

# Ethanol Consumption Modifies Dendritic Cell Antigen Presentation in Mice

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**Background:** Alcohol consumption impairs type 1 cell-mediated adaptive immune responses both in vivo and in vitro. The present study investigated the effect of alcohol consumption on antigen-presenting cell (APC) populations and cytokine production.

**Methods:** BALB/c were fed ethanol-containing, pair-fed isocaloric liquid control, or solid diets for 11 days. Macrophage and dendritic cell (DC) populations were isolated by paramagnetic bead separation and used to present ovalbumin (OVA) to highly purified syngeneic CD4<sup>+</sup> T cells derived from DO11.10 T cell receptor transgenic mice in coculture. DC isolated from diet-fed mice were also used to present OVA to highly purified CD4<sup>+</sup> T cells derived from antigen-naïve DO11.10Rag2<sup>-/-</sup> mice that are devoid of memory T cells. In vitro cytokine responses, interleukin (IL) -2, IL-6, IL-12, IL-13, IL-17A, and interferon- $\gamma$  (IFN- $\gamma$ ) were measured by enzyme-linked immunosorbent assay. Flow cytometry measured cell surface molecule expression.

**Results:** Alcohol consumption impairs delayed hypersensitivity responses (type 1) and enhances serum IgE levels (type 2). CD11c<sup>+</sup> DC, but not F4/80<sup>+</sup> macrophages, support cytokine responses by purified CD4<sup>+</sup> T cells. CD11c<sup>+</sup> DC derived from ethanol consuming BALB/c mice show diminished ability to support IFN- $\gamma$  responses by purified CD4<sup>+</sup> T cells derived from DO11.10 or DO11.10Rag2<sup>-/-</sup> mice. Subset analysis indicates that of the 3 “conventional” DC subsets found in mouse spleens, CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs are both responsible for OVA presentation and susceptible to the effects of ethanol. Ethanol consumption does not overtly alter the percent of splenic DC, but does increase the surface density of CD11c on these cells. Data show that cocultures containing purified CD4<sup>+</sup> T DO11.10 cells and APC derived from alcohol-consuming mice show decreased IL-6, IL-12, IL-17A, and IFN- $\gamma$  and increased IL-13 cytokine production in response to OVA stimulation.

**Conclusions:** Ethanol alters CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC function, affecting cytokines responsible for adaptive immune responses. A unifying hypothesis for the underlying mechanism(s) of ethanol's effect upon adaptive immune function is proposed.

**Key Words:** Alcohol, Type 1 Immune Response, Dendritic Cells, CD11c, Cytokines.

ALCOHOL ABUSE AFFECTS most organ systems (Nagy, 2004; Room et al., 2005). Alteration in both innate and adaptive immune systems seen in alcohol abusers (Nagy, 2003) is accompanied by increased frequency and severity of infections (Cook, 1998; Roselle et al., 1995). The innate immune system recognizes a limited number of pathogen-associated molecules that trigger conserved pattern recognition receptors that trigger inflammatory responses to infection (Janeway, 2001). Cells of the adaptive immune system generate a wide range of surface receptors expressed on lymphocytes that respond to antigen encounter by clonal expansion, cellular differentiation/maturation, and the production of effector molecules (cytokines, antibodies, etc.) that

both amplify and direct cells and molecules of the innate immune system (Cooper and Alder, 2006). In humans, chronic alcohol consumption impairs cell-mediated immune responses and increases immunoglobulin levels (Cook, 1998; Gonzalez-Quintela et al., 2004; Spies et al., 2004). Likewise, in experimental animal models, ethanol consumption impairs cell-mediated immunity and enhances some antibody responses (Jayasinghe et al., 1992; Jerrells and Sibley, 1995; Starkenburg et al., 2001). Although alcohol has long been known to affect immune responses (Rush, 1784/1814), the underlying mechanism(s) have been elusive (Cook, 1998).

Cells of the innate immune system initiate many adaptive immune responses. Encountered microbes or antigens are internalized; microbial peptides produced by proteolytic cleavage are bound by major histocompatibility complex II (MHC II) molecules and displayed on the surfaces of dendritic cells (DCs) or macrophages. These “professional” antigen-presenting cells (APCs) interact with antigen-specific receptors on CD4<sup>+</sup> T helper (Th) cells that recognize both peptide and MHC II. APC-CD4<sup>+</sup> Th cell interactions are critical in determining the subsequent type 1 or type 2 nature of the effector immune response. In humans and rodents, type 1 and type 2 immune responses show defined patterns of cyto-

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kine secretion (Fiorentino et al., 1989; Romagnani, 1994). Secretion of interleukin-12 (IL-12) by APC and interferon- $\gamma$  (IFN- $\gamma$ ) by CD4<sup>+</sup> Th1 cells is associated with type 1 responses that lead to cell-mediated immune responses such as delayed-type hypersensitivity (DTH) (Macatonia et al., 1993, 1995). Both IL-4 and IL-10 are associated with type 2 responses (Mosmann and Sad, 1996; Romagnani, 1994) and up-regulate antibody responses (Mosmann and Sad, 1996). Ethanol consumption diminishes type 1 IL-12 and IFN- $\gamma$  (Mandrekari et al., 2004; Peterson et al., 1998b; Starkenburg et al., 2001) production, but up-regulates type 2-dependent IgE responses in both humans and mice (Gonzalez-Quintela et al., 2004; Latif et al., 2002; Starkenburg et al., 2001). Studies in both humans and mice locate ethanol's effect on diminished type 1 cytokine secretion to the influence of APC on CD4<sup>+</sup> T cell maturation.

Not all APC function with equal efficacy. Lysosomes within macrophages degrade most antigens to the point that only a relatively small amount is available for presentation on the cell surface as MHC II-peptide complexes (Pulendran et al., 2001). B cells lacking the relevant antigen-specific surface immunoglobulin are inefficient at stimulating naïve CD4<sup>+</sup> T cells (Itano and Jenkins, 2003). In contrast, mature DCs contain specialized MHC II-rich compartments that allow them to express 10 to 100 times more MHC II-peptide complexes on their cell surfaces than macrophages or B cells, making DCs very efficient stimulators of T cells (Inaba et al., 1997). The functional plasticity of DC "tunes" the adaptive immune response according to the nature of an ongoing infection (d'Ostiani et al., 2000). DCs are dynamically regulated by maturational stimuli, kinetics of activation, the nature of the antigen/pathogen, and by both internal (e.g., cytokine) and external (pharmacological) chemical stimuli, events that affect type 1 or type 2 immune responses (Banchereau and Steinman, 1998). Immature DC arise from hematopoietic precursors in the bone marrow and give rise to distinct DC lineages that may bias the induction of type 1 or type 2 responses in both humans and mice (Kalinski et al., 1999; Liu, 2001; Maldonado-Lopez and Moser, 2001; Moser and Murphy, 2000; Pulendran et al., 1999). Three "conventional" CD11c<sup>+</sup> DC subsets have been identified in mouse spleens based upon CD4 and CD8 $\alpha$  expression (Maldonado-Lopez et al., 1999; Schnorrer et al., 2006; Shortman and Naik, 2007). Data from several laboratories argue that CD8 $\alpha$ <sup>+</sup> DC favor development of type 1 responses and CD8 $\alpha$ <sup>-</sup> favor type 2 responses (Maldonado-Lopez et al., 1999; Moser and Murphy, 2000), yet others suggest that it is the nature of the stimulating antigen that determines the "Th-determining potential" of the DC and not its ontogeny (Huang et al., 2001; Manickasingham et al., 2003). In this study, we show that CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DC isolated from ethanol-consuming BALB/c mice show a diminished capacity to support type-1 cytokine responses by ovalbumin (OVA) -specific, T cell-transgenic DO11.10 T cells.

## MATERIALS AND METHODS

### Animals

Female BALB/c mice were purchased from the Charles River, Wilmington, MA and were held for a 2-week acclimation period prior to use. BALB/c syngeneic, T cell receptor (TCR) transgenic DO11.10 mice (Jackson Labs, Bar Harbor, ME) were bred and maintained at Northwestern University. Founders of a DO11.10Rag2<sup>-/-</sup> breeding colony were the kind gift of Dr. Stephen D. Miller, Northwestern University. All mice were 8 to 10 weeks of age at the initiation of experiments. Northwestern University's vivarium is fully accredited by the American Association for the Accreditation of Laboratory Animal Care and the Northwestern University Animal Care and Use Committee approved animal protocols used in these studies.

### Diets and Feeding

Mice were fed a solid diet (SD) consisting of laboratory chow (7022 NIH-07 diet; Harlan TekLad, Madison, WI) and water ad libitum, a liquid ethanol diet containing 30% ethanol-derived calories (LED30) or pair-fed a liquid control diet (LCD). Modified Lieber-DeCarli liquid diets (Lieber and DeCarli, 1989; Lin, 1989; Schodde et al., 1996) that meet the American Institute of Nutrition (AIN) standards for rodents were compounded in our laboratory. Briefly, the diets contained sucrose (91 g/l), corn oil (12.8 g/l, item 401150; Dyets Inc., Bethlehem, PA), AIN mineral mixture 76 (8.93 g/l, item 200000; Dyets Inc.), AIN vitamin mixture 76A (2.55 g/l, item 300050, Dyets Inc.), DL-methionine (0.77 g/l, item 402950; Dyets Inc.), choline bitartrate (0.51 g/l, item 400750; Dyets Inc.), xanthine gum (2 g/l, item 402525; Dyets Inc.), and casein hydrolysate (51 g/l, item 12855; US Biochemical, Cleveland, OH). Some mice were fed diets containing ethanol at 45.8 g/l (LED30). Additional sucrose (12.5 g/l) was added to the LCD to isocalorically substitute for ethanol. All liquid diets contained 1 kcal/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 liquid diet-fed groups and were replaced with the appropriate liquid diet. Mice were fed fresh diet daily in new 50 ml plastic disposable plastic tubes fitted with an autoclaved neoprene stopper and sipper tube. Mice were pair-fed; i.e., intake by the LED-fed mice determined the amount of LCD that mice were fed on the subsequent day. This ethanol feeding regimen typically results in blood alcohol levels in the 0.1 to 0.15% range.

