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ALCOHOL-MEDIATED POLARIZATION OF TYPE 1 AND TYPE 2 IMMUNE RESPONSES

Omar Latif [1], Jeffrey D. Peterson [2], Carl Waltenbaugh [3]

[1] Stanford University School of Medicine, Stanford, CA 94305, Department of Pharmacology, [2] CuraGen, Inc., Branford, CT 06405, [3] Northwestern University Medical School, Chicago, IL 60611

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1. ABSTRACT

Immune responses of alcoholics are often compromised, placing them at increased risk for frequent and severe infections. We demonstrate, using a murine model that parallels human alcoholism, that ethanol consumption polarizes adaptive immune responses by $CD4^+$ T helper lymphocytes (Th). Alcohol impairs Th1-regulated cell-mediated, although Th2-regulated humoral responses are largely unimpaired and may be enhanced. Ethanol's effect is most pronounced during the early or cognitive phase of the immune response, when antigen-presenting cells (APC) interact with T cells. We find that alcohol does not act directly upon T cells, but upon APC. Consequences of this interaction of alcohol with APC *in vivo* are diminished Th1-mediated delayed hypersensitivity (DTH) reactions, while at the same time increased Th2-regulated serum IgE levels are seen. Further ethanol consumption leads to decrease affinity of the IgG_{2a} and IgG_{2b} Th1-regulated antibody isotypes.

2. INTRODUCTION

The effects of alcohol on the immune system may precede pathological alterations seen in other organ systems. Chronic alcohol abuse correlates with increased infection risk and disease severity (1-6), bacterial infections including pneumonia, bronchitis, tuberculosis, peritonitis, bacteremia, and endocarditis are more common in alcoholics than in the general population (2-9). Experimental animal models confirm that chronic alcohol consumption promotes lung infection in rodents (10-16).

When consumed in excess, alcohol alters normal immune function, evident in the increased frequency and severity of infections seen in alcoholic patients (1, 17). Cell-mediated and humoral immune responses are affected, impairing DTH and elevating serum immunoglobulin levels (18, 19). Likewise, in experimental animals, ethanol consumption for 7-10 d impairs cell-mediated immunity while humoral immunity is enhanced (20-22). In humans and mice, two types of $CD4^+$ T lymphocytes, termed Th1 and Th2, defined by cytokine secretion and immune function, are largely responsible for mediating cell-mediated (Type 1) and humoral (Type 2) immunity (23, 24). In general, ethanol polarizes the immune response away from Type 1 and toward Type 2 (22, 25).

Cell-mediated immunity is important for resistant to infection by intracellular parasites and for allograft rejection. Grafts between individuals that differ at the major and minor histocompatibility loci are rejected. Major histocompatibility complex (MHC) differences usually lead to acute reject and minor histocompatibility differences lead to more prolonged chronic rejection. In mice, the male-specific minor histocompatibility antigen, Hya, provides a well-characterized system for the study of chronic graft rejection. Females of mouse strains with the $H2^b$ haplotype (*e.g.*, C57BL/6 strain) generate strong cellular immune responses against Hya-disparate grafts as measured by allograft rejection and by the development of CTL and DTH responses directed specifically against Hya (26). We showed that alcohol consumption causes a prolongation of Hya disparate skin grafts and that this is most likely due to the impairment of DTH mechanisms in ethanol-consuming graft recipients (27).

In mice, alcohol consumption also causes a rapid alteration in leukocyte populations (28-32). B cells rapidly decline in number even after a few days alcohol consumption (32). Paradoxically, loss of B cells occurs precisely at the time when serum immunoglobulin and antibody levels are unaffected or enhanced (22, 32). Splenic T cells do not overtly decline in ethanol-fed mice (32) although little is known about the effect of ethanol on a particular (clonotypic) T cell population in alcohol consuming mice. Antigen presenting cells (APC) influence the development of Type 1 and Type 2 adaptive immune responses. Ethanol affects APC (*e.g.*, dendritic cells, macrophages, or B cells) function (33, 34) by decreasing IL-12, a cytokine central to many Type 1 responses (35). Ethanol consumption by BALB/c APC donor mice decreases both IL-12 production and subsequent ovalbumin- (OVA) stimulated IFN-γ production by purified T cells from αβ T cell receptor (TCR) transgenic mice (DO11.10, ref 36).

Dietary ethanol decreases overall splenic cellularity in experimental animals by as much as 50% with greatest losses occurring in B and T lymphocyte populations (28, 31, 37, 38). Previous studies from our laboratory and others have shown that ethanol impairs CD4 T-cell dependent, cell-mediated immunity, whereas antibody responses are largely unaffected (22, 34, 39, 40). CD4 lymphocytes regulate both antibody isotype [*i.e.*, IgG_{2a} responses are regulated by Th1, whereas IgE is regulated by Th2 (41)] and antibody affinity (42, 43) through the production of regulatory cytokines. Here we show that ethanol consumption decreases DTH and IFN-γ while at the same time, Type 2 IgE responses are augmented. Ethanol also decreases the relative affinity of an antibody response to a chemically defined epitope. The decrease in affinity is apparent only for IgG_{2a} and IgG_{2b}, Type 1-associated isotypes. IgG_1 anti-hapten responses and antibody responses of all isotypes tested to the carrier molecule are unaffected.

3. MATERIALS AND METHODS

3.1. Animals

Female BALB/c mice were purchased from the Small Animal Production Unit, National Cancer Institute, Frederick, MD, and were held for a two week acclimation period prior to use. OVA-specific αβ TCR transgenic D011.10 mice (44), syngeneic with BALB/c mice, were bred and maintained in the Northwestern University vivarium. Mice were 8-10 weeks of age at the initiation of experiments. All animal protocols met with prior approval of the Northwestern University Animal Care and Use Committee.

