Published in final edited form as:

Vaccine. 2013 December 17; 32(1): 54–61. doi:10.1016/j.vaccine.2013.10.076.

Moderate alcohol consumption enhances vaccine-induced responses in rhesus macaques

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Abstract

We have recently shown that chronic alcohol consumption in a rhesus macaque model of ethanol self-administration significantly modulates the serum cytokine profile. In this study, we extended these observations by investigating the impact of chronic ethanol exposure on the immune response to Modified Vaccinia Ankara (MVA). All animals were vaccinated with MVA before ethanol exposure to ethanol and then again after 7 months of 22 h/day of “open-access” drinking of 4% (w/v) ethanol. Our results indicate that animals whose blood ethanol concentration (BEC) chronically exceeded 80 mg/dl had lower CD4 and CD8 T cell proliferation as well as IgG responses following MVA booster than control animals. In contrast, relatively moderate drinkers whose BEC remained below 80 mg/ml exhibited more robust MVA-specific IgG and CD8 T cell responses than controls. To begin to uncover mechanisms underlying the differences in MVA-specific responses between the three groups, we analyzed plasma cytokine levels and microRNA expression in peripheral blood mononuclear cells following MVA booster. Our findings suggest that moderate ethanol consumption results in higher levels of antiviral cytokines and an expression profile of microRNAs linked to CD8 T cell differentiation. In summary, moderate alcohol consumption enhances recall vaccine responses, whereas chronic alcohol intoxication suppresses this response.

Keywords

Ethanol; MVA; Vaccine; Macaque; MicroRNA; Cytokine; T cell; Antibody

1. Introduction

Alcohol use disorders are associated with increased susceptibility to infection. For example, chronic alcohol abusers are 3–7 times more susceptible to acute bacterial pneumonia and

have a higher incidence of Hepatitis B virus (HBV), Hepatitis C virus (HCV), *Mycobacterium tuberculosis* and *Corynebacterium diphtheriae* (reviewed in [1]). In addition, multiple studies have demonstrated reduced seroconversion in alcoholic patients receiving HBV vaccine [2]. These observations were recapitulated in several animal studies. For instance, mice chronically exposed to ethanol generate reduced CD4 and CD8 T cell responses, with diminished proliferation, cytotoxicity and T-helper 1 (Th1) cytokine production following immunization with HCV antigen [3,4]. Similarly, chronic ethanol exposure also results in ablated CD8 T cell responses and a shift to Th2 responses in mice following infection with *Listeria monocytogenes* [5,6]. Ethanol also inhibits allogeneic T cell responses (reviewed in [7]). Finally, in rhesus macaques, multiple studies have demonstrated ethanol consumption significantly inhibits the control of Simian Immunodeficiency virus replication [8–10]. However, no study to date has compared the effects associated with chronically drinking intoxicating versus moderate amounts of ethanol as defined by a BEC cut off of 80 mg/dl [11].

Analyzing the effects of varying daily doses of ethanol attained by alcohol consumers is extremely difficult in the clinical setting. Moreover, although several animal models are available, none have employed an oral ethanol self-administration approach that mimics the daily ingestion patterns of alcoholic drinking phenotypes. These dose-effects may be particularly critical, since a biphasic impact of alcohol on life expectancy has been described, with moderate drinkers having a lower relative risk of mortality than either those that abstain from alcohol entirely or drink heavily (reviewed in [12]).

In this study, we leveraged a rhesus macaque model of alcohol self-administration [13] where, following an induction period, the monkeys have “open-access” and can choose to drink ethanol or water for 22 h/day with food available as three meals. Our model allows each animal to voluntarily drink alcohol, and thus there are individual differences in intake. Risk factors in this monkey model for heavy drinking include sex, age at the onset of drinking, social status, and HPA axis response to stressors [13–15]. In this longitudinal design, we determined the impact of precisely measured ethanol intakes on immune responses to MVA booster vaccination. Data presented here demonstrate that heavy alcohol consumption associated with daily BEC exceeding 80 mg/dl broadly suppresses antigen-specific T cell and antibody responses. However, moderate alcohol consumption associated with BECs below 80 mg/dl improved peak T and B cell responses compared to control animals without ethanol exposure.