### Delayed-Type Hypersensitivity

Female BALB/c mice were fed LED30, pair-fed LCD, or were fed SD for the duration of the experiment. Mice were primed subcutaneously with 100  $\mu$ g OVA (Fraction V; Sigma, St Louis, MO) in complete Freund's adjuvant on dietary day 4. Six days later mice were anesthetized with sodium pentobarbital (60 mg/kg), baseline ear thickness was measured using a Mitutoyo dial thickness gauge, and 20  $\mu$ g OVA in 10  $\mu$ l phosphate-buffered saline was injected intradermally into the dorsal surface of the ear using a syringe fitted with a 30-gauge needle. Antigen-induced ear swelling (expressed in units of 10<sup>-4</sup> in.) was determined 24 hours later in a blind reading.

### Cell Isolation

**CD4<sup>+</sup> T Cell Purification.** Splenic CD4<sup>+</sup> T cells were purified from antigen-naïve DO11.10 mice using a combination of antibody plus complement followed by paramagnetic bead fractionation. Briefly, mice were euthanized, their spleens aseptically removed and gently teased into running buffer [0.15 M PBS, pH 7.4, containing 0.1% bovine serum albumin (BSA)], and washed. Cells (10<sup>8</sup>/ml) were incubated with a cocktail containing  $\alpha$ MHC II (100  $\mu$ g, clone

M5/114.15.2; Biolegend, San Diego, CA),  $\alpha$ CD49b (100  $\mu$ g clone DX5; Biolegend), and  $\alpha$ CD205 (400  $\mu$ g, clone DEC 205; produced in our laboratory) for 30 minutes on ice followed by a 1 hour incubation at 37°C with complement (Low-tox; Cedarlane Laboratories, Hornby, Ontario). Cells were washed and CD4<sup>+</sup> T cells were isolated by negative selection with a paramagnetic bead kit (11415D; Invitrogen, Carlsbad, CA). We obtained approximately a 5-fold enrichment of CD3<sup>+</sup> T cells (18.3  $\rightarrow$  91.6%), >99.9% decrease in B220<sup>+</sup> B cells (57.1  $\rightarrow$  0.04%), >98% decrease in F4/80<sup>+</sup> macrophages (0.58  $\rightarrow$  0.01%), and ~93% decrease in CD11c<sup>+</sup> DC (2.02  $\rightarrow$  0.14%). Natural killer cells would account for the residual 8%. Typical yield of purified CD4<sup>+</sup> T cells is 6 to 10% of the unfractionated DO11.10 spleen cells.

**APC Isolation.** BALB/c spleens were teased, incubated in collagenase D (1 mg/ml; Roche, Indianapolis, IN) for 1 hour at 37°C, and single cells were washed in PBS containing 3% bovine calf serum and 0.1% NaN<sub>3</sub>. Cells were separated by negative selection (for CD11c<sup>+</sup> cells, item 557955; BD, San Jose, CA) or by positive selection (for CD8 $\alpha$ <sup>+</sup> cells, item 551516, BD or for F4/80<sup>+</sup> cells, clone BM8, 1  $\mu$ l/10<sup>6</sup> cells, Caltag, and item 120-000-900; Miltenyi Biotec, Auburn, CA) with paramagnetic particles. All separations were performed as according to the manufacturers' directions with the exception of the  $\alpha$ CD8 $\alpha$  particles, which were used at double the concentration. BALB/c spleens typically yielded 1 to 5% F4/80<sup>+</sup> cells and 2% CD11c<sup>+</sup> cells (approximately 2/3s were isolated as CD8 $\alpha$ <sup>+</sup> by paramagnetic beads).

### Culture

Unfractionated (50  $\times$  10<sup>3</sup>) or fractionated BALB/c spleen cells (20  $\times$  10<sup>3</sup> CD11c<sup>+</sup> or F4/80<sup>+</sup>, 14.5  $\times$  10<sup>3</sup> CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup>, or 5.5  $\times$  10<sup>3</sup> CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup>) were cocultured with (200  $\times$  10<sup>3</sup>) purified DO11.10 CD4<sup>+</sup> T cells  $\pm$  OVA (18  $\mu$ M, 160  $\mu$ g/well). Controls included unfractionated DO11.10 cells, purified DO11.10 CD4<sup>+</sup> T cells, and unfractionated BALB/c cells  $\pm$  OVA. Cells were cultured in Dulbecco's minimal essential medium: Ham's F12 in a 1:1 ratio (Hyclone, Logan, UT) supplemented with 50  $\mu$ M 2-mercaptoethanol, 3  $\mu$ M glutamine, and 1% Nutridoma (a serum substitute; Roche). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and were harvested at optimal times (24 hours for IL-2 and IL-12, 72 hours for IFN- $\gamma$ ) for enzyme-linked immunosorbent assay (ELISA) determination of cytokine levels, see below.

### Flow Cytometry

Single-cell suspensions were passaged through a 30- $\mu$ m nylon mesh, washed (staining buffer; PBS, containing 1% bovine calf serum, 2 mM EDTA, and 0.1% NaN<sub>3</sub>), and 2  $\times$  10<sup>6</sup> cells were added to individual wells of 96-well round-bottom, flexible polystyrene plates, pelleted by centrifugation (300  $\times$  g for 5 minutes). Resuspended cells were treated with Fc Block (clone 2.4 G2) to minimize nonspecific staining. Cells were labeled with an antibody cocktail containing  $\alpha$ CD3-fluorescein isothiocyanate (0.5  $\mu$ g/well, clone 145-2C11; BD),  $\alpha$ CD11c-biotin (2  $\mu$ g/well, clone N418; eBioscience, San Diego, CA), and  $\alpha$ CD8 $\alpha$ -phycoerythrin (1  $\mu$ g/well, clone 53-6.7; BD) for 20 minutes on ice then washed 3 times with staining buffer. Streptavidin-allophycocyanin (SA-APC, item 554067; BD) was added to the cell mix as a second step reagent for the biotinylated antibody for 15 minutes on ice. Washed cells were transferred through a 30  $\mu$ m nylon mesh to 12  $\times$  75 mm polystyrene tubes to a final volume of 1 ml (~2  $\times$  10<sup>6</sup> cells/ml final) and propidium iodide (Molecular Probes, Eugene, OR) added immediately prior to analysis to discriminate viable cells. A minimum of 30,000 gated events were collected on a BD FACSCalibur dual laser flow cytometer for each sample. Data were analyzed with FlowJo software (Treestar, Ashland, OR).

### Cytokine and Isotype Screening

Enzyme-linked immunosorbent assays were used to determine IFN- $\gamma$ , IL-2, IL-6, IL-12p70, IL-13, and IL-17A concentrations in tissue culture supernatants as well as serum IgE levels. Wells of 96-well Maxisorp (NUNC, Nalge Nunc International, Rochester, NY) plates were coated with capture antibody,  $\alpha$ IFN- $\gamma$  (0.25  $\mu$ g/ml, clone R4.6A2; produced in laboratory),  $\alpha$ IL-2 (0.5  $\mu$ g/ml, clone JES6-1A12; Caltag),  $\alpha$ IL-6 (1  $\mu$ g/ml, clone MP5-20F3; BD),  $\alpha$ IL-12p40 (2  $\mu$ g/ml, clone C15.6; Caltag),  $\alpha$ IL-13 (1  $\mu$ g/ml, goat  $\alpha$ mouse IL-13; R&D Systems, Inc., Minneapolis, MN),  $\alpha$ IL-17A (1  $\mu$ g/ml clone TC11-18H10; eBioscience), or  $\alpha$ IgE (1  $\mu$ g/ml, clone LO-ME-3; Caltag) for 18 hours at 4°C. Plates were blocked for 2 hours at 37°C with 2% BSA in PBS. Standards (IFN- $\gamma$ , Biolegend; IL-2, Preprotech, Rocky Hill, NJ; IL-12 and IL-17A, eBioscience; IL-13, R&D Systems; IL-6 and IgE ascites, BD), culture supernatants (cytokine assay), or sera (isotype assay) were appropriately diluted (5 to 11 dilutions for standards), and incubated for 1 hour at 37°C. The plates were washed 3 times and incubated with an appropriately diluted detecting antibody for 1 hour at 37°C. Biotinylated detection antibodies used were:  $\alpha$ IFN- $\gamma$  (0.125  $\mu$ g/ml, clone XMG1.2; Biolegend),  $\alpha$ IL-2 (0.5  $\mu$ g/ml, clone JES6-5H4; Caltag),  $\alpha$ IL-6 (1  $\mu$ g/ml, clone MP5-32C11; BD),  $\alpha$ IL-12p35 (0.5  $\mu$ g/ml, clone C17.8; Caltag),  $\alpha$ IL-13, clone 38213.11; RND);  $\alpha$ IL-17A (1  $\mu$ g/ml, clone TC11-8H4; eBioscience), and  $\alpha$ IgE (1  $\mu$ g/ml, clone LO-ME-2; Caltag). Wells were washed 6 times and incubated with horseradish peroxidase-conjugated SA (1:4,000; KPL Laboratories, Gaithersburg, MD) for 15 to 20 minutes at 37°C. The plates were washed 6 times, and TMB (BioFX, Owings Mills, MD) was added. Chromogenic development was stopped by the addition of 0.18 M H<sub>2</sub>SO<sub>4</sub>. Plates were read in a SpectraMax<sup>190</sup> plate reader (Molecular Devices, Palo Alto, CA) at 450 nm. SoftMax Pro (Molecular Devices) curve fitting software determined concentrations. Data were further analyzed as described in the Statistical Analysis section, below.

