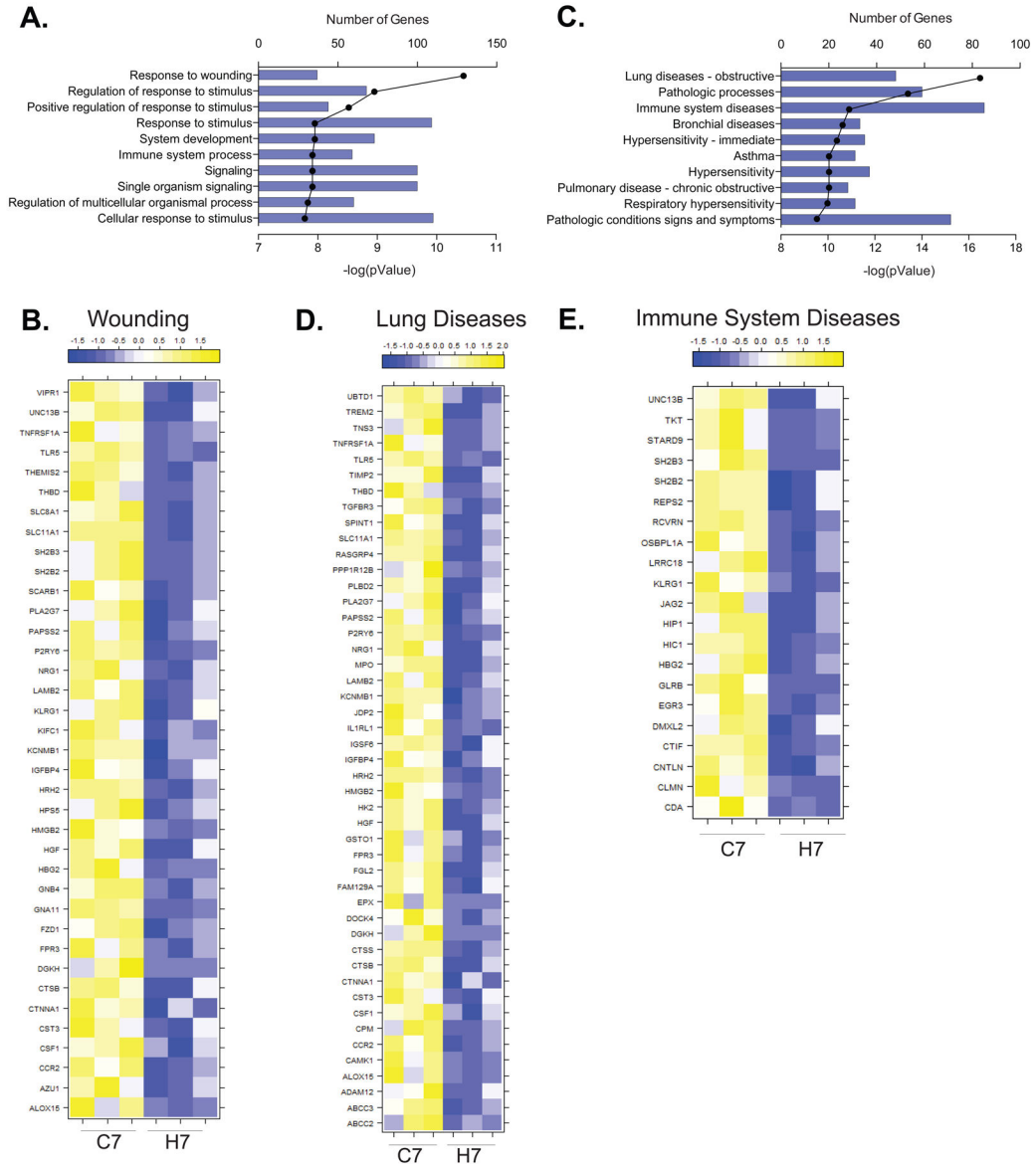


Figure 3. Alcohol abuse uniquely down-regulates additional genes that promote wound healing and contribute to obstructive lung diseases