2. Materials and methods

2.1. Animals and sample collection

This study was carried out under strict accordance with the recommendations outlined 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 studies were approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee (IACUC).

Twelve male rhesus macaques (5–6 years of age) were used in this study. Prior to alcohol induction, all monkeys were trained to present their leg for awake venipuncture and blood sampling from the femoral vein [16]. The animals were vaccinated with 10^8 pfu MVA intradermally/intramuscularly as previously described [17] prior to the induction of ethanol self-administration (designated d0) and again after 7 months of 22 h open access to ethanol (designated d0b). Blood samples were collected without anesthesia at the time points indicated. Peripheral blood mononuclear cells (PBMC) and plasma were isolated by centrifugation over histopaque (Sigma, St Louis, MO) as per manufacturer’s protocol.

PBMC were cryopreserved in Fetalplex™ Animal Serum Complex (Gemini Bio-Products, West Sacramento, CA)/DMSO.

2.2. Ethanol self-administration protocol

Integrated into each monkey's housing cage was an operant panel that allowed for precise measurement of fluid and food consumption (see [13] for details). We used a schedule-induced polydipsia procedure to establish self-administration of 4% (w/v; diluted in water) ethanol in macaques as described previously [13]. Following this induction period (4 months), the monkeys were allowed a choice between 4% ethanol or water for 22 h/day and all fluid and food consumed during these daily "open-access" sessions was recorded for the duration of the study. Four age/sex-matched co-housed control animals were given a calorically matched daily maltose–dextrose solution. BEC was measured every 5th day throughout the study by headspace gas chromatography from a blood sample taken 7 h into the 22 h drinking session. The average daily alcohol intakes during the open-access period for each animal ranged from 1.8 to 3.3 g/kg ($n = 8$) as detailed in Table S1.

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.10.076>.

2.3. Measuring MVA-specific T and B cell responses

Immune phenotyping and analysis of T and B cell proliferation was assessed exactly as described previously [18]. Frequency of MVA-specific T cell responses were determined using intracellular cytokine staining as previously described [17]. The gating strategy used is summarized in Fig. S1. All flow cytometry samples were acquired with LSRII instrument (Beckton, Dickinson) and analyzed using FlowJo software (TreeStar, Ashland, OR). Antiviral plasma IgG titers were determined using ELISA with plates coated with VV-viral lysate as previously described [17]. Endpoint titers were calculated using log–log transformation of the linear portion of the generated curve, with 0.1 OD units as cut-off.

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2.4. Measurement of circulating cytokines

Plasma samples (stored at -80°C) were thawed and diluted 1:2 in serum matrix for use with the 28-plex multiplex non-human primate magnetic bead panel as per manufacturer's instructions (Millipore Corporation, Billerica, CA).

2.5. RNA isolation, cDNA synthesis and microRNA analysis

Total PBMC RNA was isolated as described previously [19]. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). MicroRNA expression was determined using Taqman® microRNA assays (Applied Biosystems). MicroRNA expression levels for each sample were normalized to control U6 miRNA, using ΔCt calculations [20].

2.6. Statistical analysis

Repeated measure ANOVA was used for longitudinal analyses, with contrast t -test used for pair-wise comparisons at each time point. False discovery rate (FDR) adjustment was used to adjust for multiple comparisons. Plasma cytokine levels and microRNA expression were compared using the non-parametric Mann Whitney U test.

3. Results

3.1. Ethanol self administration

Over the nine months of ethanol self-administration, mean daily ethanol intake varied markedly between animals and ranged from 1.8 to 3.3 g/kg/day (Table S1). Examination of these values as well as of the BEC during the booster vaccination period revealed that animals' ethanol consumption segregated into two groups. Animals in the first group had a mean daily intake of 3.05–3.39 g/kg/day and an average BEC level of 90–126 mg, and were designated as heavy drinkers. Animals in the second group consumed an average of 1.7–2.23 g/kg/daily, had a BEC of 22.3–48.8 mg and were designated as 'moderate' drinkers (Table 1).

