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Mechanisms by which chronic ethanol feeding limits the ability of dendritic cells to stimulate T cell proliferation

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Abstract

Background—As initiators of immune responses, dendritic cells (DCs) are required for antigen (Ag) specific activation of naïve T cells in the defense against infectious agents. The increased susceptibility to and severity of infection seen in chronic alcoholics could be due to impaired DC initiation of naïve T cell responses. Specifically, these DC may not provide adequate Signals 1 (Ag presentation), 2 (costimulation), or 3 (cytokine production) to these T cells.

Methods—Using the Meadows-Cook murine model of chronic alcohol abuse, the ability of ethanol (EtOH)-exposed DC to stimulate T cell proliferation, acquire and process Ag, express costimulatory molecules, and produce inflammatory cytokines was assessed.

Results—Normal naïve T cells primed by EtOH-exposed DCs showed decreased proliferation *in vitro* and *in vivo*, compared to water-fed control mice. These EtOH-exposed DC, after activation by CpG or TNF α , were less able to upregulate costimulatory molecules CD40, CD80, or CD86, and produced less IL-12 p40, TNF α and IFN α than DC from water-fed mice. TLR9 and TNF receptor expression were also reduced in/on EtOH-exposed DC. No evidence of defective Ag acquisition or processing as a result of EtOH feeding was identified.

Conclusions—Inadequate proliferation of normal T cells following stimulation by EtOH-exposed DC is likely a result of diminished Signal 2 and Signal 3. Lack of adequate inflammatory stimulation of EtOH-exposed DC due to diminished receptors for inflammatory mediators appears to be at least partially responsible for their dysfunction. These findings provide a mechanism to explain increased morbidity and mortality from infectious diseases in alcoholics, and suggest targets for therapeutic intervention.

Keywords

dendritic cells; mouse; cytokines; antigen processing; costimulatory molecules

INTRODUCTION

Increased incidence and severity of a wide variety of infections have long been observed in chronic alcoholic individuals (Cook, 1998; Lau et al., 2009; MacGregor and Louria, 1997; Szabo, 1999; Zhang et al., 2008). Chronic ethanol (EtOH) exposure impairs T cell responses to antigenic challenge in both humans and animal models (Chang and Norman, 1999;

Geissler et al., 1997; Gluckman et al., 1977; Gurung et al., 2009; Meyerholz et al., 2008; Snyder et al., 1978; Tonnesen et al., 1992). These observed deficiencies are clearly due in part to intrinsic EtOH-induced T cell defects; however, failure of EtOH-exposed dendritic cells (DC) to appropriately activate T cells could also play a significant role in suboptimal T cell function. Chronic EtOH-induced defects in DC stimulatory capacity following activation have not previously been systematically studied.

As initiators of immune responses, DC play a critical role in the defense against infectious agents. They are required for the antigen (Ag) specific activation of naïve T cells, a step that initiates adaptive immune responsiveness to invading organisms. The process by which maximal activation of T cells occurs involves a series of interactions between DC and T cells. When encountering a pathogen in an inflammatory environment, DC capture and process Ag from the organism and present processed Ag to T cells in association with major histocompatibility complex (MHC) Class I and/or Class II. This step, Signal 1, assures that only T cells specific for the pathogen are activated. Signal 1 alone is insufficient to ensure naïve T cell activation. In fact, it will result in T cell refractoriness to activation if the Ag is not presented in the context of costimulatory molecule interactions (Signal 2) (Schwartz, 2006). Costimulatory molecules fall into two major families: the immunoglobulin family, which includes DC CD86 and CD80 interactions with T cell CD28, and the TNF family, which includes DC CD40 interactions with T cell CD154. The latter interaction is particularly important to stimulate CD8⁺ T cell responses if CD4⁺ T cell help is unavailable (Hernandez et al., 2007; Schuurheis et al., 2000). Finally, differentiation of T cells into subsets with specialized effector functions is driven by DC-secreted cytokines (Signal 3). Suboptimal ability of DC to provide Signals 1, 2, and/or 3 would be expected to result in diminished Ag-specific T cell activation.

