Published in final edited form as:

Alcohol Clin Exp Res. 2012 February ; 36(2): 310–317. doi:10.1111/j.1530-0277.2011.01624.x.

The Impact of Alcohol on BCG-Induced Immunity Against *Mycobacterium tuberculosis*

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Abstract

Background—Alcoholics are at heightened risk for developing active tuberculosis. This study evaluates chronic alcohol consumption in a murine model of vaccination with *Mycobacterium bovis* Bacille-Calmette Guérin (BCG) and subsequent pulmonary infection with virulent *Mycobacterium tuberculosis*.

Methods—BALB/c mice were administered the Lieber-DeCarli liquid ethanol diet (LED) or pair-fed the liquid control diet (LCD) for three weeks either before or after subcutaneous vaccination with *M. bovis* BCG. At least three weeks after BCG vaccination, groups of mice on the above diets were challenged with intratracheal infection with *M. tuberculosis* H37Rv. Lung mycobacterial burden, and lung and lung-associated lymph node CD4⁺ lymphocyte production of TB-specific interferon (IFN)- γ were assayed. Popliteal lymph node lymphocytes from both dietary regimens undergoing BCG vaccination (in the absence of *M. tuberculosis* infection) were also evaluated for PPD-induced interferon (IFN)- γ production by ELISpot assay.

Results—Mice begun on alcohol prior to vaccination with *M. bovis* BCG demonstrated impaired control of pulmonary challenge with virulent *M. tuberculosis*, as well as impaired lung CD4⁺ and popliteal lymph node T cell IFN- γ responses. If BCG vaccination was delivered prior to initiation of alcohol feeding, the mice remained protected against a subsequent challenge with *M. tuberculosis*, and BCG-induced immunity was not impaired in either the lung or the popliteal lymph nodes.

Conclusions—Alcohol consumption blunts the development of the adaptive immune response to *M. bovis* BCG vaccination, which impairs the control of a secondary challenge with *M. tuberculosis*, but only if the alcohol exposure is begun prior to BCG vaccination. These results provide insight into mechanisms by which alcohol consumption impairs antimycobacterial immunity, including in response to vaccination and subsequent pathogenic challenge.

Keywords

Alcohol; *M. tuberculosis*; BCG vaccination; CD4⁺ lymphocytes; IFN- γ

Introduction

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB), currently infects one-third of the world's population, with a rising incidence rate (0.4%/year) (WHO, 2010). The vast majority of the TB-infected population has latent infection, in which the host's antimycobacterial cell-mediated immunity (previously established at the inception of infection) maintains the infection in a state of chronic persistence. During latent infection, viable organisms are present in relatively low burden, but those organisms fail to progressively replicate, due to effective adaptive Th1 immunity. During this post-infectious latent period, the infection may "reactivate", with the development of active progressive disease (Wayne and Sohaskey, 2001). This progression occurs annually in eight million people globally (approximately 10% of those infected), and two million of those die of the disease (WHO, 2010). Many questions remain unanswered regarding the mechanism(s) of the shift from the latent infection to active TB, but numerous factors are thought to be associated with the breakdown of the latent state and the conversion to active disease, including age, immunosuppressive conditions (e.g., chronic corticosteroid use, human immunodeficiency virus infection, organ transplantation), diabetes mellitus, renal failure, silicosis, post-gastrectomy state, and injection drug use (ATS/CDC, 2000).

Effective vaccination against *M. tuberculosis* remains a challenge. For decades, many countries have administered a live vaccine of the attenuated strain, *M. bovis* Bacille Calmette-Guérin (BCG) to children, but protection from this vaccine is suboptimal. It confers protection against disseminated *M. tuberculosis* at younger ages (especially meningitis), but fails to protect against adult infection or reactivation disease (Flynn, 2004). In murine models, BCG vaccination confers partial protection against virulent *M. tuberculosis*, and is used as a model for development of adaptive immunity, as well as a benchmark for experimental candidate vaccines (Mason et al., 2001; Grode et al., 2005; Hinchey et al., 2007; Goonetilleke et al., 2003).