3.2. Impact of chronic ethanol self-administration on lymphocyte homeostasis

We first determined the impact of ethanol on the frequency of circulating T and B cell subsets in peripheral blood. Total white blood cell counts as well as circulating lymphocyte, monocyte and neutrophil numbers were stable during both the ethanol induction and open access (self-administration) phases of our study (Fig. S2). Similarly, the frequency of CD4 T cell, CD8 T cell and CD20 B cells within PBMC remained stable and did not differ between control and experimental groups (Fig. S3). We also found no differences in the relative frequency of naïve and memory T or B cell subsets during either the induction or open access phase of ethanol exposure (Fig. S4).

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3.3. Effect of chronic alcohol self-administration on T cell response to vaccination

We next determined the impact of chronic ethanol consumption on the immune response to Modified Vaccinia Ankara (MVA), which elicits potent cell-mediated and humoral immunity in macaques [21]. Animals were primed with MVA prior to ethanol induction and then boosted after 11 months of daily alcohol consumption (4 month induction + 7 months of self-administration), allowing vaccine responses to be longitudinally measured in animals both before and after ethanol exposure (shown schematically in Fig. 1). Both average daily ethanol intake (g/kg/day) and blood ethanol concentration (BEC, units mg %) during MVA boost are summarized in Table 1.

Proliferation and clonal expansion of antigen-specific lymphocytes are critical for mediating protective immunity and the development of immunological memory. Therefore we compared the kinetics and magnitude of T cell proliferation following MVA administration between ethanol and control animals by measuring changes in the frequency of Ki67+ central memory (CD28+CD95+) or effector memory (CD28–CD95+) T cells. Nuclear protein Ki67 is expressed during all active phases of cell division, but is absent in quiescent cells and during DNA repair [22]. During MVA priming (prior to ethanol self-administration), kinetics and magnitude of T cell proliferative responses within all three groups were comparable, with a peak proliferative burst detected 7 days post vaccination amongst CD4+ and CD8+ CM and EM T cells (Fig. 2A–D). In contrast, following MVA booster (after 9 months of open access to ethanol), heavy drinkers exhibited diminished peak proliferative responses (d7b) relative to controls (Fig. 2A–D). Moderate drinkers mounted a proliferative response of equivalent magnitude and kinetics to controls.

We also determined the frequency of MVA-specific T cells following vaccination by measuring the percentage of T cells producing TNF α + and/or IFN γ + cells following stimulation with MVA using intracellular cytokine staining (ICS) (Fig. 3A–D). As

anticipated, the frequency of antigen-specific CD4 and CD8 T cell was equivalent in all animals during primary vaccination and prior to ethanol exposure (d0–d56). Following booster vaccination, heavy drinkers exhibited a barely detectable MVA-specific T cell response within the CD4 EM and CD8 CM subsets (Fig. 3B and C). In contrast, moderate drinkers exhibited a significantly greater peak frequency of MVA-specific CD8 EM T cells than that detected in either controls or heavy drinkers or (Fig. 3D).

3.4. Impact of ethanol on B cell response to vaccination

To characterize the impact of ethanol on B cell response to MVA, we compared kinetics and magnitude of proliferative burst following vaccination in memory (CD27+IgD–) and marginal-zone like (MZ-like; CD27+IgD+) B cells. Following primary MVA vaccination and prior to ethanol induction, all three groups generated B cell proliferative bursts of comparable kinetics and magnitude (Fig. 4A and B) as well as indistinguishable IgG titers (Fig. 4C). In contrast, following the MVA-booster, heavy drinkers exhibited the lowest frequency of Ki67+ memory B cells (day 7b), whereas moderate drinkers exhibited the biggest proliferative burst (Fig. 4A). Similarly, proliferation within the MZ-like B cells was highest in moderate drinkers and lowest in heavy drinkers. As described for B cell proliferation, heavy drinkers generated significantly lower IgG titers than either moderate drinkers or controls following administration of the MVA booster (Fig. 4C), whereas the magnitude of MVA-specific IgG responses in moderate drinkers was significantly higher than that of controls.