### Statistical Analysis

Delayed-type hypersensitivity and ELISA data are reported as the arithmetic mean  $\pm$  the standard error of the mean and the Student's *t*-test assessed statistical significance. Statistical analyses were performed using JMP software (SAS Institute, Cary, NC).

## RESULTS

### Ethanol-Consumption Perturbs In Vivo Immune Responses

Female BALB/c mice were fed modified Lieber-DeCarli diets (Lin, 1989) as LED30 or pair-fed LCD or continued with the SD for 11 days—a feeding regimen previously shown to consistently alter type 1 and type 2 immune responses both in vivo and in vitro without eliciting overt liver pathology. On dietary day 4, mice were immunized with 100  $\mu$ g OVA in complete Freund's adjuvant and 6 days later (dietary day 9) their ears were injected with 20  $\mu$ g OVA in PBS. After 24 hours (dietary day 10) OVA-induced ear swelling was measured as an indicator of DTH. OVA-specific DTH responses of the LED30 group were significantly impaired compared with SD- and LCD-fed mice (Fig. 1). In a parallel experiment, sera from unimmunized diet-fed BALB/c mice were prepared and ELISA determined total IgE levels on dietary day 10. Results indicate that sera from mice-fed LED30 showed significantly enhanced total IgE levels compared with LCD- and SD-fed groups (Fig. 1). These data indicate that alcohol

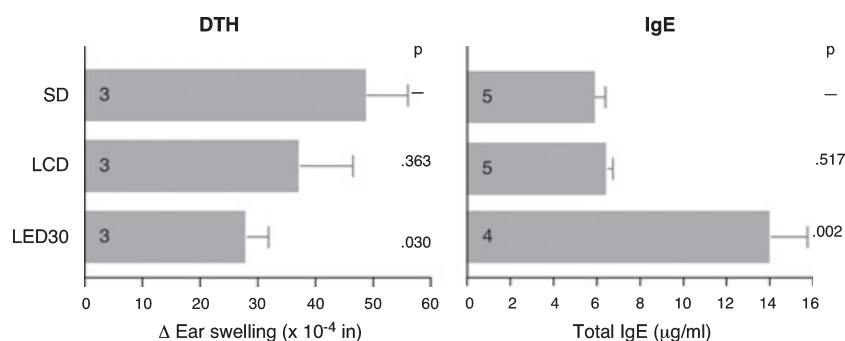


**Fig. 1.** Ethanol consumption impairs DTH and enhances IgE responses in BALB/c mice. Female BALB/c mice were fed a liquid diet containing 30% ethanol-derived calories (LED30), pair-fed a liquid control diet (LCD) that isocalorically substitutes sucrose for ethanol, or fed a solid diet (SD) of lab chow and water ad libitum. Left panel: Mice were immunized with fowl- $\gamma$ -globulin (F $\gamma$ G) on dietary day 4, F $\gamma$ G was injected into the pina on dietary day 10, and change in ear thickness ( $\Delta$  ear swelling) was measured 24 hours later. Number of mice per group is indicated within the bar. Right panel: Diet-fed mice were unimmunized and bled on dietary day 11, and serum IgE levels were determined by ELISA.

consumption impairs a type 1 cell-mediated (DTH) response and enhances type 2 a humoral (IgE) response.

#### Antigen-Presenting Cells Are Required for Cytokine Responses In Vitro

Unfractionated, naïve DO11.10 spleen cells ( $400 \times 10^3$ ) were cultured  $\pm$  OVA for 24 to 72 hours. OVA-stimulated cultures (Table 1, group B) produce peak levels of IL-12, IL-

2, and IFN- $\gamma$  at indicated 24 (IL-12 and IL-2) and 72 hours (IFN- $\gamma$ ); unstimulated cultures (group A) produced minimal or baseline cytokine levels. CD4<sup>+</sup> T cells normally constitute 20 to 25% of unfractionated DO11.10 splenic leukocytes—cultures containing  $400 \times 10^3$  unfractionated spleen cells contain  $\sim 100 \times 10^3$  CD4<sup>+</sup> T cells. We find that OVA-stimulated cocultures containing 5 to  $50 \times 10^3$  APC and  $200 \times 10^3$  purified CD4<sup>+</sup> T cells produce cytokine levels that approximate those of unfractionated DO11.10 spleen cells.

**Table 1.** Antigen-Presenting Cell Populations That Support Cytokine Production in Coculture

| Group | Cell source & treatment <sup>a</sup> | APC <sup>b</sup><br>x10 <sup>3</sup> | CD4 <sup>+</sup> c<br>x10 <sup>3</sup> | OVA <sup>d</sup><br>$\mu$ M | IL-12 <sup>e</sup><br>pg/ml | p      | IL-2 <sup>e</sup><br>pg/ml | p      | IFN- $\gamma$ <sup>e</sup><br>pg/ml | p      |
|-------|--------------------------------------|--------------------------------------|----------------------------------------|-----------------------------|-----------------------------|--------|----------------------------|--------|-------------------------------------|--------|
| A     | DO11.10 spleen cells                 | none                                 | 400                                    | 0                           | 252 $\pm$ 9.8               |        | 9 $\pm$ 0.5                |        | 44 $\pm$ 17.6                       |        |
| B     | DO11.10 spleen cells                 | none                                 | 400                                    | 18                          | 1,209 $\pm$ 202.1           |        | 3,323 $\pm$ 83.6           |        | 32,178 $\pm$ 8261.0                 |        |
| C     | Purified CD4 <sup>+</sup> T cells    | none                                 | 200                                    | 0                           | 45 $\pm$ 6.0                |        | 7 $\pm$ 1.0                |        | <11                                 |        |
| D     | Purified CD4 <sup>+</sup> T cells    | none                                 | 200                                    | 18                          | 540 $\pm$ 50.1              |        | 83 $\pm$ 4.8               |        | 696 $\pm$ 88.2                      |        |
| E     | BALB/c spleen cells                  | 50                                   | none                                   | 18                          | 51 $\pm$ 9.9                |        | 6 $\pm$ 0.2                |        | <11                                 |        |
| F     | BALB/c spleen cells                  | 50                                   | 200                                    | 0                           | 144 $\pm$ 18.1              |        | 10 $\pm$ 0.7               |        | 31 $\pm$ 8.2                        |        |
| G     | BALB/c spleen cells                  | 50                                   | 200                                    | 18                          | 1,334 $\pm$ 72.4            | > .001 | 1,564 $\pm$ 62.3           | > .001 | 14,364 $\pm$ 1302.0                 | > .001 |
| H     | CD11c positive                       | 20                                   | 200                                    | 18                          | 6,304 $\pm$ 292.1           | > .001 | 3,447 $\pm$ 243.5          | > .005 | 38,007 $\pm$ 1749.6                 | > .001 |
| I     | CD11c depleted                       | 50                                   | 200                                    | 18                          | 1,242 $\pm$ 91.6            | > .001 | 2,586 $\pm$ 105.2          | > .005 | 11,415 $\pm$ 1345.7                 | > .001 |
| J     | F4/80 positive                       | 20                                   | 200                                    | 18                          | 967 $\pm$ 93.7              | > .020 | 769 $\pm$ 51.9             | > .001 | 4,985 $\pm$ 847.3                   | > .004 |
| K     | F4/80 depleted                       | 50                                   | 200                                    | 18                          | 1,420 $\pm$ 155.8           | > .020 | 2,755 $\pm$ 112.4          | > .001 | 14,292 $\pm$ 2692.2                 | > .004 |
| L     | Dual depleted                        | 50                                   | 200                                    | 18                          | 1,015 $\pm$ 63.0            |        | 1,984 $\pm$ 105.5          |        | 7,751 $\pm$ 1553.6                  |        |

<sup>a</sup>Unfractionated DO11.10 spleen cells ( $400 \times 10^3$ ) respond to 18  $\mu$ M OVA by producing IL-12 (24 hours), IL-2 (24 hours), and IFN- $\gamma$  (72 hours). Cytokine responses of isolated, purified DO11.10 CD4<sup>+</sup> T cells (group D) and unfractionated BALB/c spleen cells (group E) to OVA are greatly impaired. Purified DO11.10 CD4<sup>+</sup> T cells ( $200 \times 10^3$ ) were cultured with unfractionated (groups F and G), CD11c-positive or -depleted (groups H and I), F4/80-positive or -depleted (groups J and K), or BALB/c spleen cells depleted of both CD11c<sup>+</sup> and F4/80<sup>+</sup> cells (group L).