Alcohol consumption has been identified as a risk factor for active tuberculosis (Borgdorff et al., 1998; Buskin et al., 1994; Friedman et al., 1987; Hudolin, 1975; Kline et al., 1995). Due to confounding host and/or lifestyle factors that factor into alcohol consumption, it has been difficult to discern the specific contribution of alcohol to active TB in human studies. In a murine model, alcohol disrupts the containment of a pulmonary challenge with the virulent strain *M. tuberculosis* H37Rv (Mason et al., 2004). Vaccination of mice with *M. bovis* BCG, via generation of adaptive antimycobacterial immunity, affords partial protection against a subsequent challenge with *M. tuberculosis* H37Rv (Mason et al., 2001). We examined the effects of two regimens of chronic alcohol feeding in a murine model utilizing vaccination with BCG prior to pulmonary challenge with *M. tuberculosis* H37Rv. We found that varying the timing of alcohol feeding in relation to BCG vaccination differentially impacts the development of BCG immunity, and also the outcome from a subsequent lung infection with *M. tuberculosis*. These findings may, in part, explain the heightened propensity for alcoholics to develop active disease after infection with *M. tuberculosis*. They also may have broader relevance for the role of alcohol in vaccine efficacy.

Materials and Methods

Animals

Specific pathogen-free BALB/c mice (NCI Animal Program, Frederick, MD) were used for these experiments. Animals were housed in the LSUHSC Biocontainment Level-3 Laboratory and experiments were performed in the Biocontainment Level-3 Laboratory in accordance with appropriate safety precautions recommended by the CDC (DHH, 2009). All

animal procedures were approved by the LSUHSC Institutional Animal Care and Use Committee and the LSUHSC Institutional Biosafety Committee. All data at day 14 and beyond represent groups of $n \geq 4$ unless otherwise stated, and results shown are representative experiments that were reproduced in replicate experiments.

Chronic alcohol diet

Mice consumed the Lieber-DeCarli liquid ethanol diet containing 36% ethanol (LED Group; Dyets #710260, Bethlehem, PA), or were pair-fed an isocaloric liquid control diet (LCD Group; Dyets #710027, Bethlehem, PA). Animals were fed their respective liquid diet for 5/7 days and given chow ad lib for 2/7 days. Alcohol-consuming animals were given water containing 20% (w/v) ethanol on the two chow days. This dietary regimen was begun either three weeks before or three weeks after vaccination with *M. bovis* BCG, and was continued until the end of the experiment. Serum alcohol concentrations (random morning sample) were measured. Twenty-one days after either BCG vaccination (in the LED/BCG group) or alcohol diet initiation (three weeks following BCG vaccination in the BCG/LED group), mice were challenged with *M. tuberculosis* H37Rv.

Vaccination with *M. bovis* BCG

M. bovis BCG was obtained from ATCC (Rockville, MD, catalog #35734). All mice were administered BCG subcutaneously ($\sim 4 \times 10^5$ cfu) in the left hind footpad either three weeks prior (BCG/LED group) or subsequent to the initiation of the alcohol diet (LED/BCG group), which in both situations was a minimum of three weeks prior to pulmonary inoculation with *M. tuberculosis* H37Rv (Baldwin et al., 1998).

M. tuberculosis H37Rv infection

M. tuberculosis H37Rv (Colorado State University School of Veterinary Medicine #UN2814, Fort Collins, CO) was prepared and inoculated into mice intratracheally (50–100 cfu) as previously described (Mason et al., 2004). At serial time points (Days 7, 14, 21, and 28) after inoculation, mice were sacrificed and lungs were quantitatively cultured. *M. tuberculosis* organism burden was then compared between the LED and LCD groups.