(A) Bar graph displaying the 10 most significant GO terms to which the 186 DEGs downregulated with heavy ethanol consumption (H7) compared to controls only (C7). Line represents the $-\log(p\text{ value})$ for each GO term. (B) Heatmap of DEGs between H7 and C7 mapping to the GO term “response to wounding”. (C) Bar graph displaying the 10 most significant Diseases by Biomarker to which the 186 DEGs downregulated with heavy drinking (H7) compared to controls (C7) only. Line represents the $-\log(p\text{ value})$ for each disease category. (D) Heatmap of the DEGs between H7 and C7 mapping to the “lung diseases-obstructive” disease category. (E) Heat map of the 21 DEGs between H7 and C7 mapping to “immune system diseases”.

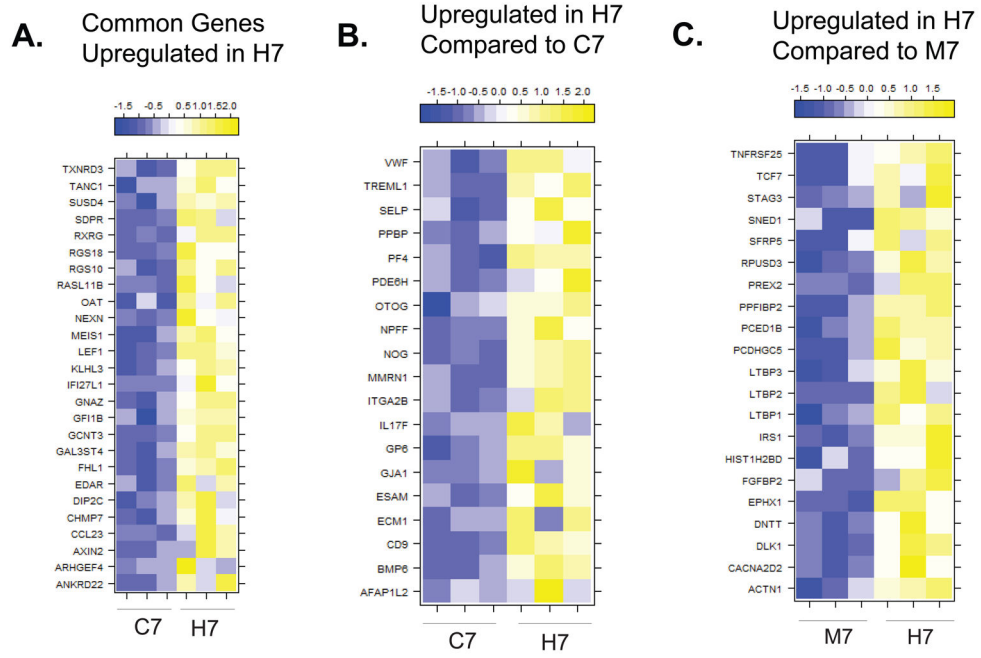


Figure 5. Alcohol abuse upregulates genes that interfere with wound healing and contribute to cancer
 Heatmaps of the DEGs upregulated with heavy ethanol consumption (H7) compared to: (A) both controls (C7) and moderate (M7) drinkers; (B) controls (C7) only and mapped to the GO term “response to wounding”; and (C) moderate (M7) drinkers only and mapped to the GO term “neuro-ectodermal tumors”.