3.2. Diets and feeding

Mice were fed a solid diet consisting of laboratory chow (TekLad 7022, NIH-07 diet, Harlan TekLad, Madison, WI) and water *ad libitum*, a liquid ethanol diet (LED), or pair-fed a liquid control diet (LCD), as previously described (22). Liquid diets that meet the American Institute of Nutrition (AIN) standards for rodents were compounded in our laboratory. Briefly, the laboratory-formulated diets contained sucrose (7.84 g/l, United Sugar, Corp., Minneapolis, MN), corn oil (1.28 g/l, Dyets, Bethlehem, PA, item 401150), AIN mineral mixture 76 (893 mg/l, Dyets, 200000), AIN vitamin mixture 76A (255 mg/l, Dyets, 300050), *DL*-methionine (77 mg/l, Dyets, 402950), choline bitartrate (51 mg/l, Dyets, 400750), xanthine gum (200 mg/l, Dyets, 402525), and casein hydrolysate (5.1 g/l, US Biochemical, Cleveland, OH, 12855). BALB/c mice were fed diets containing ethanol at 45.8 g/l (30% ethanol-derived calories, LED30). The LCD isocalorically substituted sucrose for ethanol. All liquid diets contained 1 kcal/ml (4.184 kJ/ml) and provided 20% protein-, 11% fat (corn oil)-, and 66% carbohydrate (sucrose and/or ethanol)-derived calories. Solid laboratory chow and water were removed from the groups fed liquid diets and were replaced with the appropriate liquid diet. Mice were pair-fed; *i.e.*, caloric intake by the LED-fed mice determined the caloric intake for the LCD-fed mice for the subsequent day.

3.3. Immunization for antibody responses

Fowl γ globulin (FγG, lot #167, Rockland Laboratories, Gilbertsville, PA) and bovine serum albumin (BSA, Midwest Scientific, St. Louis, MO) were conjugated with 5-dimethylamino-1-napthalenesylfonyl chloride (dansyl chloride, DNS, Acros Organics, Fisher Scientific, 11585-0010). Briefly, 100 ? l of a 10-40 mg/ml freshly prepared solution of DNS in N,N-dimethylformamide

(DMF, Sigma, D-4254) was added to 10 mg carrier protein (FγG or BSA) dissolved in 1 ml 0.15M phosphate buffered saline (PBS, pH 7.2). The reactants were incubated for 2 h in the dark at 22°C. DNS-protein conjugates were separated from DMF and unreacted DNS by gel filtration on a 1.5 x 30 cm Sephadex G-25 column (Sigma) equilibrated with PBS and the first eluting fluorescent (330 nm excitation 530 nm emission) fraction collected. The DNS-protein molar ratio was calculated from absorbance at 330 nm (DNS) and 280 nm (BSA or FγG) and correction was made for spectral overlap. A broad range (DNS_1-BSA to DNS_{62}-BSA) of hapten-BSA conjugates was prepared for ELISA (enzyme-linked immunosorbent assays) and DNS_6-FγG was prepared for immunization.

Preliminary experiments showed that BALB/c mice made very strong primary antibody responses to FγG, but rather weak responses to the DNS hapten. We previously reported (45, 46) that boosting mice with antigen 3-5 d after the initial antigen priming would result in a robust antibody response. In the experiments reported here, groups of 5 BALB/c mice were fed SD, LCD, or LED30 and remained on the diets throughout the experimental protocol. The mice were then immunized subcutaneously with 100 μg DNS_6-FγG emulsified in complete Freund's adjuvant (CFA) on dietary day 4, then boosted 4 d later (dietary day 8) with 100 μg DNS_6-FγG in PBS containing aluminum-magnesium hydroxide gel (Maalox, Rorer Pharmaceuticals, Inc., Ft. Washington, PA). Mice were bled and sera were prepared on dietary day 14, 10 days after antigen priming and 6 d after antigen boosting.

3.4. Assessment of delayed hypersensitivity

Female BALB/c mice were fed LED30, pair-fed LCD or solid laboratory chow and water for the entirety of the experiments. On dietary day four the mice were primed subcutaneously with 100 μg FγG emulsified in complete Freund's adjuvant (H37Ra, Difco Laboratories, Detroit, MI) as previously described (34). Six days after immunization, mice were anesthetized with sodium pentobarbital (60 mg/kg), baseline ear thickness was measured using a Mitutoyo dial thickness gauge (model 7326, 47), and 20 μg FγG in 10 μl PBS was injected intradermally into the dorsal surface of the ear using a syringe fitted with a 30-gauge needle. Antigen-induced ear swelling, determined 24 h later in a blind reading, is expressed in units of 10^{-4} inches (47).

3.5. Cytokine and antibody analysis

An enzyme-linked immunosorbent assay (ELISA) was used to determine cytokine concentrations in tissue culture supernatants. Briefly, wells of 96-well Maxisorp (NUNC) plates were coated with anti-IL-2 (Caltag Laboratories, Burlingame, CA, RM9120) or anti-IFN-γ (Caltag, RM9110) antibody overnight, then blocked with PBS containing 2% BSA. Standards, recombinant IL-2 (Peptrotech, Rocky Hill, NJ, 212-12) or recombinant IFN-γ?Peptrotech, 31505?and diluted culture supernatants were incubated for 2 h at 37°C on the coated plates. The plates were washed and biotinylated anti-IL-2 (Caltag, RM90215) or anti-IFN-γ (Caltag, RM90015) was added for 1 h at 37°C. The plates were washed and horseradish peroxidase-conjugated streptavidin (KPL Laboratories, Rockville, MD) was added. After washing 100 μl 3,3',5,5'-tetramethylbenzidine peroxidase substrate TMB substrate (BioFX Laboratories, Owings Mills, MD) was added. Chromogenic development was stopped by the addition of 0.18M H_2SO_4. Plates were read in a SpectraMax[190] plate reader (Molecular Devices, Palo Alto, CA) at 450 nm and SoftMax Pro (Molecular Devices) curve fitting software used to determine concentrations of unknowns.

The presence of FγG and DNS-specific antibodies were determined by ELISA. Briefly, individual wells of 96-well micro plates (NUNC Maxisorp) were coated overnight at 4°C with 100 μl of a 10 μg/ml solution of FγG or DNS_x-BSA as indicated in the figures. The plates were washed three times between each step with PBS containing 0.05% Tween-20 (PBST, Sigma P-1379). The wells were blocked for 1 h at 37°C with 300 μl of PBS containing 3% (w/v) BSA, and 100 μl serial (log_2) dilutions of sera made in PBST containing 1% BSA (PBST-BSA) were added to the wells, and incubated for 1 h at 37°C. Isotype-specific, biotin-labeled goat anti-mouse antibody (anti-IgM + IgG, M30815; anti-IgG_1, M32015; anti-IgG_{2a}, M32215; IgG_{2b}, M32415, Caltag), diluted 1:2000 to 1:4000 in PBST-BSA was added to the appropriate wells for 1 h at 37°C. Following addition of 100 μl of TMB substrate, plates were processed as described above.