Isolation of lung and lung-associated lymph node (LALN) lymphocytes

Lung and LALN lymphocytes were isolated as previously described (Mason et al., 2004). CD4⁺ lymphocytes were positively selected from total lung cells and LALN cells with magnetic beads (Invitrogen Corp., Carlsbad, CA) (Anderson et al., 1993; Collins et al., 1996; Mason et al., 2001). CD4⁺ lymphocytes isolated by the magnetic bead technique were >95% pure for the LALN and lung populations when assayed by flow cytometry (data not shown).

CD4⁺ lymphocyte cytokine production

Cytokine elicitation from CD4⁺ lymphocytes was performed in vitro with *M. tuberculosis*-infected peritoneal macrophages (PMs) as previously described (Mason et al., 2004). Briefly, PMs were obtained by peritoneal lavage after elicitation with concanavalin A 96 hours previously. PMs were plated at 2×10^5 cells/well, allowed to adhere, infected with *M. tuberculosis* H37rv (MOI 5:1) overnight, and incubated in culture for 24 hours. After this time, LALN or lung CD4⁺ T cells (1×10^5 /well) were added to the wells for 48 hours. Control wells were included. After the incubation, supernatants were harvested and saved at -80°C for cytokine assay.

After the in vitro incubation, supernatants were and saved at -80°C and subsequently assayed for IFN- γ and IL-10 by ELISA (R&D Systems, Minneapolis, MN). The lower limits

of detection for these assays were IFN- γ : 2 pg/ml; and IL-10: 4 pg/ml. Cytokine levels were then compared between the LED and LCD groups.

Isolation of popliteal lymph node cells and IFN- γ ELISpot Assay

Three weeks after BCG vaccination and alcohol feeding (BCG/LED and LED/BCG groups), mice were sacrificed and the left hind leg popliteal lymph nodes were dissected, removed, and dispersed into single cell suspensions. Cells were plated in 96-well murine IFN- γ ELISpot plates (R&D Systems, Minneapolis, MN) at 1×10^6 cells/well and stimulated with PPD 5 ug/ml (Mycos Research, Loveland, CO) for 48 hours in a 37°C/5%CO₂ incubator. Control wells contained cells in media that were not stimulated. After incubation, wells were washed three times with ELISpot wash buffer and one time with cold deionized water to lyse any remaining cells. Detection antibody was added for four hours followed by streptavidin-AP for two hours, then BCIP/NBT Chromogen was added for one hour to develop spots. The numbers of spots were enumerated and compared between the groups. In one assay, CD8+ T cells were positively selected and removed from the PLN cells using magnetic beads (Invitrogen, Carlsbad, CA), enriching the resulting PLN cells for CD4+ T cells (in order to exclude CD8+ T cells as the major source of IFN- γ). Spot numbers were then compared between the groups.

Statistical Analysis

Differences between BCG/LED and LED/BCG groups were analyzed by ANOVA, with a Bonferroni Dunn follow-up test. A p value of ≤ 0.05 was considered significant (Bourke et al., 1985).

Results

Chronic alcohol diet

Animals on the LED diet consumed ≈ 8 ml/liquid diet/day and gained weight throughout the course of the experiments, though slightly more slowly than the mice on the LCD. Based on dietary consumption, the caloric intake of our mice was 8–9 kcal/day, which is at the lower end of the normal range for adult BALB/c mice (Subcommittee on Laboratory Animal Nutrition, 1995). Serum alcohol levels (random morning sample obtained immediately after the 12-hour dark cycle ended) were measured by the LSUHSC Alcohol Research Center Core Laboratory with an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA) on representative mice (n=15) on the LED diet for 4–6 weeks, with a median concentration of 39 mg/dl (range 19–130 mg/dl). Serum albumin levels were also assayed at the sacrifice time points (after 5 – 9 weeks of alcohol or control diet administration), and all mean values of both the LED and LCD groups fell within or slightly above the reference range for mice (2.5–3.0 gm/dl) (Research Animal Resources, 2004).