<sup>b</sup>Number of antigen-presenting cells (APC) added at culture initiation.

<sup>c</sup>Unfractionated DO11.10 spleen cells ( $400 \times 10^3$ ; groups A and B) or purified DO11.10 CD4<sup>+</sup> T cells ( $200 \times 10^3$ ) were added to each culture well.

<sup>d</sup>Cultures were stimulated with 18  $\mu$ M OVA or unstimulated at culture initiation as indicated.

<sup>e</sup>Supernatants from 24-hour (IL-12 and IL-2) and 72-hour (IFN- $\gamma$ ) cultures were assayed by ELISA. Data represent the arithmetic mean  $\pm$  standard error of the mean for 7 to 9 cultures. Statistical significance between indicated data pairs determined by Student's *t*-test.

Addition of OVA to antigen-naïve BALB/c spleen cells does not stimulate cytokine secretion (group E). Coculture of unfractionated BALB/c splenic cells ( $50 \times 10^3$ ) and purified DO11.10 CD4<sup>+</sup> T cells ( $200 \times 10^3$ ) restores cytokine responsiveness to OVA stimulation (group G). To determine whether different APC populations would support cytokine production, CD11c<sup>+</sup> DCs, F4/80<sup>+</sup> macrophages, or cell populations depleted of these cells were used as APCs. Data show that  $20 \times 10^3$  CD11c<sup>+</sup> DCs (group H) support cytokine production better than  $50 \times 10^3$  CD11c-depleted BALB/c spleen cells (group I). Conversely,  $50 \times 10^3$  F4/80-depleted spleen cells (group K) support cytokine production better than  $20 \times 10^3$  enriched F4/80<sup>+</sup> macrophages (group J). Cocultures containing APC populations depleted of both CD11c<sup>+</sup> and F4/80<sup>+</sup> cells (group L) are still capable of supporting cytokine production (group L) indicating that additional APC populations also support cytokine production in this system.

#### CD11c<sup>+</sup> Cells Support Cytokine Production by Naïve T Cells

Individual T cells in transgenic and normal individuals may coexpress TCRs of several specificities (He et al., 2002; Padovan et al., 1993; Zhou et al., 2004) and raises the distinct possibility that some DO11.10 CD4<sup>+</sup> T cells although OVA-naïve, may be of a memory phenotype due to prior stimulation by environmental antigens (Zhou et al., 2004). Because the recombinase gene 2 in DO11.10Rag2<sup>-/-</sup> mice is genetically inactivated, these mice produce T cells that express only the transgenic TCR and cannot produce immunoglobulins or other TCRs. Therefore, OVA-naïve DO11.10Rag2<sup>-/-</sup> mice lack memory T cells. Coculture of purified DO11.10Rag2<sup>-/-</sup> CD4<sup>+</sup> T cells with CD11c<sup>+</sup> cells from SD-fed BALB/c mice produces IL-12, IL-2, and IFN- $\gamma$  in response to OVA stimulation (Table 2, group A) demonstrating the antigen-naïve

CD4<sup>+</sup> T cells respond to OVA or cause APCs to respond to OVA. Cocultures containing CD11c<sup>+</sup> cells isolated from mice-fed LED30 for 10 days show significantly impaired production of the effector cytokine IFN- $\gamma$  (group C) compared with SD or LCD controls. In contrast, IL-2 production by cocultures containing LED30-derived CD11c<sup>+</sup> cells is not impaired. Decreased production of the APC-derived cytokine, IL-12, is seen by LED30 group, but this decrease is of borderline significance.

#### Percentages of Cells Expressing CD11c<sup>+</sup> Are Unchanged, but CD11c Surface Expression Is Altered by Ethanol Consumption

Integrin  $\alpha_x$  (CD11c) noncovalently associates with  $\beta_2$  integrin to form a CD11c/CD18, p150/90 kDa heterodimer expressed on DCs (Metlay et al., 1990). Female BALB/c mice were fed diets for 11 days and their spleens were analyzed by flow cytometry to determine whether ethanol consumption alters CD11c surface expression by DCs, Fig. 2. The percentage of CD3<sup>+</sup>CD11c<sup>+</sup> leukocytes (B cell rich, lower left quadrant) is decreased in spleens of LED30-consuming mice consistent with a decrease B cell numbers in chronic alcoholics and in the spleens of alcohol-consuming mice (Cook, 1998). The percentage (upper left quadrant) of CD11c<sup>+</sup> spleen cells is not altered for any of the 3 diets consumed, however, ethanol consumption increases the expression of CD11c by DCs (vertical shift in median channel fluorescence; Fig. 2, right panels). Alcohol consumption appears not to overtly alter CD8 $\alpha$  expression by CD11c<sup>+</sup> DCs.

#### Ethanol Consumption Alters Antigen Presentation by Explanted CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs

BALB/c mice were fed SD, LED30, or pair-fed LCD for 10 days, euthanized, and their (un)fractionated spleen cells

**Table 2.** Cytokine Responses of DO11.10 Rag2<sup>-/-</sup> Cells in Coculture

| Group | Dietary source <sup>a</sup> |               | Purified CD4 <sup>+</sup> | OVA <sup>b</sup> | IL-12 <sup>c</sup> |      | IL-2 <sup>c</sup> |      | IFN- $\gamma$ <sup>c</sup> |      |
|-------|-----------------------------|---------------|---------------------------|------------------|--------------------|------|-------------------|------|----------------------------|------|
|       | of CD11c <sup>+</sup>       | $\times 10^3$ | $\times 10^3$             | $\mu\text{M}$    | pg/ml              | p    | pg/ml             | p    | pg/ml                      | p    |
| A     | SD                          | 20            | 200                       | 18               | 2,094 $\pm$ 386.6  | .502 | 409 $\pm$ 87.3    | .190 | 5,801 $\pm$ 1865.8         | .935 |
| B     | LCD                         | 20            | 200                       | 18               | 1,760 $\pm$ 293.6  |      | 621 $\pm$ 127.8   |      | 5,455 $\pm$ 1175.6         |      |
| C     | LED30                       | 20            | 200                       | 18               | 1,189 $\pm$ 203.5  |      | 467 $\pm$ 77.5    |      | 1,330 $\pm$ 297.8          |      |
| D     | none                        |               | 200                       | 18               | 20 $\pm$ 0.5       |      | <156              |      | <391                       |      |

<sup>a</sup>Paramagnetic bead-purified CD11c<sup>+</sup> spleen cells ( $20 \times 10^3$ ) from female BALB/c mice fed SD, LED30, or pair-fed LCD for 10 days and used as antigen-presenting cells in 24 to 72 hours coculture with  $200 \times 10^3$  purified CD4<sup>+</sup> T cells (antibody cocktail + complement followed by paramagnetic bead negative separation) isolated from the spleens of DO11.10Rag2<sup>-/-</sup> mice. Culture of purified CD4<sup>+</sup> DO11.10Rag2<sup>-/-</sup> T cells + OVA without exogenous APC (group D) served as a control for the purity of the CD4<sup>+</sup> T cell preparation.

<sup>b</sup>Cultures were stimulated with 18  $\mu\text{M}$  OVA.

<sup>c</sup>Supernatants from 24 h cultures were assessed for IL-12 and IL-2 and from 72 h cultures for IFN- $\gamma$ . (Co)cultures without OVA produced <324pg/ml IL-12, <37pg/ml IL-2, and <391pg/ml IFN- $\gamma$ . Data represent the mean  $\pm$  SEM for 9 cultures per group.

<sup>e</sup>Statistical significance assessed by two-tailed Student's *t* test; *p*  $\leq$  .05 is considered significant.