Single-cell cytokine secretion was visualized by a modification of ELISA, the enzyme-linked immunospot (Elispot) assay. Briefly, sterile 96-well flat-bottom PVDF membrane plates (Unifilter 350, Whatman 7770-001) were coated with sterile anti-IFN-γ (PharMingen, 554430) antibody diluted in PBS, incubated overnight at 4°C, then blocked for 2 h at 37°C with PBS containing 1% BSA. Single cell suspensions of 250,000 viable, unimmunized BALB/c spleen cells were stimulated with anti-CD3. After 24 h culture, the plates were washed to remove cells and culture supernatants and incubated with biotinylated anti-IFN-γ (Caltag, RM90015) overnight at 4°C. After washing, alkaline phosphatase labeled anti-biotin antibody (Vector Laboratories, Burlingame, CA, SP-3020) was added to each well and incubated for 2 h at 22°C then washed extensively. Spots were developed by the addition of NBT/BCIP (BioFX, BCID-0100-01) chromogenic substrate to each well. Spot development was stopped after 5-20 min by washing with distilled water. Plates were air dried then spots were read and enumerated on an automated reader (C.T.L. Analyzers, Cleveland, OH).

3.6. Data analysis

Sera of all three dietary groups for a given isotype and antigen-coating were assayed on the same 96-well plate to minimize plate-to-plate variation. ELISA data were analyzed and the effective reciprocal dilution of the sera that would cause 50% maximal binding (ED_{50}) was determined. ED_{50} values were calculated by fitting data to a sigmoidal curve with variable slope using Prism software (GraphPad Software, Inc., San Diego, CA). Data are

Table 1. Ethanol consumption impairs DTH responses in BALB/c mice

Strain	Diet	n	FgG Immunization	FgG Challenge	Ear Swelling ($x\ 10^{-4}$ in)			p
			dietary day 4	dietary day 10	arith mean	±	SEM	
BALB/c	SD	5	100 µg	20 µg	37.8	±	3.73	—
BALB/c	LCD	5	100 µg	20 µg	31.9	±	2.13	0.184
BALB/c	LED30	4	100 µg	20 µg	15.9	±	2.83	0.0002
BALB/c	SD	2	naïve	20 µg	1.0	±	0.88	<0.001

Naïve female BALB/c mice were fed LED30, pair-fed LCD, or remained on SD and water *ad libitum*. On dietary day 10, the mice were bled and total IgE levels determined by ELISA. Data represent the arithmetic mean ± SEM. Statistical significance determined by Student's *t* test.

expressed as $LogED_{50}$ times/divided by the log standard error of the mean. In all cases, the coefficient of determination (R^2), which measures how well the theoretical curve fits the data, is > 0.997 for all calculations. Both Student's *t* tests to obtain two-tailed P values and one-way analysis of variance (ANOVA) of the $LogED_{50}$ values were performed the GraphPad Prism statistical software. Data are reported as the arithmetic mean ± SEM. Statistical significance was assessed by two-tailed Student's *t*-test using *JMP* software from the SAS Institute (Cary, NC).

4. RESULTS

To assess the effect(s) of alcohol on immune function, female BALB/c mice were fed a liquid diet containing 30% ethanol-derived calories (LED30), pair-fed a liquid control diet (LCD) that isocalorically substitutes sucrose for ethanol, or fed a solid chow diet (SD) and water *ad libitum*. Mice were fed their respective diets throughout the experiment, consuming between 8-12 kcal (ml) liquid diet/day/mouse. On dietary d 4 all mice, except naïve, were subcutaneously immunized with 100 µg fowl γ globulin (FγG) emulsified in complete Freund's adjuvant (CFA). Six d later (dietary d 10) the mice were anesthetized, basal ear thickness determined, and their ears were injected intradermally with 20 µg FγG in 10 µl 0.15M phosphate buffered saline (PBS), pH 7.2. Twenty-four h later (dietary d 11) change in ear thickness was determined, Table 1. Statistical significance was determined using a two-tailed Student's *t* test. The naïve group assesses antigen non-specific ear swelling. LED30 consumption significantly (*p <0.001*) diminishes FγG-specific DTH responses compared to either SD or LCD fed mice. Slightly diminished DTH responses in the LCD compared to the SD group reflect caloric limitation imposed by pair feeding, however, this difference is not statistically significant. These data show that ethanol diminishes Th1-mediated antigen-specific DTH.

In conventional mice, immune responses are often assessed following immunization with antigen emulsified in CFA approximately one week previously. Adjuvant usage, nature of the antigen, and route of antigen administration influence the nature of the ensuing immune response (48, 49). The availability of transgenic (Tg) mice opened new possibilities for the precise localization of ethanol-induced impairment(s) of immune function. To determine the effect of ethanol on the immune response in the absence of *in vivo* immunization and adjuvant, we developed a co-culture system (36) illustrated in Figure 1. The BALB/c syngeneic, DO11.10 mouse strain expresses an αβ T cell receptor (TCR) specific for ovalbumin (OVA). Approximately 85% of the $CD4^+$ T cells in DO11.10 mice bear a Tg TCR, as identified by the clonotypic antibody KJ1-26 (44), and are naïve, pathway uncommitted Th0 cells. Stimulation by OVA in the context of IA^d and the appropriate costimulatory signals results in the maturation of Th0 cells into Th1 or Th2. Immunization is not required for the development of a vigorous 'DTH' response in DO11.10 mice (22). Using this system, we demonstrated that although T cell mediated immune responses are profoundly affected, alcohol acts directly upon the APC, which, in turn influences T cell function (36, 50). Using this BALB/c-DO11.10 co-culture system, we found that ethanol indirectly affects T cell function through its direct action upon APC (36, 50). Decreased IL-12 secretion by APC (36, 51) impairs IFN-γ production (36, 50). In our hands, OVA-stimulated co-cultures of APC from ethanol-consuming mice with normal DO11.10 T cells show little to no decrease in IL-2 or IL-4 production, but marked inhibition of IFN-γ (36). Flow cytometric analysis revealed that significant intracellular cytokine staining occurred only in $KJ1\text{-}26^+$ DO11.10 T cells. Co-cultures containing APC from alcohol-consuming animals show significant inhibition of IFN-γ production (data not shown).