3.5. The impact of alcohol on circulating cytokine levels

In order to begin dissecting the mechanism underlying the enhanced CD8 and IgG responses in moderate drinkers, we measured circulating levels of multiple cytokines and chemokines using a 28-plex panel on days 0b, d7b, and d14b of our study (Fig. 5). While our analysis was not sufficiently powered to find significant differences between the groups, we did observe a global trend toward elevated plasma levels of IL-2 and IL-15 (lymphocyte proliferation); IL-12 and TNF α (T cell activation and effector function); and RANTES and MIG (immune cell recruitment) in moderate drinkers compared to heavy drinkers and controls at all time points. Although IL-12 concentrations were similar between moderate drinkers and controls, mean IL-12 concentrations were lowest in heavy drinkers at all time points analyzed.

3.6. The impact of alcohol exposure on miRNA induction

MicroRNAs have emerged as potent regulators of lymphocyte proliferation and effector function (reviewed by [23]). Therefore, we next determined whether differences in microRNA expression underlie those in B/T cell responses. Specifically, we measured fold induction of a panel of microRNAs known to regulate lymphocyte differentiation and activation [24–33]: miRs 181a, 146a-5p, 155-5p, 221-3p, 17-3p, 17-5p, 21-5p, 29a, 150, 125b and 190 between days 0b and 7b of MVA boosting (Table S2). Our sample size was too small to detect statistically significant differences, but we were able to discern several microRNAs that exhibited a trend of highest expression amongst heavy drinkers, including miR146a-5p, 17-3p, 17-5p, 21-5p, 150, 125b and 190 (Fig. 6). One heavy drinking animal (26148) consistently displayed the strongest increase of these microRNAs. By contrast, moderate drinkers exhibited a trend of lowest fold induction of multiple microRNAs at d7b, notably miR 155-5p, 17-5p, 29a and 150.

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4. Discussion

In this study, we examined the impact of chronic ethanol self-administration on lymphocyte frequency and response to vaccination in rhesus macaques. Overall, chronic ethanol self-administration did not modulate the frequency or subset distribution of circulating lymphocytes. This observation is consistent with some reports of chronic ethanol exposure in rodents, NHPs and humans [8,34,35]. Nonetheless, other studies have reported significant perturbations of both the number and frequency of peripheral T and B cells with either acute or chronic alcohol exposure (reviewed by [34,36]). It has been suggested that ethanol-induced lymphocyte depletion is mediated by the action of stress-induced glucocorticoids, particularly corticosterone in rodents, on developing lymphocytes (reviewed by [34]). In this cohort of rhesus macaques, we observed a reduction in circulating cortisol levels with chronic ethanol consumption, which could explain the stability of lymphocyte numbers (Table S1).

As previously described in humans and animal models [1,2,4,6,8,37–40], our results indicate *heavy* chronic ethanol consumption impairs several key aspects of vaccine-elicited immune responses including T and B cell proliferation, T cell cytokine production and antibody secretion. Importantly our data suggest that recall responses to vaccines received prior to alcohol consumption might also be compromised. The most surprising and interesting aspect of our study is the increased frequency of antigen-specific T cells and antibodies in *moderate* drinkers compared to controls following booster vaccination. Although lymphocyte proliferation following booster MVA vaccination was comparable in moderate drinkers and controls, peak frequency of MVA-specific CD8+ EM T cells and IgG responses were significantly higher in moderate drinkers compared to control animals.

There are relatively few studies on putative benefits of moderate ethanol consumption on immune function (reviewed by [41]) but the potentiating effects of moderate alcohol consumption on recall MVA responses mirror reports of reduced all cause mortality following moderate alcohol consumption in humans [42–45]. Importantly, the enhancements T cell and antibody responses were most evident acutely post vaccination, during a period where replication of most pathogens would have been at its peak level. Our data are in line with those reported in an earlier study where rats consuming low alcohol doses generated a more robust delayed cutaneous hypersensitivity (DCH) response whereas high doses were associated with reduced DCH responses [46]. Similarly, low alcohol dose in rats was associated with increased clearance of *Mycobacterium bovis* whereas high alcohol dose was associated with decreased bacterial clearance [46]. Data from two epidemiological studies also suggest that moderate alcohol consumption (for 1 year or more) may reduce the incidence of common cold [47,48].