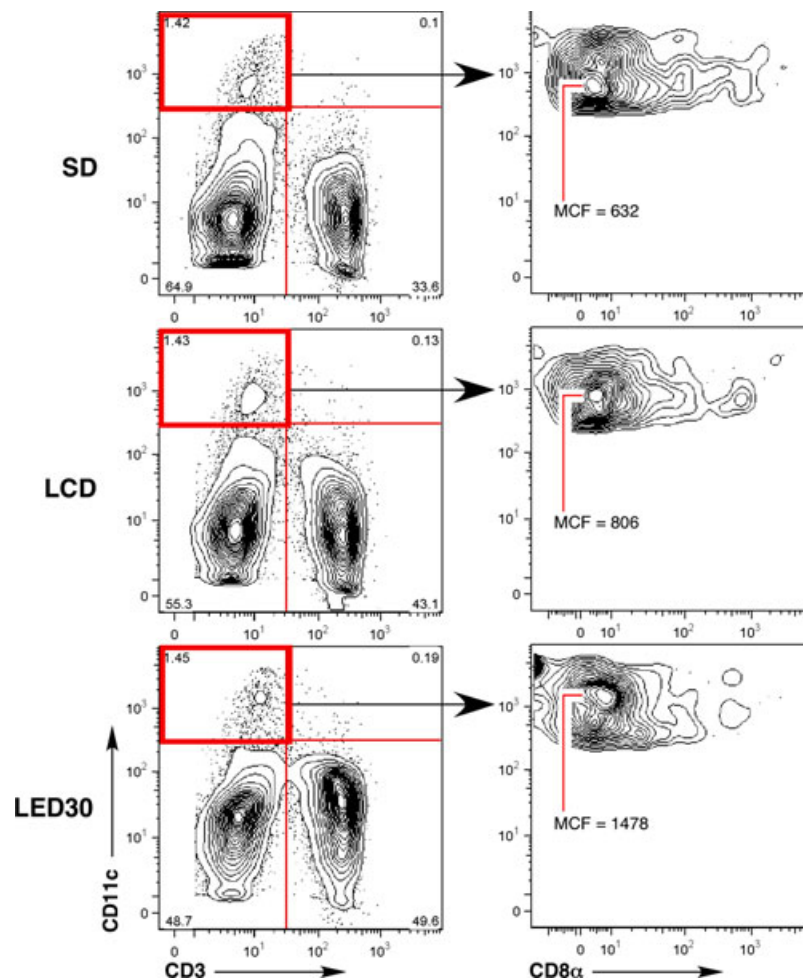


**Fig. 2.** Ethanol consumption increases dendritic cell (DC) CD11c expression. Female BALB/c mice were fed a liquid diet containing 30% ethanol-derived calories (LED30), pair-fed an isocaloric liquid control diet (LCD), or continued with a solid diet (SD) and water ad libitum for 10 days. Spleen cells were analyzed by flow cytometry. Leukocytes were electronically gated by forward and side scatter and propidium iodide-stained cells (dead cells) were excluded from analysis. Left panels: Cells were stained for CD11c (integrin  $\alpha_x$ ) found most prominently on DC and CD3 found on T cells. T cells (upper and lower right quadrants) and non-T cells (upper and lower left quadrants) are seen. CD11c<sup>+</sup> DC are found in the upper left quadrant and compose ~1.4% of the leukocyte populations in all dietary groups. Right panels: CD8 $\alpha$  expression by CD11c<sup>+</sup>CD3<sup>-</sup> (thus excluding CD8<sup>+</sup> T) cells is shown. Median channel fluorescence (MCF) for CD11c expression is indicated. Data for a single representative mouse from each dietary group are shown. This and similar analysis has been performed for 6 or more mice per dietary group and in 3 or more experiments.

were used as a source of APC in 24 to 72 hours coculture with purified DO11.10 CD4<sup>+</sup> T cells  $\pm$  OVA. Data in Table 3 show that coculture of  $200 \times 10^3$  purified DO11.10 CD4<sup>+</sup> T cells with  $50 \times 10^3$  unfractionated spleen cells from diet-fed mice as a source of APC support IL-12 production. Because F4/80<sup>+</sup> (macrophages) do not support IL-12 production (Table 1) and CD11c<sup>+</sup> DCs comprise <2% of the spleen cell population (see Fig. 2) and in this system, cocultures with unfractionated spleen cells contain <1,000 CD11c<sup>+</sup> cells and are at limiting quantities to produce IL-12 (data not shown). Addition of  $20 \times 10^3$  purified CD11c<sup>+</sup> DCs results increased IL-12 production. DCs isolated from LED30-fed mice produced significantly less IL-12 compared with DCs isolated from SD mice. DCs were further separated into CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> fractions, added to  $200 \times 10^3$  purified DO11.10 CD4<sup>+</sup> T cells in the proportions isolated ( $14.5$  and  $5.5 \times 10^3$ , respectively) from the CD11c<sup>+</sup>

population. Data in Table 3 clearly show that CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup>, but not CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup>, DCs support or produce IL-12 and that IL-12 production by DCs explanted from ethanol-consuming mice is impaired. Similar patterns are seen for the T cell cytokines IL-2 (Table 4) and IFN- $\gamma$  (Table 5). Because DTH effector function is IFN- $\gamma$  dependent, the pronounced inhibition of this cytokine in cocultures containing explanted CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs suggests a direct causal link between ethanol consumption and inhibition of cell-mediated immune function.

#### *APCs From Ethanol-Consuming Mice Support Enhanced IL-13 Secretion*

Interleukin-13, a type-2 cytokine, is recognized for its ability to promote both IgE class switching and to promote fibrogenesis (Wynn, 2003, 2004). To assess whether alcohol-

**Table 3.** IL-12 Responses by 24-Hour Cocultures of Purified CD4<sup>+</sup> T Cells With APC Fractions From Diet-Fed Mice

| Diet  | APC <sup>a</sup>                   |               |                      |                                 |                |         |                                         |                                                                                                    |         |
|-------|------------------------------------|---------------|----------------------|---------------------------------|----------------|---------|-----------------------------------------|----------------------------------------------------------------------------------------------------|---------|
|       | Unfractionated spleen <sup>b</sup> |               |                      | CD11c <sup>+</sup> <sup>c</sup> |                |         | CD11c <sup>+</sup> subsets <sup>d</sup> |                                                                                                    |         |
|       | Cells × 10 <sup>3</sup>            | pg/ml         | p-value <sup>e</sup> | Cells × 10 <sup>3</sup>         | pg/ml          | p-value | Cells × 10 <sup>3</sup>                 | pg/ml                                                                                              | p-value |
| SD    | 50                                 | 1,413 ± 142.8 | —                    | 20                              | 5,315 ± 1243.7 | —       | 14.5<br>5.5                             | CD8 <sub>z</sub> <sup>+</sup><br>CD8 <sub>z</sub> <sup>−</sup><br>17,448 ± 1265.2<br>2,049 ± 317.0 | —       |
| LCD   | 50                                 | 675 ± 49.8    | 0.003                | 20                              | 2,590 ± 601.9  | 0.061   | 14.5<br>5.5                             | CD8 <sub>z</sub> <sup>+</sup><br>CD8 <sub>z</sub> <sup>−</sup><br>8,521 ± 1242.3<br>1,189 ± 301.4  | <0.001  |
| LED30 | 50                                 | 829 ± 73.7    | <0.001               | 20                              | 2,117 ± 451.7  | 0.046   | 14.5<br>5.5                             | CD8 <sub>z</sub> <sup>+</sup><br>CD8 <sub>z</sub> <sup>−</sup><br>3,596 ± 326.4<br>992 ± 150.0     | <0.001  |

<sup>a</sup>Spleen cells from BALB/c mice-fed SD, LED30, or pair-fed LCD for 10 days were used as a source for APC in 24 hours coculture with 200 × 10<sup>3</sup> purified DO11.10 CD4<sup>+</sup> T cells + 18 μM OVA as antigen. Cocultures without OVA produced <258 pg IL-12/ml. Culture of purified DO11.10 CD4<sup>+</sup> T cells + OVA without exogenous APC produced 434 ± 57.3 pg IL-12/ml.

<sup>b</sup>Cocultures contained 50 × 10<sup>3</sup> unfractionated spleen cells, 200 × 10<sup>3</sup> purified CD4<sup>+</sup> T cells, and OVA. Data are represented as the arithmetic mean ± SEM for 6 to 18 cultures.

<sup>c</sup>Cocultures contained 20 × 10<sup>3</sup> enriched CD11c<sup>+</sup> spleen cells, 200 × 10<sup>3</sup> purified CD4<sup>+</sup> T cells, and OVA. Data are for 9 to 12 cultures.

<sup>d</sup>Cocultures contained 14.5 × 10<sup>3</sup> CD11c<sup>+</sup>CD8<sub>z</sub><sup>+</sup> or 5.5 × 10<sup>3</sup> CD11c<sup>+</sup>CD8<sub>z</sub><sup>−</sup> spleen cells, 200 × 10<sup>3</sup> CD4<sup>+</sup> T cells, and OVA. Data are for 6 cultures. Numbers of APC added to culture reflect the proportion of CD8<sub>z</sub><sup>+</sup> and CD8<sub>z</sub><sup>−</sup> isolated from the CD11c<sup>+</sup> population. Boxed values indicate responsive (*p* < 0.001) population of the CD11c<sup>+</sup> subsets.

<sup>e</sup>Statistical comparison by two-tailed Student's *t*-test; *p* ≤ 0.05 is considered significant.

APC, antigen-presenting cell; IL, interleukin; LCD, liquid control diet; OVA, ovalbumin; SD, solid diet.