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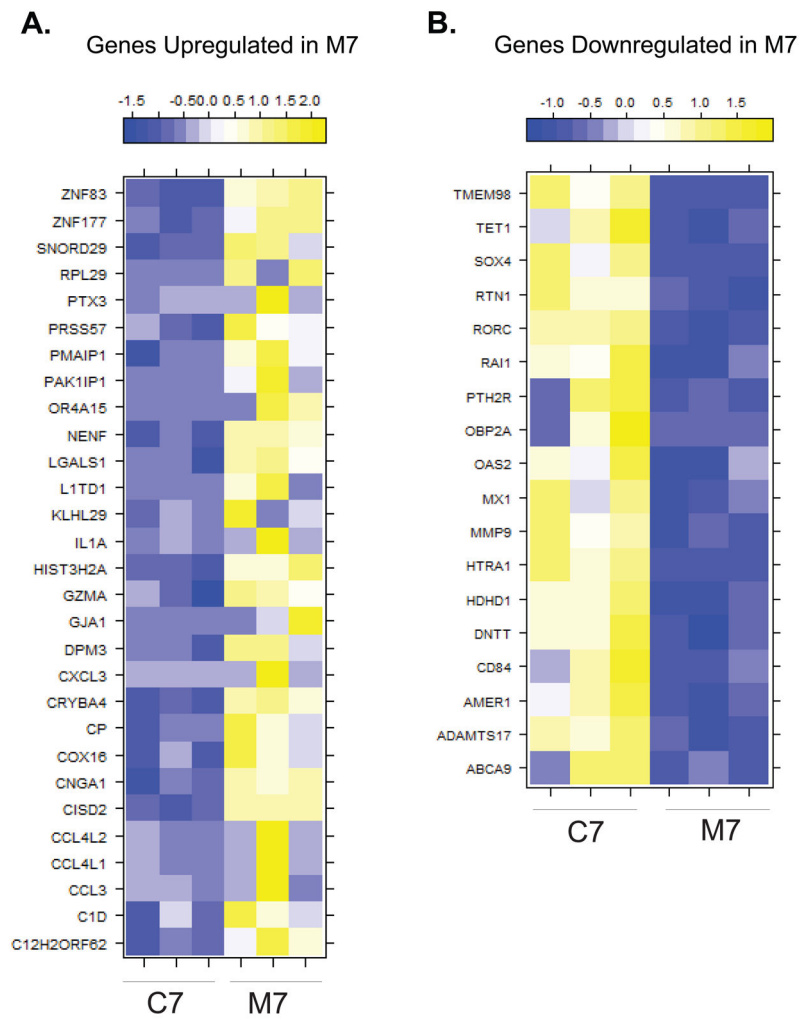


Figure 6. Moderate ethanol consumption modulates genes associated with immune response
 (A) Heatmap of the DEGs uniquely activated with moderate drinking (M7) compared to controls (C7). (B) Heatmap of the DEGs uniquely repressed with moderate drinking (M7) compared to controls (C7).