Splenic DC can be divided into two major subsets, which are specialized to promote specific aspects of T cell function. For example, conventional DC (cDC) are expert at Ag presentation (including crosspresentation) and IL-12 production, leading to CD8⁺ T cell activation and CD4⁺ Th1 differentiation (Lopez-Bravo and Ardavin, 2008). Plasmacytoid DC (pDC) are major producers of IFN α following viral infection, leading to cytotoxic T lymphocyte activation (Barchet et al., 2005).

Short term EtOH consumption decreases the ability of DC to promote Ag-specific T cell responses in humans (Mandrekar et al., 2004; Szabo et al., 2004) as well as in mice (Heinz and Waltenbaugh, 2007). Some evidence already exists that chronic EtOH feeding also decreases certain aspects of DC function. Dendritic cells from human alcoholics with cirrhosis have decreased production of IL-1 β and TNF α relative to non-alcoholic control patients (Laso et al., 2007a). Chronic EtOH feeding in macaques decreases costimulatory molecule expression on DC following *in vitro* stimulation with an inflammatory cytokine cocktail (Siggins et al., 2009). Similarly, DC from mice chronically fed EtOH in the drinking water have decreased IL-12 production in response to CpG, as well as diminished ability to stimulate non-EtOH exposed (hereafter referred to as normal) allogeneic T cell proliferation (Lau et al., 2006). Finally, DC from mice fed EtOH long-term as part of a liquid (Lieber-DeCarli) diet also had reduced allostimulatory activity and impaired ability to produce TNF α , IL-12, IFN γ , and IL-6 following lipopolysaccharide (LPS) or poly I:C stimulation (Aloman et al., 2007). The degree to which corticosterone-induced stress affected DC function in the latter model remains unclear.

The present study was undertaken to systematically investigate the ability of DC from EtOH fed mice to provide Signals 1, 2, and 3, in order to gain a more precise definition of the lesion(s) in EtOH exposed DC activation of normal T cells. The Meadows-Cook method of EtOH administration in the drinking water was utilized, as this model allows long-term

EtOH feeding and avoids the potential confounding effect of corticosterone induced immune suppression (Cook et al., 2007). These mice routinely reach blood EtOH levels as high as 400 mg/dl after nocturnal feeding and drinking, and return to very low or undetectable levels by late afternoon (Song et al., 2002). The results provide evidence for multiple defects in DC ability to provide Signals 2 and 3, without evidence for a defect in Signal 1. These findings identify specific targets for which interventions to improve DC function in alcoholics could be developed.

MATERIALS AND METHODS

Mice and EtOH feeding regimen

Six to seven wk old female C57Bl/6 (CD45.2) mice were obtained from the National Cancer Institute (Frederick, MD). Pep3bBoy/J (CD45.1 congenic mice on a C57Bl/6 background) (Shen et al., 1985), ovalbumin (OVA) specific T cell receptor (TCR) transgenic (Tg) H-2K^b restricted (OTI) and I-A^b restricted (OTII) mice (Barnden et al., 1998; Hogquist et al., 1994) were obtained from in-house breeding colonies. All mice were maintained in the specific pathogen-free facility at the University of Iowa, and all animal procedures were approved by the animal care use committee at the University of Iowa.

After a 1 wk acclimation period, control and EtOH fed groups of C57Bl/6 mice were created by random separation of mice from the same lot. Pharmaceutical grade EtOH (AAPER Alcohol, Shelbyville, KY) was provided in double distilled drinking water as the sole water source at 10% (w/v) for 2 d, 15% for 5 d, and 20% for 4 to 16 wk. All time points for EtOH consumption are reported as duration on 20% EtOH. The mice were provided laboratory chow *ad libitum* in the bedding in all cases, and age-matched controls were given the same double-distilled water as that used for mixing the EtOH solutions. Mice maintained on this regimen demonstrate normal weight gain without elevations in blood corticosterone levels (Cook et al., 2007).