**Table 4.** IL-2 Responses by 24-Hour Cocultures of Purified CD4<sup>+</sup> T Cells With APC Fractions From Diet-Fed Mice

| Diet  | APC <sup>a</sup>                   |               |                      |                                 |               |         |                                         |                                                                                             |         |
|-------|------------------------------------|---------------|----------------------|---------------------------------|---------------|---------|-----------------------------------------|---------------------------------------------------------------------------------------------|---------|
|       | Unfractionated spleen <sup>b</sup> |               |                      | CD11c <sup>+</sup> <sup>c</sup> |               |         | CD11c <sup>+</sup> subsets <sup>d</sup> |                                                                                             |         |
|       | Cells × 10 <sup>3</sup>            | pg/ml         | p-value <sup>e</sup> | Cells × 10 <sup>3</sup>         | pg/ml         | p-value | Cells × 10 <sup>3</sup>                 | pg/ml                                                                                       | p-value |
| SD    | 50                                 | 1,093 ± 126.9 | —                    | 20                              | 1,003 ± 160.2 | —       | 14.5<br>5.5                             | CD8 <sub>z</sub> <sup>+</sup><br>CD8 <sub>z</sub> <sup>−</sup><br>2,065 ± 186.3<br>81 ± 7.9 | —       |
| LCD   | 50                                 | 748 ± 114.9   | 0.058                | 20                              | 733 ± 71.2    | 0.130   | 14.5<br>5.5                             | CD8 <sub>z</sub> <sup>+</sup><br>CD8 <sub>z</sub> <sup>−</sup><br>817 ± 92.0<br>97 ± 13.4   | <0.001  |
| LED30 | 50                                 | 665 ± 82.9    | 0.005                | 20                              | 596 ± 71.6    | 0.032   | 14.5<br>5.5                             | CD8 <sub>z</sub> <sup>+</sup><br>CD8 <sub>z</sub> <sup>−</sup><br>520 ± 34.9<br>52 ± 4.6    | <0.001  |

<sup>a</sup>Spleen cells from BALB/c mice-fed SD, LED30, or pair-fed LCD for 10 days were used as a source of APC in 24-hour cocultures with 200 × 10<sup>3</sup> purified DO11.10 CD4<sup>+</sup> T cells + 18 μM OVA as antigen. Cocultures without OVA produced <36 pg IL-2/ml. Culture of purified DO11.10 CD4<sup>+</sup> T cells + OVA without exogenous APC produced 51 ± 6.4 pg IL-2/ml.

<sup>b</sup>Cocultures contained 50 × 10<sup>3</sup> unfractionated spleen cells, 200 × 10<sup>3</sup> CD4<sup>+</sup> T cells, and OVA. Data are shown for 18 to 24 cultures.

<sup>c</sup>Cocultures contained 20 × 10<sup>3</sup> enriched CD11c<sup>+</sup> spleen cells, 200 × 10<sup>3</sup> CD4<sup>+</sup> T cells, and OVA. Data are shown as the arithmetic mean ± SEM for 24 cultures.

<sup>d</sup>Either 14.5 × 10<sup>3</sup> CD8<sub>z</sub><sup>+</sup> or 5.5 × 10<sup>3</sup> CD8<sub>z</sub><sup>−</sup> spleen cells, 200 × 10<sup>3</sup> CD4<sup>+</sup> T cells, and OVA. Data are shown as the arithmetic mean ± SEM for six 24-hour culture supernatants. Numbers of APC added to culture reflect the proportion of CD8<sub>z</sub><sup>+</sup> and CD8<sub>z</sub><sup>−</sup> isolated from the CD11c<sup>+</sup> population. Boxed values indicate significantly responsive (*p* < 0.001) population of the CD11c<sup>+</sup> subsets.

<sup>e</sup>Statistical comparison by two-tailed Student's *t*-test, where *p* ≤ 0.05 is considered significant.

APC, antigen-presenting cell; IL, interleukin; LCD, liquid control diet; OVA, ovalbumin; SD, solid diet.

consumption influences IL-13 production in our coculture system, female BALB/c mice were fed SD, LED30, or pair-fed LCD for 10 days and their unfractionated spleen (400 × 10<sup>3</sup>) cells were used as a source of APC and cultured together with purified DO11.10 CD4<sup>+</sup> T (50 × 10<sup>3</sup>) cells ± 18 μM OVA for 72 hours. Data in Fig. 3, show an increase of nearly 50% in IL-13 secretion by cocultures containing purified DO11.10 CD4<sup>+</sup> T cells and BALB/c spleen cells derived from ethanol-consuming (LED30) mice compared cultures containing APC derived from SD or LCD consuming BALB/c mice.

#### *Cocultures Containing DCs From Ethanol-Consuming Mice Show Diminished IL-17A and IL-6 Secretion*

Recently, a distinct lineage of IL-17A-secreting, effector CD4<sup>+</sup> T (Th17) cells, distinct from Th1, Th2, and Treg, has been described (Harrington et al., 2005; Stockinger and Veldhoen, 2007). As a member of the IL-17 cytokine family [IL-17A, B, C, D, E (i.e., IL-25), and F], IL-17A is a potent proinflammatory cytokine shown to have a role in autoimmunity, stimulates neutrophils, and is important in host defense against extracellular bacteria (Bettelli et al., 2007;

**Table 5.** IFN- $\gamma$  Responses by 72-Hour Cocultures of Purified CD4<sup>+</sup> T Cells With APC Fractions From Diet-Fed Mice

| Diet  | APC <sup>a</sup>                   |                    |                      |                                 |                   |         |                                         |                                                                                        |         |
|-------|------------------------------------|--------------------|----------------------|---------------------------------|-------------------|---------|-----------------------------------------|----------------------------------------------------------------------------------------|---------|
|       | Unfractionated spleen <sup>b</sup> |                    |                      | CD11c <sup>+</sup> <sup>c</sup> |                   |         | CD11c <sup>+</sup> subsets <sup>d</sup> |                                                                                        |         |
|       | Cells $\times 10^3$                | pg/ml              | p-value <sup>e</sup> | Cells $\times 10^3$             | pg/ml             | p-value | Cells $\times 10^3$                     | pg/ml                                                                                  | p-value |
| SD    | 50                                 | 7,739 $\pm$ 1705.9 | —                    | 20                              | 3,034 $\pm$ 919.8 | —       | 14.5<br>5.5                             | CD8 $_{\alpha}^{+}$<br>CD8 $_{\alpha}^{-}$<br>11,161 $\pm$ 1534.2<br>1,873 $\pm$ 494.0 | —       |
| LCD   | 50                                 | 3,957 $\pm$ 820.3  | 0.152                | 20                              | 1,335 $\pm$ 130.4 | 0.081   | 14.5<br>5.5                             | CD8 $_{\alpha}^{+}$<br>CD8 $_{\alpha}^{-}$<br>9,326 $\pm$ 816.6<br>664 $\pm$ 380.1     | 0.316   |
| LED30 | 50                                 | 1,397 $\pm$ 196.1  | <0.001               | 20                              | 627 $\pm$ 295.7   | 0.021   | 14.5<br>5.5                             | CD8 $_{\alpha}^{+}$<br>CD8 $_{\alpha}^{-}$<br>4,951 $\pm$ 638.0<br>424 $\pm$ 162.0     | 0.004   |

<sup>a</sup>Spleen cells from BALB/c mice-fed LED30, pair-fed LCD, or fed SD for 10 days were used as a source for APC and cocultured together with  $200 \times 10^3$  purified DO11.10 CD4<sup>+</sup> T cells together with 18  $\mu$ M OVA as antigen. Data from OVA-stimulated 72-hour cultures are shown. Cocultures without OVA produced <150 pg IFN- $\gamma$ /ml. Culture of purified DO11.10 CD4<sup>+</sup> T cells + OVA without exogenous APC produced 481  $\pm$  102.5 pg IFN- $\gamma$ /ml.

<sup>b</sup>Cocultures contained  $50 \times 10^3$  unfractionated spleen cells and  $200 \times 10^3$  CD4<sup>+</sup> T cells + OVA. Data are shown as the mean  $\pm$  SEM for 6 to 18 cultures.

<sup>c</sup>Cocultures contained  $20 \times 10^3$  enriched CD11c<sup>+</sup> spleen cells and  $200 \times 10^3$  CD4<sup>+</sup> T cells + OVA. Data are shown as the mean  $\pm$  SEM for 12 cultures.

APC, antigen-presenting cell; IFN, interferon- $\gamma$ ; LCD, liquid control diet; OVA, ovalbumin; SD, solid diet LED30, liquid ethanol diet.

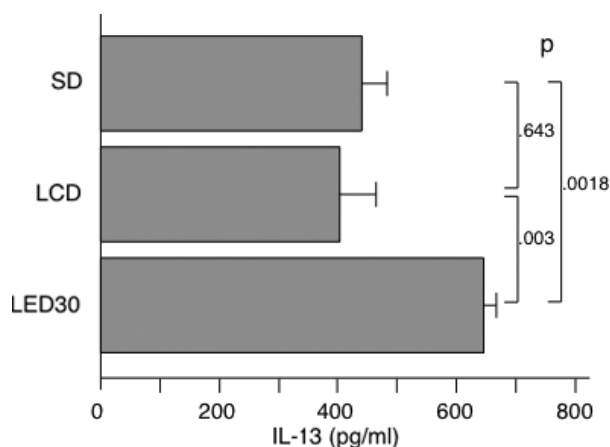


**Fig. 3.** Ethanol consumption enhances IL-13 secretion. Female BALB/c mice were fed LED30, pair-fed LCD, or continued SD and water ad libitum for 10 days. BALB/c spleen cells ( $400 \times 10^3$ ) were used as a source for APC in coculture with  $50 \times 10^3$  purified DO11.10 T cells  $\pm$  18  $\mu$ M OVA as antigen. ELISA was used to determine IL-13 content of 72-hour culture supernatants and is indicated as the arithmetic mean  $\pm$  SEM for 12 cultures per data group. Cultures of purified DO11.10 CD4<sup>+</sup> T cells without exogenous APC and cocultures without antigen produced <78 pg IL-13/ml. Statistical significance between indicated data pairs determined by Student's *t*-test.