Alcohol consumption effects on BCG-induced protection against *M. tuberculosis*

Vaccination with BCG prior to intratracheal inoculation with *M. tuberculosis* confers significant protection (≥ 1 log-fold) against growth of *M. tuberculosis* in the lung, as previously reported (Mason et al., 2001), but alcohol consumption prior to vaccination significantly impairs BCG-induced protection in the LED/BCG mice at 21 and 28 days after *M. tuberculosis* inoculation (Figure 1A), with 2–3-fold higher organism burdens at those times. The challenge was performed in triplicate, confirming an alcohol-induced *M. tuberculosis* growth disparity in the lung with consistently significantly higher organism burden (replicate data not shown). However, when BCG vaccination was administered three weeks prior to the initiation of alcohol feeding (BCG/LED) (and *M. tuberculosis* was inoculated three weeks after beginning the alcohol diet), culture results revealed similar

control of *M. tuberculosis* in the lungs of the alcohol and control diet mice over the same time course (Figure 1B). Thus, in order for alcohol to adversely impact BCG-induced protection, alcohol consumption had to be ongoing at the time the vaccine was administered. If alcohol was begun after vaccination, it failed to alter BCG-induced protection against *M. tuberculosis*.

CD4+ lymphocyte cytokine production

At the serial time points at which the lungs were cultured for *M. tuberculosis* burden, as well as at the Day 35 time point, lung and LALN were harvested and CD4+ lymphocytes isolated as previously described. CD4+ lymphocytes isolated from LALN and lung from both the LED/BCG and BCG/LED groups were assayed for elicited cytokine production after stimulation by *M. tuberculosis*-infected PMs as described above. Figures 2A & 2B show elicited IFN- γ production from the both sites by LED/BCG and LCD/BCG groups and Figures 2C & 2D show the same for the BCG/LED and BCG/LCD groups. There is significantly less IFN- γ elicited from the LED/BCG lung CD4+ lymphocytes at the Day 21 time point as compared to the LCD/BCG group, with trends to less IFN- γ from the LED/BCG cells at the 28 and 35 day time points as well (Figure 2B), corresponding to the time frame when there is discrepancy in bacterial growth between the groups (see Figure 1). However, in the BCG/LED group (Figures 2C & 2D), there was no decrement in IFN- γ production by the CD4+ LALN or lung T cells compared to the BCG/LCD group. In fact, there was heightened IFN- γ production by the CD4+ T cells from both sites in the BCG/LED group. Thus, IFN- γ production, elicited in vitro from equal numbers of LALN and lung CD4+ lymphocytes, is significantly less in the LED/BCG group at the Day 21 time point, but is unimpaired in the BCG/LED group. Figure 3A shows IL-10 elicited from lung CD4+ T cells from the LED/BCG and LCD/BCG groups, and reveals no detectable upregulation of IL-10 production by the LED/BCG group. There are no differences between the groups, and therefore, no demonstrable shift to a CD4+ lymphocyte Th2 profile over this time frame was evident. Figure 3B reveals IL-10 elicited from the lung CD4+ T cells from the BCG/LED and BCG/LCD groups. Similarly to the IFN- γ data in Figure 2, the BCG/LED group has higher IL-10 levels than does the BCG/LCD group at several of the time points. However, despite the increased IL-10 production, IFN/IL-10 ratios favored a Th1 T cell bias at all times from both groups, due to the concurrently heightened IFN- γ levels from the BCG/LED group. This is consistent with the higher bacterial clearance in the BCG/LED group.