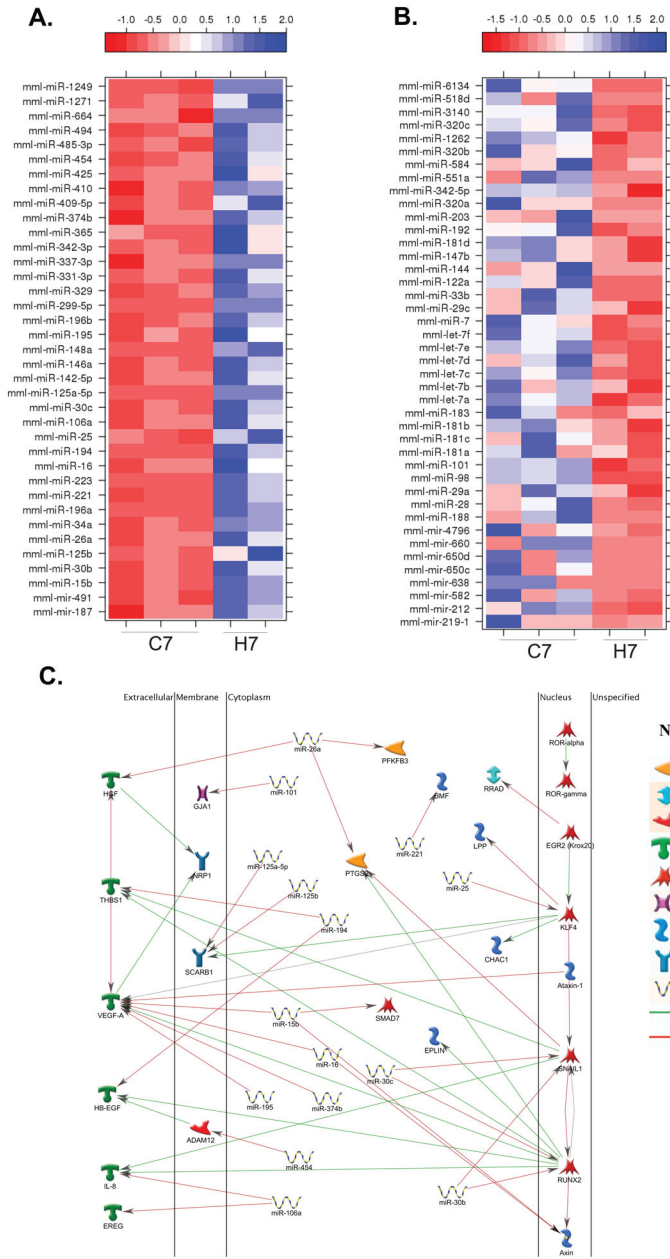


Figure 7. Heavy ethanol consumption changes expression of several microRNA
 Heatmaps of the (A) upregulated and (B) downregulated microRNAs with heavy drinking (H7) compared to controls (C7). (C) Network image of a subset of the differentially expressed microRNAs and their mRNA targets that were both differentially expressed in our study.

Table 1mRNA-miRNA pairs in our dataset¹

Differentially Expressed MicroRNAs	Differentially Expressed mRNA Targets
<i>Down-regulated</i>	<i>Up-regulated</i>
miR-144	AXIN2
miR-203	AFAP1L2, ROBO2
miR-183	ROBO2
miR-29c	SH3PXD2A
miR-29a	SH3PXD2A
miR-101	GJA1
miR-29b	SH3PXD2A
<i>Up-regulated</i>	<i>Down-regulated</i>
miR-26a	HGF, PTGS2, NTN4, RP2, RAB3IP, CHAC1, PFKB3, DAPK1
miR-25	GRHL1, KLF4, FAM20C
miR-142-5p	GAS7, BVES, ATP13A3, PRRG4, RTN1, LPP, PRDM8, NRG1
miR-374b	GAS7, VEGFA, ATXN1, DUSP6, DUSP8
miR-125b	SCARB1, CCR2, FAM129B, C19orf39
miR-410	RORA, SMAD7, RAPGEF2, SASH1, SNAI1, PTX3
miR-485-3p	BAIAP2
miR-16	SMAD7, TNFSF13B, VEGFA, ATP13A3, RASGEF1B
miR-425	FSCN1
miR-30c	SH2B3, SNAI1, RUNX2, HLX, EAF1, RRAD
miR-106a	LIMA1, NTN4, OSM, WFS1, EGR2, EREG, DOCK4, FAM129A, RORC, IL8
miR-342-3p	ZAK, NEURL1B
miR-494	IRAK3, ZFHX3
miR-125a-5p	SCARB1, CCR2, FAM129B, C19orf38
miR-148a	NRP1, B4GALT5, SESTD1
miR-15b	SMAD7, TNFSF13B, VEGFA, ATP13A3, RASGEF1B, AATK
miR-221	BMF, NRG1
miR-365	KCNQ1
miR-454	ZAK, ATXN1, RTN1, ADAM12, ACSL1, WDFY3, MB21D2, TMEM170B
miR-195	SMAD7, TNFSF13B, VEGFA, ATP13A3, RASGEF1B, AATK
miR-329	ATXN1
miR-34a	CLEC10A, REPS2
miR-30b	SH2B3, SNAI1, RUNX2, HLX, EAF1, RRAD
miR-194	HBEGF, THBS1, ZFHX3
miR-223	OLFM1, SLC8A1

¹ Table shows the downregulated miRNAs that target upregulated mRNAs as well as the upregulated miRNAs that target downregulated mRNAs in our datasets.