Antibodies

The following monoclonal antibodies were used for four-color flow cytometric analyses and/or T cell purification: N418, a hamster anti-mouse CD11c (Metlay et al., 1990); 6B2, a rat IgG2a anti-mouse CD45R (B220) (Morse et al., 1982); M1/70, a rat IgG2b anti-mouse CD11b (Mac-1) (Springer et al., 1979); Ter119, a rat IgG2b anti-mouse Ly-76 (Ikuta et al., 1990); 536.72, a rat IgG2a anti-mouse CD8 (Ledbetter and Herzenberg, 1979); 34.1.2S, a mouse IgG2a anti-mouse MHC Class I (Ozato et al., 1982); G1-1, a rat IgG2b anti-mouse CD86 (Freeman et al., 1993); and 1C10, a rat IgG2a anti-mouse CD40 (Heath et al., 1994) were derived from hybridomas in the laboratory and conjugated to biotin, Cy5, or FITC using standard procedures. Phycoerythrin (PE) anti-MHC class II (I-A/I-E; M5/114.15.2), PECy5.5 anti-CD11c (N418); APC anti-CD4 (L3T4), PE anti-IL-12 p40 (C17.8), PE anti-TNF α (MP6-XT22), PE anti-IL-6 (MP5-20F3), PE anti-TLR4 (MTS510) and FITC anti-TLR9 (M9.D6) were purchased from eBioscience (San Diego, CA). FITC anti-IFN α (RMMA-1) was purchased from PBL InterferonSource (Piscataway, NJ). PE anti-TNFR1 (55R-286) and PE anti-TNFR2 (HM102) were purchased from Abcam (Cambridge, MA).

Polyclonal purified rat IgG (Jackson ImmunoResearch, West Grove, PA) was used as an isotype control for all experiments, as the majority of antibodies used were rat, and comparisons between polyclonal rat, mouse, and hamster IgG showed equivalent background staining (data not shown). All samples were incubated with anti-CD16/32 (clone 2.4G2) (Unkeless, 1979) and rat serum (Pel-Freez Biologicals, Rogers, AR) during flow cytometric staining to counteract background binding to Fc γ R.

Cell preparation

For DC isolation, spleens were dissociated by injecting 1 ml of 25 µg/ml Liberase Blendzyme III (Roche Diagnostics, Indianapolis, IN) and 20 µg/ml DNase I (Roche Diagnostics) and incubating for 20 min at 37° C. For measurement of MHC Class I/II and TLR9/TLR4/TNF receptor (TNFR) levels, splenocyte suspensions were centrifuged through FicoLite LM (Atlanta Biologicals, Lawrenceville, GA) and mononuclear cells were recovered from the interface. For measurement of DC costimulatory molecules and intracellular cytokines, splenocyte suspensions were enriched for DC using Optiprep (Axis-shield, Norway) density gradients. For T cell stimulation and Ag processing experiments, DC were purified by positively selecting CD11c⁺ cells on AutoMACS (Miltenyi Biotec, Auburn, CA). The purity was verified to be greater than 90% by flow cytometric staining.

For T cell proliferation assays, splenocyte suspensions were subjected to Fico-Lite (Atlanta Biologicals, Norcross, GA) density gradient followed by negative selection (depletion of B220⁺, CD11c⁺, CD11b⁺, and Ter119⁺ cells). The purity was verified to be greater than 90% by flow cytometric staining.

In vitro T cell proliferation assay

Varying numbers of DC from EtOH fed or control mice were pulsed with OVA protein or OVA peptide 258-264 or 323-339 (Biosynthesis, Lewisville, TX) for 2 hr, washed extensively, and cultured with 2×10^5 T cells from OTI or OTII mice for 72 h. T cell proliferation was measured by ³H-thymidine incorporation following an additional 18 h incubation.