Popliteal Lymph Node (PLN) Cell IFN- γ ELISPOT Assay

Murine IFN- γ ELISPOT assays were performed with PLN cells stimulated with PPD from LED/BCG and BCG/LED mice and their respective control diet mice. Figures 4A & 4B reveal, in representative ELISPOT wells, that the PLN cell production of IFN- γ from the LED/BCG mice was significantly impaired compared to the LCD/BCG (and Chow/BCG) control mice. However, IFN- γ spot-forming cells in the BCG/LED mice were not different from the BCG/LCD (nor the BCG/Chow) control mice (Figure 4B). Removal of the CD8+ T cells by selection from the total PLN cells (CD4+CD8- group, thereby enriching the population for CD4+ lymphocytes) had no significant influence on the results. Control (unstimulated) wells had a mean of six spots. These results confirm that the effect of alcohol consumption on impairing IFN- γ production is only evident when alcohol is present at the time of BCG vaccination. When alcohol administration is begun after vaccination, the production of IFN- γ from the regional lymph nodes is unimpaired. See Table 1 for group results.

Discussion

M. bovis BCG vaccination confers partial protection against the growth of *M. tuberculosis* in the lungs of mice (Baldwin et al., 1998; Brooks et al., 2001; Mason et al., 2001). BCG-induced protection is related to earlier, more robust LALN and lung CD4⁺ Th1 lymphocyte responses after *M. tuberculosis* inoculation as compared to unvaccinated mice (Mason et al., 2001). Here we report that chronic alcohol consumption can ablate this protection in mice, but importantly, that the timing of the alcohol exposure in relationship to vaccination is critical in determining whether the protection is intact or not. When alcohol consumption is started prior to BCG vaccination, it adversely alters *M. tuberculosis* growth control, ablating the vaccine protection. If alcohol is begun after BCG vaccination, there is no significant impact on protection. We also demonstrate that locoregional CD4⁺ lymphocyte production of IFN- γ the Th1 cytokine critical to mycobacterial growth control (Cooper et al., 1993; Flynn et al., 1993), is differentially affected by the timing of the alcohol consumption in relation to the vaccination. This effect is demonstrable both in the regional popliteal lymph nodes after BCG vaccination, and in the lung after *M. tuberculosis* challenge of vaccinated mice. Our work is consistent with the primary effect of alcohol occurring at the time of initiation of adaptive immunity at the DC/CD4⁺ T cell juncture, potentially adversely affecting both the DC and the CD4⁺ T cell.

There are limited reports of the effects of alcohol consumption on vaccination. Most have evaluated the response to hepatitis B vaccination as determined by seroconversion, and the reported alcohol effects are varied: most studies report alcohol use as a risk for impaired seroconversion (Hagedorn et al., 2010; Kim et al., 2008; Wang and Lin, 2007). However, others report either no impairment in vaccine serologic response in alcohol-consuming subjects (Kulkarni et al., 2006), or that alcoholics can mount antibody responses to vaccination, but that with continued consumption, responses may not be as durable as in non-consuming subjects (Nalpas et al., 1993). In many of these reports, quantitation of alcohol consumption is poorly described, making the studies difficult to compare. Other investigators found decreased lymphocyte proliferation and serologic responses to tetanus toxoid vaccination in offspring of nonhuman primate mothers that were fed weekly doses of ethanol while pregnant (Grossman et al., 1993). Thus, alcohol is capable of altering the development of immunity.

Serological responses to vaccination were the primary outcome of these reports, but for *M. tuberculosis* infection, the T cell arm of adaptive immunity is most relevant. Studies have shown the impact of alcohol on this arm of the immune system as well. Fan et. al. noted reduced proliferation of ethanol-unexposed CD4⁺ and CD8⁺ T cells after stimulation by ethanol-exposed, OVA peptide-stimulated DCs, which was partially restored by stimulation with a greater amount of peptide (Fan et. al., 2011). DC antigen capture and processing was intact, but costimulatory molecule upregulation and cytokine production were depressed by ethanol. Gurung and colleagues reported that CD8⁺ T cells from ethanol-exposed mice had dampened responses to *Listeria monocytogenes* peptide epitopes (as compared to CD4⁺ T cells), including proliferation and cytokine production (Gurung et. al., 2009).