E), whereas macrophages (F4/80<sup>+</sup>, line F) and CD11c-MHC II<sup>+</sup> (i.e., B cells, line G) do not. In marked contrast, cocultures containing spleen cells from LED30-fed BALB/c mice show significantly diminished IL-17A secretion in response to OVA (line K). Both IL-6 and transforming growth factor (TGF) - $\beta$ 1 are required for the induction of Th17 cells. Culture supernatants from these same cocultures were assessed to determine whether alcohol affect IL-6 secretion. Similar to the IL-17A pattern, cocultures containing spleen cells from LED30-consuming mice (Table 6, line K) were significantly diminished compared with cocultures containing spleen cells from SD- (line D) or LCD- (line I) BALB/c mice. Collectively, these data suggest that as a consequence of diminished IL-6 secretion may be a decrease in the generation of IL-17A-secreting Th17 cells.

## DISCUSSION

Ethanol consumption alters immune function in both humans and mice, but the mechanisms for this alteration have been elusive. Although alcohol consumption profoundly affects T cell regulated adaptive immune responses, T cells appear to be indirect targets. Bridging both the innate and adaptive immune systems, phagocytic leukocytes destroy microbial invaders or antigens and the resulting proteolytic peptides bound to MHC class II are displayed on their cell surfaces for recognition by antigen-specific CD4<sup>+</sup> T cells. Several laboratories, including our own, have shown that alcohol consumption affects APC function (Israel et al., 1992; Lau et al., 2006; Mandrekar et al., 2004; Mikszta et al., 1995; Schirren et al., 1997; Szabo et al., 1993; Waltenbaugh and Peterson, 1997). In this study, we show that alcohol consumption affects the manner or context in which antigen is presented by CD11c<sup>+</sup>CD8 $_{\alpha}^{+}$  DCs. Although these DCs normally account for <2% of the splenic leukocyte population, APCs from alcohol-consuming mice either directly or indi-

Kolls and Linden, 2004; Stockinger and Veldhoen, 2007). In a pulmonary *Klebsiella pneumoniae* infection model, alcohol consumption decreases IL-17A both in vivo in lung lavage fluid and in vitro by spleen cells (Shellito et al., 2001). Data in Table 6 (line A) show that unseparated DO11.10 spleen cells produce IL-17A in response to OVA stimulation, whereas purified DO11.10 CD4<sup>+</sup> T cells do not (line B). Coculture of unfractionated spleen cells from SD- or LCD-fed BALB/c mice with purified DO11.10 CD4<sup>+</sup> T cells require OVA stimulation for IL-17A secretion (lines D and I). Coculture of CD11c<sup>+</sup> DCs from SD mice with purified CD4<sup>+</sup> T cells support IL-17A production in response to OVA (line

**Table 6.** IL-17A and IL-6 Responses by 72-Hour Cocultures Containing APCs From Diet-Fed Mice

| Dietary source of BALB/c APC <sup>a</sup> |       |                                                 | DO11.10 spleen cells <sup>b</sup> | IL-17A <sup>c</sup> pg/ml |                     |                     | IL-6 <sup>c</sup> pg/ml |                |                     |
|-------------------------------------------|-------|-------------------------------------------------|-----------------------------------|---------------------------|---------------------|---------------------|-------------------------|----------------|---------------------|
|                                           |       |                                                 |                                   | No antigen                | 18 $\mu$ M OVA      | <i>p</i>            | No antigen              | 18 $\mu$ M OVA | <i>p</i>            |
| A                                         |       | None                                            | Unseparated                       | 176 $\pm$ 30.4            | 9,674 $\pm$ 1,607.5 | <.0001 <sup>d</sup> | 35 $\pm$ 2.1            | 859 $\pm$ 76.1 | <.0001 <sup>d</sup> |
| B                                         |       | None                                            | Purified CD4 <sup>+</sup> T       | 136 $\pm$ 29.7            | 144 $\pm$ 31.4      | .847                | 22 $\pm$ 2.4            | 22 $\pm$ 2.1   | .701                |
| C                                         | SD    | Unfractionated                                  | 0                                 |                           | 113 $\pm$ 35.3      |                     |                         | 23 $\pm$ 1.6   |                     |
| D                                         | SD    | Unfractionated                                  | Purified CD4 <sup>+</sup> T       | 112 $\pm$ 35.4            | 1,438 $\pm$ 99.3    |                     | 19 $\pm$ 1.8            | 140 $\pm$ 15.0 |                     |
| E                                         | SD    | Purified CD11c <sup>+</sup>                     | Purified CD4 <sup>+</sup> T       |                           | 1,457 $\pm$ 295.0   |                     |                         | 121 $\pm$ 29.5 |                     |
| F                                         | SD    | Purified F4/80 <sup>+</sup>                     | Purified CD4 <sup>+</sup> T       |                           | 256 $\pm$ 58.8      |                     |                         | 36 $\pm$ 3.8   |                     |
| G                                         | SD    | Purified CD11c <sup>+</sup> MHC II <sup>+</sup> | Purified CD4 <sup>+</sup> T       |                           | 381 $\pm$ 162.4     | .0702               |                         | ND             | .06                 |
| H                                         | LCD   | Unfractionated                                  | 0                                 |                           | 112 $\pm$ 35.4      |                     |                         | 25 $\pm$ 2.8   |                     |
| I                                         | LCD   | Unfractionated                                  | Purified CD4 <sup>+</sup> T       | 142 $\pm$ 42.9            | 1,058 $\pm$ 187.3   | <.0001              | 21 $\pm$ 2.0            | 99 $\pm$ 11.2  | .0002               |
| J                                         | LED30 | Unfractionated                                  | 0                                 |                           | 119 $\pm$ 34.6      | .0075               |                         | 35 $\pm$ 9.8   | .005                |
| K                                         | LED30 | Unfractionated                                  | Purified CD4 <sup>+</sup> T       | 146 $\pm$ 42.6            | 536 $\pm$ 38.1      |                     | 25 $\pm$ 4.4            | 60 $\pm$ 5.4   |                     |

<sup>a</sup>Fifty thousand unfractionated or  $20 \times 10^3$  purified spleen cells from naïve female BALB/c mice-fed LED30, pair-fed LCD, or continued on SD and water for 10 days used as APC.

<sup>b</sup>Cultures contained  $200 \times 10^3$  CD4<sup>+</sup> purified T cells or  $400 \times 10^6$  unseparated spleen cells from DO11.10 mice  $\pm$  OVA as indicated.

<sup>c</sup>IL-17A and IL-6 levels were determined in supernatants of 72-hour cultures by ELISA.

<sup>d</sup>Statistical comparison for IL-17A or IL-6 between OVA stimulated and unstimulated (lines A and B) and between OVA-stimulated cocultures containing unfractionated APC from the 3 dietary groups (lines D, I, and K) was assessed by two-tailed Student's *t*-test;  $p \leq 0.05$  is considered significant.

APC, antigen-presenting cell; ELISA, enzyme-linked immunosorbent assays; IL, interleukin; LCD, liquid control diet; MHC, major histocompatibility complex; OVA, ovalbumin; SD, solid diet; ND, not determined.

rectly influence the secretion of IL-6, IL-12, IL-13, IL-17A, and IFN- $\gamma$  production in cocultures containing purified DO11.10 CD4<sup>+</sup> T cells. The altered pattern of cytokine secretion and effector cell responses suggests an underlying mechanism for the effect(s) of ethanol on immune function.

Initiation of alcohol consumption at the time of immunization or thereafter has little or no effect upon the immune response (Starkenbourg et al., 2001). In most experimental models, subjects begin alcohol consumption days or weeks prior to the antigen exposure, suggesting that critical alterations occur prior to antigenic encounter. Alcohol alters APC function (Girouard et al., 1998; Peterson et al., 1998a; Szabo et al., 1996, 2001). T cells isolated from alcohol-consuming mice show near normal responsiveness when cultured with APC derived from SD- or LCD-fed mice. T cells derived from SD- or LCD-fed mice, in contrast, when cocultured with APC derived from ethanol-consuming mice show marked impairment of IFN- $\gamma$  production, a type 1 cytokine (Peterson et al., 1998a,b).

Because DCs are efficient lymphocyte stimulators (Banche-reau and Steinman, 1998), residual DC in purified CD4<sup>+</sup> T cell populations can pose technical difficulties when evaluating function of exogenous APC. In preliminary experiments (data not shown), we found neither isolation by paramagnetic beads (positive or negative isolation using beads supplied by 3 different manufacturers) nor treatment with a cocktail of antibody (MHC II, CD49b, and CD205) plus complement sufficiently removed contaminating APC, because CD4<sup>+</sup>-enriched splenic T cells from DO11.10 mice—produced IL-12, IL-2, and IFN- $\gamma$  in response to OVA. Sequential treatment using antibody plus complement followed by paramagnetic bead negative selection produced DO11.10 CD4<sup>+</sup> T cell populations sufficiently free from contaminating APC

that would produce only minimal cytokine responses to OVA (Table 1). Coculture of purified DO11.10 CD4<sup>+</sup> T cells with APC isolated from syngeneic BALB/c mice indicates that CD11c<sup>+</sup> DCs, but not F4/80<sup>+</sup> macrophages support most of the cytokine responses to OVA. Cocultures of BALB/c spleen cells depleted of both CD11c<sup>+</sup> and F4/80<sup>+</sup> cells and purified CD4<sup>+</sup> T cells, show some responsiveness to OVA indicating that either a few contaminating CD11c<sup>+</sup> DCs remain or that other APC may be responsible. In vivo studies indicate that B cells do not usually interact with naïve CD4<sup>+</sup> T cells because they lack the proper antigen-specific immunoglobulin, less than 1% of B cells produce cell surface, peptide-MHC II complexes, and B cells are rarely found in T cell rich areas of lymph nodes, so that B cell interaction naïve T cells is unlikely to occur (Itano and Jenkins, 2003).