Antigen uptake and processing assays

Dendritic cells were incubated with 20 µg/ml FITC-OVA (Invitrogen, Eugene, OR) or 500 µg/ml 40kDa FITC-dextran (Invitrogen) or 200 µg/ml DQ-OVA (Invitrogen) for 0, 5, 15 or 30 min, and then thoroughly washed. Fluorescence was detected by flow cytometry.

In vivo T cell proliferation assay

On day 1, 5×10^7 splenocytes of OTI or OTII mice were labeled with 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) and transferred intravenously (i.v.) into syngeneic (C57Bl/6) mice. On day 2, 5×10^6 DC from EtOH fed or control mice were pulsed with 100 nM OVA peptide 258-264 or 323-339 for 2 hours, washed extensively, and transferred i.v. into the same recipients. On day 5, residual CFSE labeling in Tg CD8⁺ or CD4⁺ T cells were studied.

Costimulatory molecule induction

Mice were injected i.v. with TNFα (4 µg; PeproTech, Rocky Hill, NJ) or with CpG 1826 (25 µg; Coley, Wellesley, MA) in phosphate buffered saline (PBS) 24 h before harvesting spleens. Controls were injected with PBS alone. CD40, CD80 and CD86 expression were detected by flow cytometric analysis.

Intracellular cytokine induction and staining

Splenic DC preparations were cultured with 1 µg/ml CpG 1826 or 0.5 µg/ml TNFα for 6 h. 10 ng/ml Brefeldin A (BD, San Jose, CA) was added in culture to retain cytokine intracellularly. Cells were harvested and stained with MHC Class II and CD11c followed by fixation with 1× FACS lysing solution (BD, San Jose, CA). Cells were then permeabilized using 0.5% saponin buffer followed by staining with anti-cytokine antibodies.

Flow Cytometric Analysis

After staining, cells were fixed with 1% formaldehyde in 1.25X PBS. Data were acquired on a BD FACSCalibur or FACS Canto II (BD Biosciences, San Diego, CA) equipped with CellQuestPro software (BD Biosciences) and analyzed using FlowJo 8.5.2 or 8.8.6 software (TreeStar Inc, Ashland, OR). Dead cells were excluded by low angle and orthogonal light scatter. Spectral overlaps between FITC and PE, PE and Cy5.5PE and Cy5 and Cy5.5PE were corrected by manual compensation on singly stained positive controls. At least 50,000 cells were collected per sample.

Statistics

Unless otherwise indicated, the *p* values for differences between EtOH and control groups were calculated from the two tailed unpaired Student *t* test. For cytokine experiments and figures 1A and 3A, the *p* values for differences between EtOH and control groups were calculated from the two-tailed Wilcoxon matched pairs test performed on the primary data. Values of *p* <0.05 were considered significant, and are indicated in the figures by an asterisk. All statistical results were calculated using InStat (GraphPad Software, San Diego, CA).

RESULTS

Chronic EtOH feeding impairs the ability of protein-pulsed DC to stimulate normal T cell proliferation in vitro

The overall goal of this study was to systematically investigate the DC components of DC-T cell interactions, which could contribute to the decreased ability of DC from chronic EtOH fed mice to activate T cells, as measured by T cell proliferation. To confirm that the DC obtained from mice on the Meadows-Cook model of EtOH feeding indeed are unable to stimulate normal Ag-specific T cell proliferation in response to a protein Ag, DC were prepared from mice fed EtOH for 4 or 16 weeks, and water fed controls. The DC were pulsed with OVA protein and cultured with T cells from OTI or OTII mice that were never fed EtOH, at various ratios of DC:T cells. (These mice contain either CD8⁺ (OTI) or CD4⁺ (OTII) T cells that are Tg for T cell receptors specific for OVA peptides 258-264 or 323-339 presented in association with MHC Class I or II respectively.) EtOH exposed DC (hereafter EtOH DC) showed a decreased ability to promote the proliferation of OVA-specific CD4⁺ or CD8⁺ T cells (Figure 1A). The magnitude of the loss in T cell proliferation was similar between CD4⁺ and CD8⁺ T cells.