Spies and colleagues evaluated cell-mediated immunity in long-term alcoholics undergoing elective gastrointestinal surgery, and found that alcoholic patients had significantly reduced delayed type hypersensitivity (DTH) responses to skin testing with a panel of common antigens, as well as reduced Th1:Th2 T cell ratios and IFN- γ :IL-10 ratios (from lipopolysaccharide-stimulated whole blood cells) that failed to increase post-operatively, as occurred in the nonalcoholics (Spies et al., 2004). The alcoholics also had significantly higher rates of post-operative infections, especially pneumonia. Thus, alcohol exposure may

impair the serological and/or cellular immune response to antigenic challenge, of which the cellular responses may have significant import for infection with *M. tuberculosis*.

To further dissect the early effects of alcohol on the inflammatory events at the juncture of innate and adaptive immunity, Heinz et al. studied development of adaptive immunity in a murine model of ovalbumin (OVA) sensitization. Mice fed an alcohol diet for eleven days had impaired CD4⁺ T cell Th1 cytokine production, as well as diminished DTH response to OVA but enhanced Th2 (IL-13) cytokine production (Heinz and Waltenbaugh, 2007). CD11c⁺ dendritic cell (DC) profiles were further evaluated in this model, and were found to be the most potent inducers of CD4⁺ T cell cytokines, but also to be adversely affected when derived from alcohol-consuming mice. Alcohol-exposed DCs resulted in significantly reduced CD4⁺ T cell IFN- γ and IL-12, but unimpaired IL-2 production. Other investigators have also described numerous adverse effects of alcohol on DC number and function, including reduced antigen presentation, reduced T cell stimulation, deranged cytokine production, and decreased ability to polarize Th1 T cells (Siggins et al., 2009; Edsen-Moore et al., 2008; Lau et al., 2006; Mandrekar et al., 2004; Szabo et al., 2004; Miksza et al., 1995). Thus, ethanol may lead to protean effects on immune cells, including DCs and T cells. Our work is consistent with many features of the described alterations.

We also noted in mice in which BCG protection remains intact, lung CD4⁺ T cell production of IFN- γ and IL-10 is of greater magnitude than that of the nonalcohol-consuming control mice. There are numerous reports in the literature of acute alcohol consumption downregulating proinflammatory cytokines, such as TNF, IL-1 and IL-6 (Szabo et al., 1995; Nelson et al., 1989a; Nelson et al., 1989b; Lin et al., 1998; Stoltz et al., 2000; D'Souza et al., 1989), but also that subacute or chronic consumption may upregulate inflammatory cytokines, including tumor necrosis factor (Enomoto et al., 2001; Kamimura and Tsukamoto, 1995), IL-1 and transforming growth factor (TGF)- β (Martinez et al., 1992; Spies et al., 2004), IL-6 (Hong et al., 2002), IL-10, IL-12, and IFN- γ (Szabo, 1998; Plackett et al., 2005; Zisman et al., 1998). The etiology of inflammatory cytokine upregulation is not completely clear, but several mechanisms have been postulated. Chronic alcohol may increase the permeability of the gut, and allow endotoxin to breach the intestinal barrier, gaining access to the portal circulation (Mason et al., 1998); alcohol may enhance Kupffer cell expression of CD14 and heightened TNF production early after alcohol begins (Enomoto et al., 2001); uptake and degradation of plasma cytokines may be variably affected by alcohol and may influence cytokine clearance rates (Deaciuc et al., 1996); and/or dysregulation of plasma glucocorticoid levels by alcohol may result in an imbalance of regulatory and proinflammatory cytokines, favoring the proinflammatory cascade (Sipp et al., 1993; Barber et al., 1993; Waltman et al., 1993).