Alcoholics often display enhanced serum IgG (52, 53) and IgE levels (54-56). Patients with alcoholic liver disease (ALD) and patients known to abuse alcohol for >5 years often show pronounced elevation in serum IgE (54, 55, 57-60). Elevated IgE levels in patients decline upon abstinence from alcohol (54). In experimental models, alcohol has been shown not to alter, and in some cases enhance, antibody production (39, 61, 62) including IgE (32, 63, 64). IgE responses are viewed as controlled exclusively by Th2 (65). Ten day ethanol consumption by unimmunized, naïve BALB/c mice elevates serum IgE levels, Table 2, and (32). BALB/c mice are often viewed as a Th2 strain, C57BL/6 mice, on the other hand are viewed as a prototypic Th1 strain (66). Ethanol consumption also elevates serum IgE levels in naïve C57BL/6 mice (32). Therefore, alcohol consumption affects IgE levels in both humans and experimental animal models.

The rapid appearance of serum IgE suggests a Th2 bias in alcohol-consuming mice. To determine

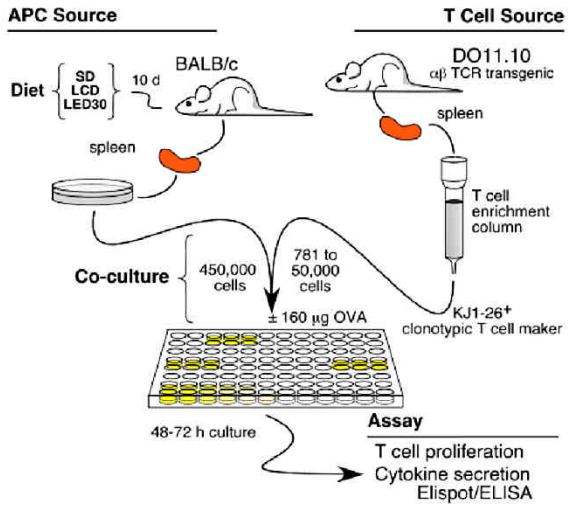

Figure 1. Co-culture of BALB/c APC and OVA-specific DO11.10 T cells. BALB/c mice were fed LED30, pair-fed LCD or remain on SD and water. Single cell suspensions of the BALB/c spleen cells were prepared from the BALB/c mice fed their respective liquid diets for 7 d and were used as a source of APC. Unimmunized BALB/c spleen cells (450,000 per culture well) rarely make even a minimal immune response to 18 μM OVA. Spleen cells from otherwise un-manipulated OVA-specific αβ TCR transgenic, DO11.10 mice were depleted of endogenous APC by passage over a T cell enrichment column. Enriched T cells, since they are depleted of APC, cannot respond to OVA without the addition of exogenous APC. Graded numbers of enriched DO11.10 T cells are added in co-culture ± 18 μM OVA, T cell proliferation (72 h) and cytokine production (24-72 h) are assessed at optimal times after culture initiation.

Table 2. Ethanol consumption for 10 d increases serum IgE levels in BALB/c mice

Strain	Diet	n	Serum IgE (μg/ml)			p
			arith mean	±	SEM	
BALB/c	SD	15	3.35	±	0.291	—
BALB/c	LCD	15	3.99	±	0.671	0.383
BALB/c	LED30	14	5.29	±	0.498	0.002

Naïve female BALB/c mice were fed LED30, pair-fed LCD, or remained on SD and water *ad libitum*. On dietary day 10, the mice were bled and total IgE levels determined by ELISA. Data represent the arithmetic mean ± SEM. Statistical significance determined by Student's *t* test.

whether ethanol consumption by APC donor mice would affect cytokine production by naïve T cells from DO11.10 OVA-TCR transgenic mice Elispot assays were performed. Table 3 shows the data from one of 4 such experiments. Naïve BALB/c mice (3/dietary group) were fed indicated diets for 10 d and their spleens were used as a source of APC to present OVA to purified DO11.10 T cells. It is important to note that the same pool of DO11.10 T cells was used in co-culture and that these T cells were never exposed to ethanol. Different numbers of DO11.10 T cells (20,000, 10,000, or 5,000) were cultured with a fixed number of spleen cells (450,000) from one of the dietary groups as a source of APC together with 18 μM OVA. Cells were co-cultured for 24-48 h on anti-cytokine coated plates. Optimal culture periods for each cytokine are indicated. Although not shown, serum IgE levels of LED30 fed BALB/c mice increased approximately 3 fold over SD and LCD fed mice. IFN-γ responses are significantly impaired in co-cultures containing APC from LED30 fed mice. No significant increase in the Th2-related cytokines, IL-4, IL-5, IL-9, and IL-13 is apparent. Among several possible explanations are 1) alcohol may alter cytokine secretion kinetics, 2) other tissues (peritoneal cells, lymph node, *etc*) may show alterations not seen in spleen, or 3) that differences in receptor expression for these cytokines may be responsible for changes in immune function.

We have consistently observed that both primary and secondary antibody responses to FγG in LED30-fed mice show ELISA titers $>10^5$ that do not differ significantly from anti-FγG titers of mice fed SD. A strong anti-FγG response reflects the immunogenic nature of FγG, which is composed of a large number of heterogeneous antigenic determinants (epitopes). To simplify the detection and analysis of ethanol-induced effects on antibody production, the response to a small number of hapten determinants conjugated to FγG was analyzed. A single chemically-defined epitope, six dansyl chloride (DNS) determinants were bound to each FγG molecule (DNS_6-FγG) and used to immunized mice fed one one of the three dietary regimens. Consistent with our previous observations, Figure 2 left panel, shows that mice from all three dietary groups produced vigorous antibody responses to the FγG carrier protein, producing statistically indistinguishable titers of $>10^5$. Figure 2, right panel, shows the antibody responses to the DNS hapten of these same sera assayed on DNS_{29}-BSA coated ELISA plates. The overall titers of the sera to the DNS are at least an order of magnitude less than the anti-FγG titer indicating that there are fewer DNS epitopes than the uncharacterized epitopes on FγG. Further, the anti-DNS binding of LED30 sera is less than that of the SD or LCD sera.