To investigate whether the observed DC defect could be overcome if the DC initially encountered higher doses of Ag, the experiments were repeated using varying concentrations of OVA protein. Figure 1B shows that DC from mice fed EtOH for 16 wk had decreased ability to promote the proliferation of CD4⁺ or CD8⁺ T cells when pulsed with 10 and 20 µg/ml OVA protein, but the EtOH DC could stimulate efficient T cell proliferation when 50 µg/ml of protein was used. Similar results were obtained from experiments using DC from mice fed EtOH for 4 wk, although the changes in OTII T cell proliferation at 10 µg/ml did not reach statistical significance. These findings indicate that at relatively low concentrations of Ag, EtOH DC are impaired in their ability to promote T cell proliferation. However, the defect is not absolute because at higher Ag concentrations this aspect of DC-T cell interaction (the ability to instigate T cell proliferation) is intact. Nevertheless, the existing defect could be sufficient to delay initiation of T cell responses early in infection, when the pathogen levels are still low and the infection is normally most easily cleared.

Chronic EtOH feeding does not lead to defective Ag uptake and processing

Protein Ag require internalization into the DC and processing into smaller fragments prior to being associated with MHC Class I or Class II for display on the cell surface to activate the T cell. Antigen acquisition in DC occurs primarily through endocytosis or macropinocytosis (Villadangos and Schnorrer, 2007). To determine whether these Ag acquisition methods are affected by exposure to EtOH (and could thus at least partially explain the reduction in T cell proliferation observed in Figure 1), splenic DC from mice fed EtOH for 16 weeks were incubated with fluoresceinated OVA or fluoresceinated dextran. As seen in Figure 2A (upper and middle panels), DC from EtOH fed mice did not show decreased ability to internalize dextran by macropinocytosis or OVA by endocytosis compared to water-fed controls.

To assess whether protein Ag processing was inhibited by EtOH, DC from EtOH fed mice were incubated with OVA labeled with the fluorescent dye BODIPY (DQ)-OVA. The DQ-OVA is endocytosed, after which the levels of fluorescence detected are proportional to the extent of DQ-OVA processing into peptides (Boonacker and Van Noorden, 2001). DC from mice exposed to EtOH for 16 wk did not show decreased ability to process OVA protein into peptides (Figure 2A, bottom panel). The final step of Ag processing by DC is the ability to display the processed peptides on the cell surface in association with MHC Class I or Class II. While measurement of specific OVA peptides associated with MHC molecules on DC from EtOH and water fed mice was not technically feasible, EtOH feeding had no effect on MHC Class I or Class II expression levels on DC (Figure 2B). Thus no evidence was found to support a defect in Ag processing and presentation as a contributory aspect of the observed decrease in the ability of EtOH DC to stimulate normal Ag specific T cell proliferation.

Chronic EtOH feeding impairs the ability of peptide-pulsed DC to stimulate normal T cell proliferation in vitro

Because no differences in protein processing could be identified between EtOH and water exposed DC, we hypothesized that even if DC were loaded directly with peptide prior to being placed with T cells, EtOH DC would remain deficient in their ability to stimulate T cell proliferation. DC prepared from mice fed EtOH for 4 or 16 weeks (and water fed controls) were pulsed with 10 nM of the appropriate OVA peptide and cultured with T cells from OTI or OTII mice that were never fed EtOH, at various ratios of DC:T cells. As when pulsed with OVA protein, EtOH DC showed a decreased ability to promote the proliferation of OVA-specific CD4⁺ or CD8⁺ T cells (Figure 3A). The magnitude of loss in T cell proliferation was again similar between CD4⁺ and CD8⁺ T cells, and at best only slightly less than the decrease in proliferation seen with OVA protein pulsed DC (Figure 1A).