In this work, we used BCG vaccination as a model for studying the impact of alcohol on development of adaptive immunity because BCG is currently the sole vaccine for *M. tuberculosis*, and has been used for decades in humans, especially in developing countries. Given at a very young age, it protects neonates and children against tuberculous meningitis and other extrapulmonary or disseminated forms of tuberculosis, but is not effective in adolescents and adults for prevention of initial infection or reactivation disease (Antas et al., 2008). As a live vaccine, immunocompromised hosts, in particular those HIV-infected, should not be administered BCG. Thus, with the burgeoning global burden of *M. tuberculosis* infection, the specter of highly drug-resistant strains emerging, in addition to the problems with BCG efficacy, the race is on for a more effective vaccine. The ideal vaccine will be effective in pre-exposure states to prevent infection, and also in the post-exposure situation (latent infection) to prevent reactivation. Some of the vaccine candidates currently in early trials are based on live BCG or *M. tuberculosis* (though these strains are attenuated and modified for greater efficacy) and are being administered to adults in ongoing

clinical trials (Kaufmann et. al, 2010). Most of the trials are being conducted in the developing world, which has the highest rates of endemic tuberculosis. These areas are fraught with significant socioeconomic problems, e.g., poverty, homelessness, malnutrition, mental illness, and often substance abuse, including alcohol. As previously mentioned, alcohol consumption has been identified as a risk factor for tuberculosis infection and active tuberculosis, as well as for greater morbidity and mortality from tuberculosis. Our results reported here shed light on the impact of alcohol on BCG vaccination efficacy in mice, especially in regard to the relative timing of alcohol consumption and BCG administration. As new vaccine candidates are being evaluated in clinical trials in adolescents and adults, our results provide the basis for consideration of current alcohol use by vaccine recipients as a potential confounding variable in vaccine efficacy.

In summary, we have demonstrated for the first time that the impact of the differential timing of alcohol consumption in relation to BCG vaccination on the efficacy of protection against a subsequent challenge with the pathogen *M. tuberculosis* in mice. Vaccination while the host is actively consuming alcohol leads to failure of vaccine protection in our model, but vaccination prior to initiation of alcohol leads to intact protection. Our findings directly correlate with production of IFN- γ by locoregional CD4⁺ T cells in *M. tuberculosis*-challenged mice, as well as by the popliteal lymph node cells after BCG vaccination alone. Thus, alcohol disrupts the development of adaptive immunity to BCG, but only if alcohol is being actively consumed at the time of the vaccination. Our findings may have relevance to efficacy of future tuberculosis vaccines, and possibly more broadly, to other vaccines as well.

Acknowledgments

The authors thank Connie Porretta for expert assistance with flow cytometry analyses.

Supported NIAAA Grant AA011760, AI058810, HL076100, T32AA007577 & The Wetmore Foundation

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Figure 1.

1A. Lung burden of *M. tuberculosis* H37Rv in mice consuming either the LCD or LED diet prior to *M. bovis* BCG-vaccination and *M. tuberculosis* infection. There are significantly more organisms present in the LED/BCG mice at 21 and 28 days, at which times the LED mice had 157% and 237% increases in cfu respectively, compared to the LCD mice.

* $p < 0.05$ LCD/BCG vs LED/BCG.

Figure 1A mean cfu $\pm$ SEM: Day 7: LCD/BCG = $2 \pm 0 \times 10^2$; LED/BCG = $1.6 \pm 0.6 \times 10^2$; Day 14: LCD/BCG = $3.5 \pm 0.6 \times 10^4$; LED/BCG = $5.4 \pm 0.7 \times 10^4$; Day 21: LCD/BCG = $1.2 \pm 0.1 \times 10^5$; LED/BCG = $2.0 \pm 0.1 \times 10^5$; Day 28: LCD/BCG = $2.9 \pm 0.6 \times 10^5$; LED/BCG = $6.9 \pm 1.4 \times 10^5$.