High versus low affinity antibody responses may be distinguished by their ability to bind to ELISA plates coated with proteins of differing degrees of haptenation (42, 43). High affinity antibodies bind equally well to ELISA plates coated with both high and low epitope density protein conjugates. Low affinity antibodies bind better to high-epitope density assay plates and less well or not at all to low-epitope density plates. Sera from SD-, LCD-, or LED30-consuming, female BALB/c mice immunized with DNS_6-FγG were assayed for their binding to FγG-, DNS_{62}-BSA-, DNS_{38}-BSA-, or DNS_{10}-BSA-coated ELISA plates. Figure 3, top row, shows that the total antibody (developed with a reagent that detects all heavy and light chains) responses of all three dietary groups bind to the FγG-coated plates with equal efficacy. Throughout these experiments, unimmunized, normal mouse serum (NMS) serves as a negative control, and never showed significant binding. The total (IgM + IgG)

Table 3. Elispot data for BALB/c mice fed diets for 10 d in a single experiment

Cytokine	Optimal Culture period	DO11.10 T cells added	Dietary Source of BALB/c APC (450,000 cells)			*p*
			SD	LCD	LED30	
IFN-g	**24 h**	20K	104.3 ± 7.51	121.7 ± 5.38	35.3 ± 9.28	0.004
		10K	69.0 ± 10.54	67.6 ± 18.89	15.7 ± 1.33	0.038
		5K	32.7 ± 4.33	41.6 ± 4.40	15.0 ± 4.72	0.015
IL-2	**24 h**	20K	124.3 ± 21.73	121.3 ± 10.84	146.3 ± 6.33	0.460
		10K	129.3 ± 12.57	120.6 ± 3.18	116.6 ± 6.69	0.583
		5K	124.0 ± 4.58	111.0 ± 5.29	83.3 ± 5.54	0.004
IL-4	**48 h**	20K	244.7 ± 15.68	256.7 ± 5.17	275.0 ± 8.14	0.208
		10K	196.3 ± 7.88	211.3 ± 6.64	210.0 ± 3.79	0.258
		5K	133.6 ± 1.20	166.3 ± 2.33	162.3 ± 7.96	0.006
IL-5	**48 h**	20K	119.0 ± 2.65	125.0 ± 3.21	123.7 ± 12.67	0.850
		10K	96.0 ± 8.08	90.3 ± 4.91	80.0 ± 0.58	0.192
		5K	64.0 ± 4.51	63.0 ± 3.51	63.0 ± 4.51	0.981
IL-9	**48 h**	20K	65.3 ± 6.74	53.0 ± 6.08	57.3 ± 6.08	0.331
		10K	91.0 ± 7.00	79.0 ± 4.62	94.3 ± 12.99	0.486
		5K	107.0 ± 29.87	136.0 ± 13.07	126.3 ± 14.84	0.625
IL-13	**48 h**	20K	256.3 ± 7.54	243.0 ± 3.06	256.3 ± 14.90	0.573
		10K	210.7 ± 4.98	184.0 ± 6.81	169.0 ± 5.00	0.006
		5K	129.6 ± 0.33	126.0 ± 6.66	112.3 ± 6.12	0.128

Naïve female BALB/c mice, 3 mice per group, were fed LED30, pair-fed LCD or were fed SD and water *ad libitum* for 10 d, euthanized, their spleens aseptically removed, and used as a source of APC. APC were held constant (450,000) were co-cultured with the indicated numbers of purified T cells from syngeneic DO11.10 mice ± 18 μM OVA on anti-cytokine coated, sterile 96-well PVDF plates as detailed in the Methods and Materials. After 24-48 h culture, the plates were processed and cytokine-specific spots (Elispots) were developed. Cultures containing DO11.10 T cells and APC, but not stimulated with OVA, produced < 10 spots per well. Statistical significance was determined by Student's *t* test.

anti-DNS responses of these sera show similarly shaped binding curves on DNS_{62}-, DNS_{38}-, and DNS_{10}-BSA coated plates. The lowest hapten conjugation ratio (DNS_{10}-BSA) revealed a difference in the anti-DNS response between the dietary groups, similar to DNS_{29}-BSA responses seen in Figure 2. To explore the possibility that dietary ethanol may influence anti-DNS activity of a particular isotype, the sera from the three dietary groups were developed in ELISAs on plates coated with BSA (10 μg/ml) of various degrees of DNS conjugation using isotype-specific secondary antisera specific for the heavy chains of IgG_1, IgG_{2a} and IgG_{2b}, (Figure 3). IgG_1 antibody responses to either FγG or DNS do not appear to differ within any of the three dietary groups and show marked similarity to the total antibody profiles for these sera. IgG_{2a} antibody responses for the three dietary groups are similar for FγG, DNS_{62}-, and DNS_{38}-binding. In marked contrast, the binding of LED30 sera to DNS_{10}-BSA is appreciably less than SD or LCD. Differences in anti-DNS antibody binding are even more pronounced for IgG_{2b}, the anti-DNS IgG_{2b} activity of LED30 sera are appreciably less than SD sera and compared to SD, in contrast, LCD responses are elevated. Collectively these data show that although the FγG responses do not differ, anti-DNS IgG_{2a} and IgG_{2b} activity of LED30 immunized mice is lower.

The relative binding affinities for both anti-F? G and anti-DNS antibodies may be determined from the binding curves in Figure 4. Non-linear regression analyses were performed on the raw ELISA data depicted in Figure 4. Curve-fitting software were used to generate theoretical binding curves by non-linear regression through the actual data points and to extend the curve to theoretical saturation. Figure 4 illustrates the curve fitting that results for the IgG_{2a} binding of SD, LCD and LED30 sera to DNS_{10}-BSA coated ELISA plates. Coefficient of determination (R^2), a measure of how well the theoretical curve fits the data, is > 0.997 in this figure and for all subsequent calculations. The effective (reciprocal) dilution of the sera that would cause 50% maximal binding (ED_{50}) is calculated from the theoretical curve and is expressed as $LogED_{50}$. Relative affinities were calculated for all the ELISA data (from Figure 2) and are illustrated graphically in Figure 5 for SD (open bars), LCD (hatched bars), and LED30 (gray bars). Only those LED30 groups indicated by black bars (DNS_{10}-BSA for IgG_{2a}, IgG_{2b} for DNS_{62}-, DNS_{38}-, and DNS_{10}-BSA) have $LogED_{50}$ values are significantly lower (*p < 0.001*), than SD sera assayed on the same ELISA plate. Therefore, ethanol-consumption down-regulates hapten-specific responses by Th1-regulated antibodies (67).

5. DISCUSSION

The effects of alcohol on the immune system may precede pathological alterations seen in other organ systems. Data show phenotypic and functional alterations occur within the first few days of alcohol consumption. In the present study, we show that alcohol diminishes Type 1 DTH and IFN-γ responses although Type 2 regulated serum IgE levels are enhanced. Further analysis of specific anti-DNS antibody shows that alcohol consumption diminished the relative affinity of Th1-regulated IgG_{2a} and IgG_{2b} anti-DNS antibodies.