Unlike immunoglobulin genes, TCR genes are not allelically excluded; a single T cell in transgenic or normal individuals may express TCRs of several specificities. OVA-naïve DO11.10 CD4<sup>+</sup> T cells could possibly be memory cells by nontransgenic TCR stimulation. Because they have an inactive recombinase 2 gene, DO11.10 Rag2<sup>-/-</sup> mice do not produce B cells and can only express the transgenic TCR on their T cells. Coculture experiments (Table 2) demonstrate that antigen-naïve CD4<sup>+</sup> T cells respond to OVA presented by CD11c<sup>+</sup> DCs and that DCs isolated from alcohol-consuming mice do not efficiently support IFN- $\gamma$  responses by these T cells. While not ruling out a possible antigen-presenting role for B cells, these data clearly indicate that ethanol impairs the ability of CD11c<sup>+</sup> cells to support IFN- $\gamma$  responses by OVA-naïve CD4<sup>+</sup> T cells.

Major histocompatibility complex II, a maker generally limited to APC, expression is decreased in alcohol-consuming humans and mice (Cook et al., 1991; Starkenburg

et al., 2001). Because MHC II serves as a restricting element for  $CD4^+$  T cells, a quantitative decrease in cell-surface expression indicates potential change in effector T cell function. Although the percentage ( $\sim 1.4\%$ ) of DCs isolated from the spleens of alcohol-consuming mice is unaltered, the  $CD11c$  cell surface density increases (Fig. 2).  $CD8_\alpha$  expression by  $CD11c^+$  DCs appears to be unaltered by alcohol consumption. Of the 3 "conventional" DC subsets found in mouse based upon  $CD4$  and  $CD8$  expression (Shortman and Naik, 2007), only  $CD11c^+CD8_\alpha^+$  cross present endogenous and viral antigens on MHC class I molecules to  $CD8^+$  T cells (Banchereau and Steinman, 1998; Shortman and Naik, 2007). That alcohol consumption does not diminish  $CD8_\alpha$  expression by  $CD11c^+$  DCs suggests that they may retain their ability to efficiently present viral antigens to MHC I-restricted  $CD8^+$  T cells. The increase in effector and memory  $CD8^+$  T cells in both human alcoholics (Cook et al., 1991; Laso et al., 1996) and in a murine chronic ingestion model (Zhang and Meadows, 2005) described in previous studies may be aided by cross presentation by  $CD11c^+CD8_\alpha^+$  DCs.

Alcohol impairs type 1 and enhances type 2 effector adaptive immune responses (reviewed by Cook, 1998). Ethanol influences cytokine responses as well. Depending upon glucocorticoid levels, trauma, severity of liver dysfunction, ethanol consumption has been shown to either augment (Deviere et al., 1989; Faunce et al., 1998; Hong et al., 2002; Sheron et al., 1991) or diminish (Irwin and Miller, 2000; von Dossow et al., 2004; Aloman et al., 2007; Rivier, 1999; Szabo et al., 1995; and this communication) IL-6 production. Alcohol consumption inhibits IL-12 (Aloman et al.,

2007; Girouard et al., 1998; Mandrekar et al., 2004; Mason et al., 2000; Peterson et al., 1998b; Zisman et al., 1998), IL-17A (Shellito et al., 2001; and this communication), and IFN- $\gamma$  secretion (Starkenburger et al., 2001; Szabo et al., 2001; Zisman et al., 1998), but enhances IL-10 (Aloman et al., 2007; Girouard et al., 1998; Mandrekar et al., 2004; Szabo et al., 1996) and IL-13 secretion (Szabo et al., 2001; Dominguez-Santalla et al., 2001; and this communication). In general, type 1-cytokine responses (e.g., IL-6, IL-12, IL-17A, and IFN- $\gamma$ ) are inhibited by alcohol consumption, but type 2 cytokines (e.g., IL-10 and IL-13) are enhanced. Levels of these cytokines produced by Th1 (IFN- $\gamma$ ) and Th17 (IL-17A) are impaired and secretion of several Th2 cytokines (IL-10 and IL-13) is enhanced. Alcohol appears to indirectly act upon effector  $CD4^+$  T cell function by directly acting upon APCs (Aloman et al., 2007; Lau et al., 2006; Mandrekar et al., 2004; Ostrovidov et al., 2002; Peterson et al., 1998a).

A unified model for the effects of alcohol on adaptive immune function is postulated based upon experimental evidence, Fig. 4. Alcohol impairs Th1-mediated delayed hypersensitivity and enhances Th2-mediated IgE responses. Impaired Th17 activity is proposed based upon, because these are the only cells, so far, known to produce IL-17 (Harrington et al., 2005) and alcohol decreases IL-17 (Shellito et al., 2001; and this communication) and IL-23 (Happel et al., 2006) production. Although required to maintain Th17 cells, IL-23 is not required for the differentiation of Th17 from naïve  $CD4^+$  T cells (Stockinger and Veldhoen, 2007). Effector  $CD4^+$  T cell differentiation is strongly influenced by the milieu in which APCs interact with precursors of effector  $CD4^+$  T cells (Thp and Th0); Fig. 4. By down-regulating IL-12 and up-regulating IL-10 APCs decrease or increase Th0 differentiation away from Th1 and toward Th2 effector function. Both IL-6 and TGF- $\beta$ 1 are required for Th17 development (Stockinger and Veldhoen, 2007). Although TGF- $\beta$ 1 is increased during ethanol consumption (Bechara et al., 2004), Th17 will not differentiate unless IL-6 is also present (Veldhoen et al., 2006). This model places APCs, and DC in particular, at the epicenter for the effect of alcohol on adaptive immune function. Recently, another member of the IL-17 family of cytokines, IL-17E also known as IL-25, has been shown to have an important regulatory role (Kleinschek et al., 2007). Unlike other members of the IL-17 cytokine family (Kolls and Linden, 2004), IL-25 promotes type 2 and inhibits type 1 adaptive immune responses (Fallon et al., 2006; Kleinschek et al., 2007) by antagonizing IL-17A secretion and promoting IL-13 production. Consequently, IL-13 inhibits IL-6 and IL-23 secretion and the subsequent development of Th17 effector T cells (Fort et al., 2001; Kleinschek et al., 2007). Moreover, an IL-25 induced rapid rise in IL-13 secretion would promote B cell class switching to IgE production (Kang et al., 2005; Owyang et al., 2006; Sharkhuu et al., 2006; Wynn, 2003). Bone marrow-derived mast-cells (Tamachi et al., 2006),  $CD4^+$  T cells (Fort et al., 2001), and alveolar mac-

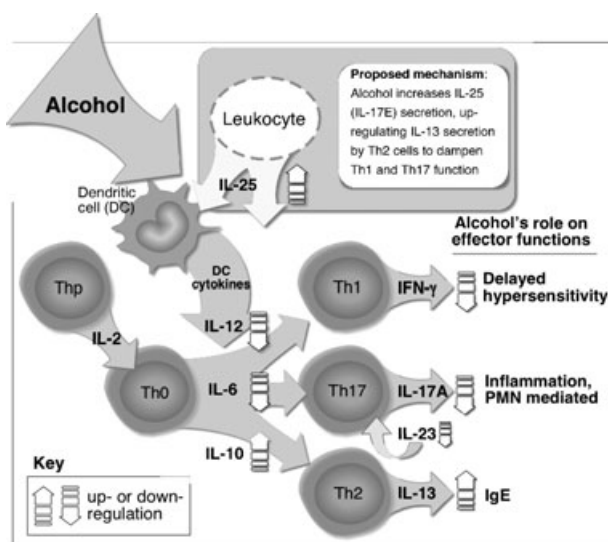


**Fig. 4.** Proposed model for the effect of alcohol on the immune system. Gray arrows indicate cytokines that influence  $CD4^+$  T cell differentiation. White vertical up or down segmented arrows indicate respective increase or decrease on cytokine quantity or on immune effector function. Hypothetical role for leukocyte secreted IL-25 is offset within the shaded rectangle. See text for details.

rophages (Kang et al., 2005) have been reported among those cells that produce IL-25. We propose that alcohol by directly acting upon DCs and/or IL-25-producing cells may induce IL-25 production to enhance Th2 effector function and to dampen Th1 and Th17 function Fig. 4.

In conclusion, the present study localizes alcohol-induced alteration of splenic immune function to “conventional” CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs in an antigen-specific model of adaptive immune function. Alcohol consumption increased CD11c cell-surface expression and decreased the capacity of these DCs to support IL-17A and IFN- $\gamma$  cytokine secretion, but enhance IL-13 production by purified CD4<sup>+</sup> T cells derived from DO11.10 transgenic mice. In addition, we demonstrate that the ethanol consumption diminishes the capacity of CD11c<sup>+</sup> DC to secrete IL-12 and IL-6 support type 1 cytokine secretion by naïve CD4<sup>+</sup> transgenic T cells. A unified hypothesis is proposed for the underlying mechanism(s) for the effect(s) of ethanol on DC and subsequent alteration of adaptive immune function.

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