To investigate whether the observed DC defect could be overcome if the DC initially encountered higher doses of the appropriate OVA peptide, the experiments were repeated using varying concentrations of peptide. The results indicate that DC from mice fed EtOH for 16 wk have decreased ability to promote the proliferation of CD4⁺ or CD8⁺ T cells when DC were pulsed with 10-50 nM OVA peptide, but EtOH DC could stimulate efficient T cell proliferation when 100 nM peptide was used (Figure 3B). Similar results were obtained from experiments using DC from mice fed EtOH for 4 wk, although the changes in OTII T cell proliferation were of a smaller magnitude and did not reach statistical significance at 20 nM. These findings indicate that at relatively low concentrations of peptide, EtOH DC are impaired in their ability to promote T cell proliferation. As in the protein Ag studies, the defect is not absolute because at higher peptide concentrations this aspect of DC-T cell interaction (the ability to instigate T cell proliferation) is intact.

Chronic EtOH feeding impairs the ability of peptide-pulsed DC to stimulate normal T cell proliferation *in vivo*

To determine if the decreased ability of EtOH DC to stimulate proliferation of Ag specific T cells also occurs during *in vivo* interactions, CFSE labeled splenocytes from OTI or OTII mice were transferred to non-EtOH fed C57Bl/6 “receptacle” mice, followed one day later by the transfer of DC from mice fed EtOH for 16 weeks or controls that had been pulsed with 100 nM OVA peptide 258-264 or 323-339 *in vitro*. Three days later, CFSE⁺ CD8⁺ or CD4⁺ T cells were identified in splenocyte populations from the receptacle mice and studied for CFSE fluorescence intensity. In this system, the only cells that encountered EtOH were the Ag pulsed DC, thus any differences between the treatment groups must be due to EtOH effect on transferred DC (and not T cells). As shown in Figure 4, stimulation with EtOH DC resulted in 1) higher numbers of Tg T cells that did not divide at all, and 2) a skewing towards lower generation numbers of the stimulated T cell population. As in the *in vitro* experiments (Figures 1 and 3), the results were similar for CD4⁺ and CD8⁺ Tg T cells. In separate experiments, non-Ag pulsed DC from EtOH and water fed mice were recovered in equivalent numbers from the spleens of receptacle mice at 30 min, 1 hour and 2 hours post transfer, indicating no difference in their ability to transit to and accumulate in the spleens of receptacle mice following i.v. transfer (data not shown). Thus the observed differences are not the result of delayed stimulation of Tg T cells due to gross defects in DC migration (although the possibility of microanatomic defects in DC migration specifically to splenic T cell zones has not been excluded).

The similarities between the results when EtOH DC were pulsed with OVA protein vs. peptide (Figure 1 vs. Figures 3 and 4) suggested that one or more function(s) of DC required for T cell stimulation other than Ag processing/presentation was being affected by EtOH. Accordingly, studies were conducted to examine the ability of DC from EtOH fed mice to provide Signal 2 (costimulation) and Signal 3 (cytokine production) to T cells.

Dendritic cell costimulatory molecule upregulation is reduced in chronic EtOH fed mice following inflammatory stimulation

To determine if costimulatory pathways are inhibited by chronic EtOH feeding, costimulatory molecule (CD40, CD80, and CD86) expression on splenic DC subsets was measured. No significant differences were found between EtOH and control DC costimulatory molecule expression in the absence of inflammatory stimulation (Figure 5). However, when 16 wk EtOH fed mice were treated *in vivo* with CpG 1836, a TLR9 agonist, their DC (both cDC and pDC subsets) did not upregulate CD40, CD80, or CD86 to the same extent as DC from water fed controls. Similar changes were seen at 36 h following stimulation, indicating that the diminished ability to upregulate costimulatory molecules was persistent and not simply a result of delayed kinetics (data not shown).