Alcohol consumption alter leukocyte populations (32). Alcohol-consuming mice tend to limit their caloric

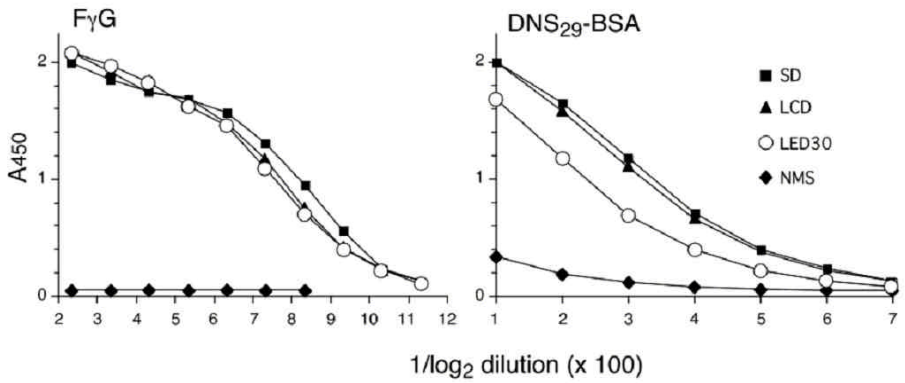

Figure 2. Ethanol consumption influences the antibody quality to a chemically-defined epitope. Female BALB/c mice were fed LED30, pair-fed LCD, or SD for 14 d as detailed in the Methods and Materials. Mice were immunized sc on dietary day 4 with 100 μg DNS_6-FγG in CFA and boosted ip on dietary day 8 with 100 μg DNS_6-FγG in Maalox. On dietary day 14, the mice were bled and their sera were assayed for total antibody (IgM + IgG) activity both to FγG and to DNS on FγG- or DNS_{29}-BSA-coated ELISA plates, respectively. Nearly identical anti-FγG responses by SD (■), LCD (▲), and LED30 (❍) are seen, whereas no binding is seen for unimmunized, normal mouse serum, NMS (◆). In contrast, binding of these same sera shows both a >10-fold decrease in antibody titer and that the anti-DNS content of the LED30 sera is less than that of the SD or LCD sera.

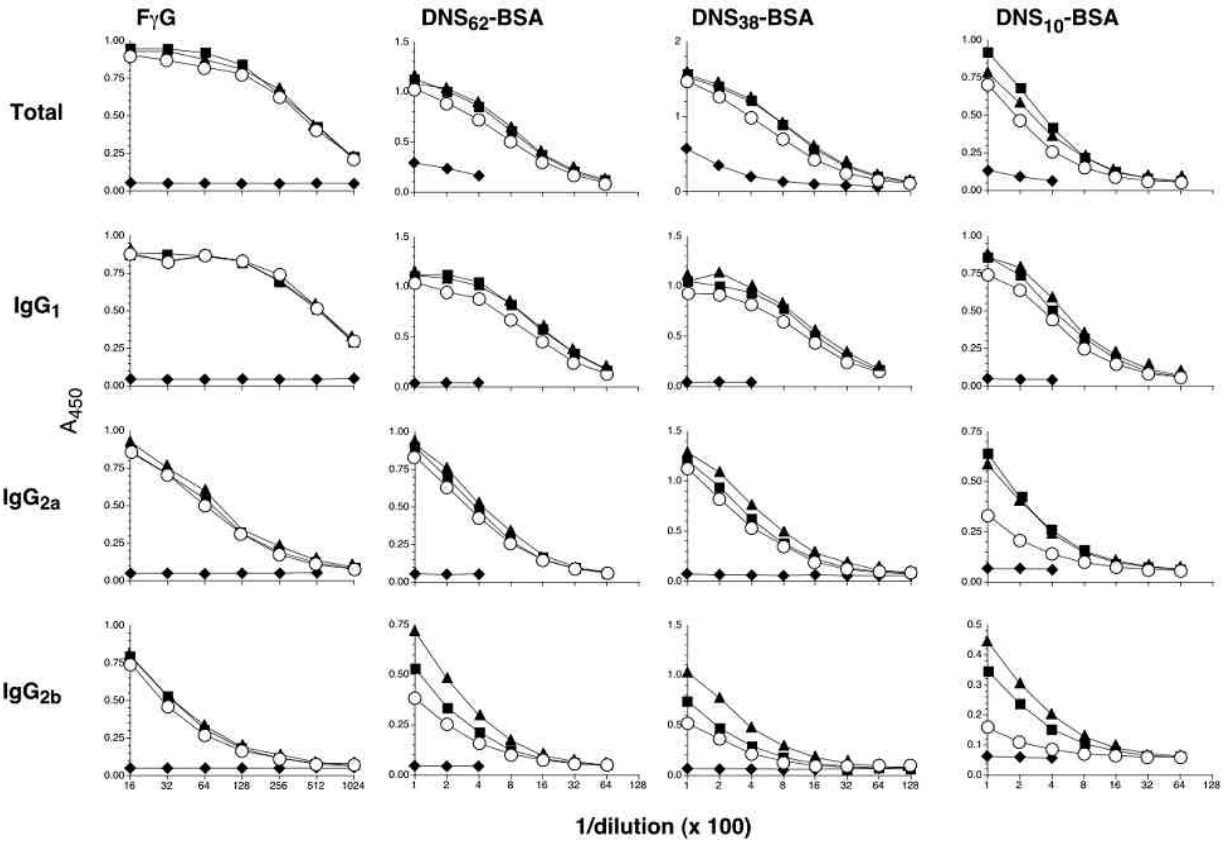

Figure 3. Alcohol consumption influences antibody affinity to a chemically-defined epitope in an isotype-specific manner. Female BALB/c mice were fed LED30, pair-fed LCD, or fed SD for 14 d as detailed in the Methods and Materials. Mice were immunized sc on dietary day 4 with 100 μg DNS_6-FγG in CFA and boosted ip on dietary day 8 with 100 μg DNS_6-FγG in Maalox. On dietary day 14, the sera from these mice were assayed on FγG, DNS_{62}-, DNS_{38}- and DNS_{10}-BSA coated (protein concentration held constant at 10 μg/ml) ELISA plates and developed with biotin-labeled, goat anti-mouse total (IgM + IgG) or isotype (IgG_1, IgG_{2a} or IgG_{2b}) specific antibodies, followed by reaction with horseradish conjugated streptavidin, and colorimetric detection of acidified TMB substrate. Data are represented as absorbencies at 450 nm versus the reciprocal sera dilution for SD (■), LCD (▲), LED30 (❍) and NMS (◆).