1B. Lung burden of *M. tuberculosis* H37Rv in mice vaccinated with *M. bovis* BCG prior to consuming either the LCD or LED diet and *M. tuberculosis* infection. There are no significant differences in the burdens in the BCG/LED and BCG/LCD mice. **Figure 1B** mean cfu $\pm$ SEM: Day 7: BCG/LCD = $1.4 \pm 0.5 \times 10^2$; BCG/LED = $3.1 \pm 1.3 \times 10^1$; Day 14:

BCG/LCD = $9.0 \pm 1.6 \times 10^3$; BCG/LED = $2.1 \pm 0.5 \times 10^3$; Day 21: BCG/LCD = $4.1 \pm 0.8 \times 10^4$; BCG/LED = $5.7 \pm 1.3 \times 10^4$; Day 28: BCG/LCD = $8.6 \pm 1.5 \times 10^4$; BCG/LED = $3.5 \pm 0.6 \times 10^4$.

Figure 2.

2A. Elicited IFN- γ levels after stimulation of LALN (**Figures 2A & 2C**) and lung (**Figures 2B & 2D**) CD4⁺ lymphocytes from LED/BCG and LCD/BCG, and BCG/LED and BCG/LCD groups by *M. tuberculosis*-infected PMs. ELISA assays performed on 48-hour cell culture supernatants. There are no differences in IFN- γ between the LCD/BCG and LED/BCG LALN CD4⁺ T cells (**Figure 2A**), but there is significantly more IFN- γ from the BCG/LED LALN CD4⁺ lymphocytes vs the BCG/LCD at 21, 28 and 35 days after *M. tuberculosis* infection (**Figure 2C**). * $p < 0.05$ BCG/LED vs BCG/LCD. For the lung CD4⁺ lymphocytes, there is significantly less IFN- γ produced by the LED/BCG CD4⁺ T cells at 21 days after *M. tuberculosis* infection, and trends to less IFN- γ at 28 and 35 days after *M. tuberculosis* infection (**Figure 2B**). * $p < 0.05$ LCD/BCG vs LED/BCG. In the BCG/LED group, there is more IFN- γ produced at all time points, significantly so at 14, 21 and 35 days following *M. tuberculosis* infection (**Figure 2D**). * $p < 0.05$ BCG/LED vs BCG/LCD.

Figure 3.

Elicited IL-10 levels after stimulation of lung CD4+ lymphocytes from LED/BCG and LCD/BCG (**Figure 3A**), and BCG/LED and BCG/LCD (**Figures 3B**) groups by *M. tuberculosis*-infected PMs. There are no differences in IL-10 levels between the LED/BCG and LCD/BCG groups in **Figure 3A**; however, in **Figure 3B** there are higher levels of IL-10 in the BCG/LED group at all time points, reaching statistical significance at 21, and 35 days after *M. tuberculosis* infection. * $p < 0.05$ BCG/LED vs BCG/LCD.

Figure 4. Representative ELISPOT assay of PPD-stimulated IFN- γ spot-forming cells in the popliteal lymph nodes of mice vaccinated with BCG. **Figure 4A:** ELISPOTs from representative mice in Chow/BCG, LCD/BCG and LED/BCG diet groups, with fewer spots present in the LED group. The assay was repeated after selection and removal of the CD8⁺ T cells from the total cells, thereby enriching the cells for CD4⁺ T cells, without differences from the total cells. **Figure 4B:** ELISPOTs from representative mice in BCG/Chow, BCG/LCD and BCG/ LED diet groups, with no fewer spots in the LED group. The assay was also repeated after selection and removal of the CD8⁺ T cells from the total cells, thereby enriching the cells for CD4⁺ T cells, with no change in the results.

Table 1

Group ELISPOT Results

		Diet Group	
	Chow	LCD	LED
<u>BCG Group</u>			
Diet/BCG	72.5±16.8	58.6±5.3	28.0±5.7 [*]
BCG/Diet	127±8.4	67±14.9	126±33.2

Data=mean±SEM; n≥5/grp

^{*}
p<0.0001 LED/BCG vs BCG/LED