The mechanisms by which moderate ethanol intake enhances recall responses are still under investigation, but in this study the augmented vaccine responses cannot be simply attributed to differences in frequency or total numbers of lymphocyte subpopulations, as these were similar in all three experimental groups. Moreover, the enhanced B cell response may be independent of enhanced CD4 T cell help, since both CD4 proliferative and cytokine responses were equivalent in moderate drinkers and controls.

To begin uncovering the mechanisms underlying improved immune responses in moderate drinkers, we analyzed plasma cytokine levels following MVA booster using a rhesus-specific 28 multiplex. Overall, plasma cytokine levels did not change greatly, but moderate drinkers showed a trend toward higher basal levels of IL-2, IL-15, TNF α , RANTES and MIG that could explain the enhanced T cell response given their role in T cell proliferation and differentiation (IL-2 and 15), effector function (TNF α) and recruitment (RANTES and

MIG). These observations are in line with previous studies reporting that moderate alcohol intake significantly increases plasma levels of multiple cytokines in humans [41].

We also investigated differences in microRNA expression between the three experimental groups. MicroRNAs can modulate gene expression through translational repression or degradation of target mRNAs and have been shown to play a critical role in regulating immune function [23]. Our analysis showed that miR-155 was strongly down-regulated in moderate drinkers following MVA boost. Mice deficient in miR-155 have a Th2-skewed immune profile [49] and impaired regulatory T cell (Treg) fitness [32]. Thus, reduced miR-155 expression in moderate drinkers could explain increased T cell proliferation (decreased regulatory function) and antibody response (increased Th2 responses). Other microRNAs showing a trend toward reduced expression following vaccination in moderate drinkers were miR-29a, miR-150 and miR-17. Down-regulation of these microRNAs was reported to promote IFN γ production [30], enhance Ig responses [31] and increase clonal expansion of T cells [50] respectively.

We also observed a trend toward increased expression of some microRNAs in heavy drinkers during MVA boost, notably miR-146a, miR-21 and miR-125b. Both miR-146a and miR-21 are both highly expressed in Treg, with miR-146a expression promoting Treg suppressor function [28,29]. In addition, miR-125b expression is known to inhibit effector T cell differentiation [27]. Thus, putative upregulation of these microRNAs could explain the reduced immune function observed in heavy drinkers. Interestingly, miR-150 and miR-17 both showed a trend toward enhanced induction in heavy drinkers, converse to their downregulation in moderate drinkers. Thus, differential expression of microRNAs in heavy versus moderate drinkers may contribute to the differential response to vaccination seen in these groups.

The small group size ($n = 4/\text{group}$) precluded us from detecting statistically significant differences in plasma cytokine and microRNA expression levels between control, heavy and moderate animals. Nevertheless, we were able to identify trends that provide a strong basis for continued efforts in this area of research using our robust self-administration nonhuman primate model, which provides a unique and powerful tool to dissect dose-dependent effects of alcohol on different organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Kevin Nusser for his expert support and help in the completion of the animal studies. This work was funded by NIH 8P51 ODO11092-53 and NIH/NIAAA R24 AA019431, U01 AA13641, U01 AA13510 and NIH/NIAAA R21AA021947.

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Fig. 1.

Schedule of ethanol induction and MVA administration. Ethanol self-administration was induced in 8 male Indian origin rhesus macaques using schedule induced polydipsia. Following a four-month induction period of escalating ethanol dose (0.5 g–1.5 g), animals were given open access to 4% ethanol for 9 months (22 h access). All animals were vaccinated with Modified Vaccinia Ankara (MVA) one month before any exposure to ethanol (priming) and again after 7 months of ethanol self-administration (booster).