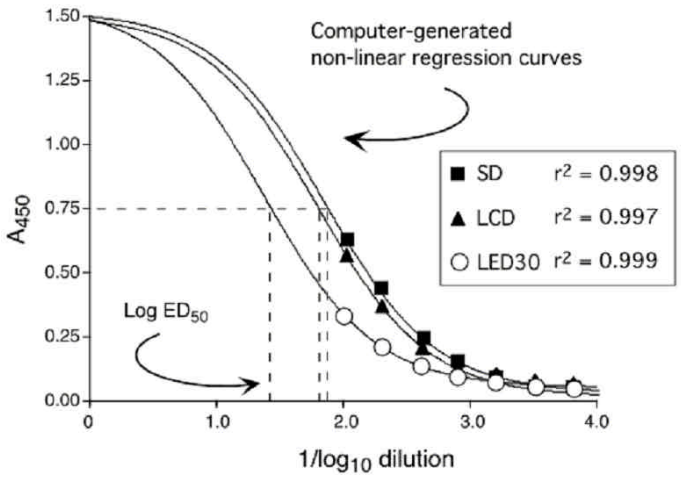

Figure 4. Calculation of the effective dilution that causes 50% maximal binding ($logED_{50}$) of sera from DNS_6-FγG immunized BALB/c mice. Sera from SD (■), LCD (▲), and LED30 (❍) mice immunized with DNS_6-FγG were allowed to bind to DNS_{10}-BSA coated ELISA plates and the plates were developed with goat anti-mouse IgG_{2a}-specific antibody. Curve-fitting software was used to fit a theoretical binding curve by non-linear regression through all the data points and to extend the curve to a theoretical saturation. Coefficient of determination (R^2) in all cases was >0.997. The log of the theoretical effective dilution of the sera that would cause 50% maximal binding ($LogED_{50}$) is calculated as the reciprocal dilution of the sera that would result in 50% maximal antibody binding.

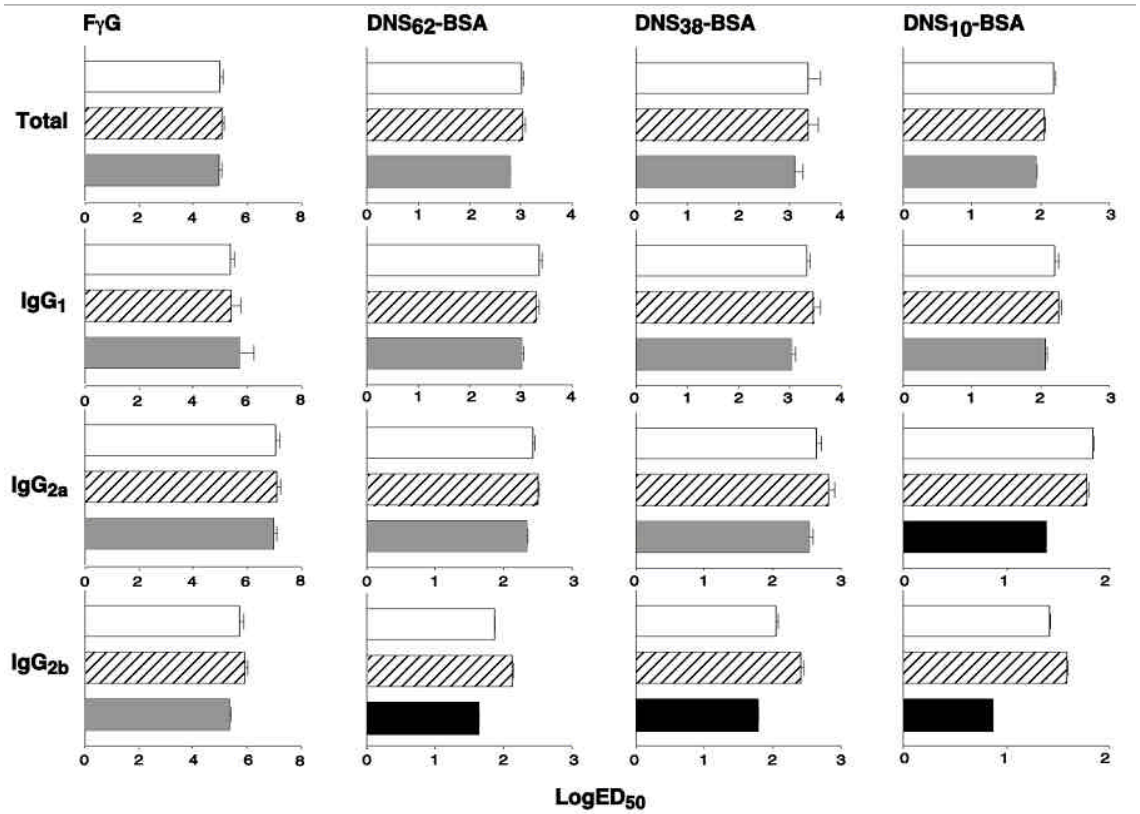

Figure 5. The affinity of anti-DNS IgG_{2a} and IgG_{2b} antibody is significantly decreased in ethanol-consuming DNS_6-FγG immunized BALB/c mice. $LogED_{50}$ values were calculated for the ELISA binding curves shown in Figure 2 as described in Figure 3. Data are represented as the $LogED_{50}$ multiplied/divided by the log standard error for SD (open bars), LCD (hatched bars), and LED30 (gray and black bars). LED30 groups indicated by black bars (DNS_{10}-BSA for IgG_{2a}, IgG_{2b} for DNS_{62}-, DNS_{38}-, and DNS_{10}-BSA) have $LogED_{50}$ values are significantly ($p < 0.001$), as determined by both Student's *t* tests to obtain two-tailed P values and one-way analysis of variance (ANOVA) lower than SD sera assayed on the same ELISA plate.

intake due to their natural aversion ethanol (68). The effect(s) of nutritional status within the context of alcohol consumption remains, as yet, largely undefined (69) and, as such, the consequences of alcohol upon immune function are still ill-defined. In fact, we recently found that mice fed liquid diets are more resistant to *Listeria monocytogenes* strain EGD, than mice fed SD (70). We recently showed that alcohol consumption profoundly decreases B cell and natural killer cell number (32). In contrast, T cells show no significant decline in number until dietary day 10, suggesting that alcohol does not grossly alter this cellular compartment. Although alcohol consumption profoundly affects T-dependent immune responses, T cells themselves do not appear to be a direct target of alcohol (36, 50, 51). Alcohol profoundly affects APC function (33, 36, 51, 71, 72). Since MHC II is a restricting element for $CD4^+$ T cells it would be logical to predict that $CD4^+$ Type 1 and/or Type 2 responses would be affected. The decline in B cells in the presence of alcohol is accompanied by a concomitant decline the amount of MHC class II expressed on this population, yet a concomitant decline in MHC II expression non-B APC populations is not seen (32). Paradoxically, although B cell populations show the greatest amount of decrease in alcohol-consuming mice, IgE, a Th2-regulated B cell product, increases in these same mice.

Ethanol consumption profoundly impairs cell-mediated immune responses,(20, 25, 73) yet alcohol's influence on humoral immune responses has been less clear. Several studies indicate that acute or chronic ingestion impairs the ability of experimental animals to make antibody in response to immunization with T-cell dependent antigens (74-76). Yet other investigations show that chronic ethanol consumption does not quantitatively alter T-cell dependent antibody responses and may even enhance the quantity of antibody produced (22, 77, 78). Unfortunately, clear clinical evidence is lacking. To help resolve the role of ethanol on humoral responses, we used an experimental model in which female BALB/c mice are fed a liquid diet containing a moderate amount of ethanol and immunized with an immunogenic protein (FγG) that was conjugated with a small number of a chemically defined epitopes generating DNS_6-FγG. Our data show that ethanol consumption does not significantly alter antibody responses to strongly immunogenic FγG, yet LED down-regulates antibody responses to DNS. Our ELISAs were designed to detect differences in relative affinity to anti-DNS antibody. A significant decrease in anti-DNS affinity is apparent only for IgG_{2a} and IgG_{2b}, isotypes which are mainly regulated by Th1 (67, 79, 80). IgG_1 anti-DNS responses and anti-total antibody (against all isotypes) responses to FγG and DNS are unaffected.

We show that ethanol consumption affects the *quality* of antibody in terms of both antibody affinity and isotype without affecting the total quantity of antibody. Our results are consistent with a model in which ethanol consumption down-regulates Th1-related cells and cytokines. Our laboratory has shown that ethanol-fed mice are impaired in their ability to develop antigen-specific DTH (22, 39) which correlates with a decreased production in Type 1 cytokines IFN-γ and IL-12, produced by macrophages/monocytes in alcohol consuming mice (51). Type 1 cells and cytokines are both important in the up-regulation of cell-mediated immunity and the down-regulation of humoral immune responses. Conversely, Type 2 responses generally up-regulate humoral immune and down-regulate cell-mediated responses. A dynamic balance between Th1-Th2 cells and their cytokines is normally seen, and over (or under) abundance of cell-mediated immunity is often accompanied by a low (or enhanced) humoral immune response. The consequences of an imbalance in CD4 Th1-Th2 regulation would be reflected not only in cell-mediated immune responses, but in antibody affinity and isotype profile in response to antigen (42, 43, 81, 82). In mice, Th1 cells regulate IgG_{2a} and IgG_{2b} antibody responses (83, 84). In contrast, Th2-associated immunity most clearly influences the development of IgG_1 and IgE antibody responses (41). Our data show that ethanol-consuming mice immunized with DNS_6-FγG display diminished affinity in anti-DNS IgG_{2a} and IgG_{2b} antibodies, whereas total and IgG_1 antibody responses to DNS and anti-FγG antibodies for all isotypes tested are not diminished. It is important to note that IgG_1 anti-DNS_6-FγG responses predominant in terms of antibody quantity, are present in quantities 5-10 fold greater than IgG_{2a} and IgG_{2b}, and are indistinguishable from the total antibody responses.

Several laboratories report marked impairment in the quantity of antibody in response to T-cell dependent antigens in rodents fed diets containing 36-37% ethanol-derived calories (38, 74) or in an acute model of binge drinking (85). In contrast, we and others find that humoral immune responses are unchanged (22, 77) or enhanced (39, 78) in ethanol-consuming mice. How can these differences be reconciled? In the present study the amount of ethanol (30% ethanol-derived calories) fed to the mice is less than the 36-37% ethanol derived calories in other laboratories that show a marked impairment in T-cell dependent antibody responses. We showed (22) that mice fed liquid diets containing 20-35% ethanol-derived calories consumed a surprisingly similar amount of ethanol per day per mouse irrespective of the dietary concentration of ethanol. Impaired DTH responses occurred for those mice fed >25% ethanol derived calories, although LED20 mice consume nearly the same total amount of ethanol per day. Dietary components (fats, carbohydrates, proteins, *etc.*) may affect alcohol catabolism. When mice ingest 36-37% ethanol-derived calories, however, both Type 1 and Type 2-regulated immune responses may be impaired. Therefore, feeding mice a diet containing 30% ethanol-derived calories, may afford a window in which Type 1 immune responses are more severely impaired than Type 2.

The nature of the immune response is strongly influenced by the initial interaction between APC and CD4 T cells. We recently showed that ethanol consumption in the period just prior to and during immunization severely impairs the subsequent development of an antigen-specific DTH response (34). Ethanol consumption initiated after this cognitive phase has no significant effect upon a DTH response (34). Furthermore, APC derived from ethanol-

consuming mice are impaired in their ability to make the macrophage-derived, Type 1 cytokine IL-12.(23) IL-12 is required for the development of Type 1 responses (23). Both IL-12 and IFN-γ are capable of inhibiting Th-2 responses and although they are down-regulated in alcohol-fed mice, humoral immune responses do not go unchecked. This suggests that alcohol consumption does not eliminate control of humoral immune responses. Rather, alcohol may alter the affinity maturation of the Th1-regulated IgG subclasses IgG_{2a} and IgG_{2b} by decreasing APC IL-12 and the concomitant Th1 IFN-γ production that drives B cell class-switching to IgG_2 and IgE.

6. ACKNOWLEDGMENTS

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Send all correspondence to: Dr Carl Waltenbaugh, Department of Microbiology-Immunology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611, Tel: 312-503-8459, Fax: 312-503-8459, E-mail: waltenbaugh@northwestern.edu