Fig. 2.

Alcohol self-administration modulates T cell proliferation. The frequency of Ki67+ central memory (CM) and effector memory (EM) CD4 (A and B) and CD8 (C and D) T cells was analyzed in peripheral blood mononuclear cells (PBMC) following primary MVA vaccination prior to ethanol induction (d0–56; prime) or booster MVA vaccination after 7 months of open access to ethanol (d0b–56b) at the time points indicated. Animals consuming a moderate alcohol dose, heavy alcohol dose and control non-drinkers are shown ($n = 4/\text{group}$). Symbols represent group means for each time point $\pm$ SEM. *Control vs heavy, #moderate vs heavy; */# $p < 0.05$, ***/### $p < 0.001$. Contrast t -test for pair wise comparisons at each time point, with FDR correction.

Fig. 3.

Moderate alcohol consumption enhances CD8 EM cytokine responses. The frequency of MVA-specific TNF α + and/or IFN γ + producing CD4 CM, CD4 EM, CD8 CM and CD8 EM T cells in PBMC were quantified using ICCS following stimulation with VV (A–D).

Animals consuming a moderate alcohol dose, heavy alcohol dose and control non-drinkers are shown ($n = 4/\text{group}$). Symbols represent group means for each time point $\pm$ SEM. †Moderate vs control, #moderate vs heavy; # $p < 0.05$, †††/### $p < 0.001$. Contrast t -test for pair wise comparisons at each time point, with FDR correction.

Fig. 4.

Heavy alcohol consumption inhibits memory B cell proliferation and MVA-specific IgG responses. The frequency of Ki67+ memory (A) and MZ-like (B) B cells was determined in PBMC following primary (d0–56; prime) or booster MVA vaccination after 7 months of open access to ethanol (d0b–56b; boost) at the time points indicated. (C) MVA-specific IgG endpoint titers were determined by ELISA at the time points indicated. Animals consuming a moderate alcohol dose (average 1.8–2.3 g/kg/day, $n = 4$), heavy alcohol dose (average 2.8–3.3 g/kg/day, $n = 4$) and control non-drinkers ($n = 4$) are shown. Symbols represent group means for each time point $\pm$ SEM. †Moderate vs control, #moderate vs heavy, *control vs heavy; †/* $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$.

Fig. 5.

Plasma cytokine levels at d0, d7 and d14 of MVA boost. The concentration of plasma cytokines (pg/ml) IL-2, IL-12, IL-15 and TNFα, as well as chemokines RANTES and MIG were analyzed at d0b, d7b and d14b of MVA boost. Controls and animals consuming a moderate or heavy alcohol dose are shown ($n = 4/\text{group}$). Bars represent group means for each time point $\pm$ SEM.

Fig. 6.

Fold induction of microRNA expression in PBMC. The relative expression of microRNAs 181a-5p, 146a-5p, 155-5p, 221-3p, 17-3p, 17-5p, 21-5p, 29a, 150, 125b and 190a was analyzed on days 0b and 7b of MVA boost. Expression levels at each time point were normalized to housekeeping microRNA U6. Each symbol represents an individual animal ($n = 4/\text{group}$).

Table 1

Mean ethanol intake during MVA boost (d0b–d56b). Mean daily ethanol intake (g/kg/day) and blood ethanol concentration (mg%) was calculated in rhesus macaques during the period of MVA booster vaccination (d0b–d56b) administered after 7 months of ethanol self-administration. Drinking behavior of rhesus macaques during MVA boost (d0b–d56b).

Animal ID	Mean daily ethanol intake (g/kg/day)	Mean BEC (mg%)	Group
26077	0	0	Control
26082	0	0	Control
26089	0	0	Control
26104	0	0	Control
25811	1.70	29.2	Moderate
25790	2.12	48.8	Moderate
26016	2.23	22.3	Moderate
25742	1.93	30.9	Moderate
25787	3.39	114.1	Heavy
26148	3.37	91.5	Heavy
25882	3.50	90.4	Heavy
26168	3.05	126.0	Heavy