To determine whether the observed alterations in DC costimulatory molecule expression as a result of chronic EtOH feeding were limited to stimulation with particular inflammatory mediators, similar experiments were performed using TNF α or LPS as the stimulatory agents. LPS was chosen as an alternative TLR agonist that acts through MyD88 dependent and independent (TRIF) pathways. TNF α was chosen to represent endogenous inflammatory mediators, as it is released from NK cells, DC and macrophages following their activation, often by pathogen associated molecular patterns (PAMPS) (Beutler and Cerami, 1989; Tsujimura et al., 2004). The ability of EtOH DC to upregulate costimulatory molecule expression in response to TNF α was also diminished in some but not all instances when compared with control DC, and the degree of the defect appeared to be less than with CpG (Figure 5). Studies of costimulatory molecule expression following CpG and TNF α stimulation conducted at 4 and 8 weeks of EtOH feeding showed trends toward decreased

expression on EtOH cDC and pDC, but no statistical differences were observed (data not shown). Responsiveness of DC costimulatory molecule upregulation in response to LPS (a TLR4 agonist) was virtually unaffected by EtOH feeding (data not shown). Thus EtOH feeding appears to variably affect the ability of DC to respond to inflammatory stimuli with costimulatory molecule upregulation. However, in most infections, multiple inflammatory mediators are present simultaneously in the environment, and such conditions often synergize to produce maximal DC activation (Gautier et al., 2005; Napolitani et al., 2005). When the ability to effectively respond to one or more mediators is missing *in vivo*, increased susceptibility to or severity of infection is observed (Bafica et al., 2006; Bafica et al., 2005; Tabeta et al., 2004; Weiss et al., 2004). Thus the observation that EtOH DC fail to respond partially to multiple individual inflammatory stimuli would be predicted to compromise their ability to provide a robust Signal 2 to T cells, and may thereby provide a mechanism underlying the failure of EtOH fed DC to stimulate normal levels of T cell proliferation.

Dendritic cell cytokine production is reduced in chronic EtOH fed mice following inflammatory stimulation

Cytokine production by DC provides Signal 3 when naive T cells are optimally stimulated during the initiation of immune responses. To determine if chronic EtOH feeding also limits the ability of DC to produce inflammatory cytokines, enriched splenic DC were cultured with media alone, CpG 1826, TNF α , or LPS. The percentages of cytokine-producing cDC or pDC were enumerated by intracellular staining (gating strategy shown in Figure 6A). Even in the absence of inflammatory stimuli, a smaller fraction of EtOH pDC (similar trend for cDC) produced TNF α than did control DC (Figure 6B). In response to stimulation with CpG 1826, these changes persisted. In addition, stimulated EtOH cDC were also less likely to produce IL-12p40, and pDC were less likely to produce IFN α . The fraction of EtOH DC producing TNF α following TNF α stimulation was not statistically lower than control DC, but IL-12p40 producing DC remained fractionally lower in EtOH vs. control mice. Following LPS stimulation, no differences in fraction of DC producing these cytokines were apparent between EtOH and control DC (data not shown). The observed alterations in cytokine production were observed after as early as 4 wk of EtOH feeding, and persisted with similar magnitude until at least 16 wk of EtOH feeding. No statistically significant differences were observed between EtOH and control DC in the amount of cytokine produced per cell, in the cytokine producing DC populations (data not shown). These results indicate that immunostimulatory cytokine production by both pDC and cDC in response to inflammatory stimulation is disrupted by chronic EtOH exposure, and provide a second mechanism by which DC from EtOH fed mice may be unable to appropriately activate naive T cells.

TLR9 and TNFR expression are downregulated in EtOH DC

By at least two measures (costimulatory molecule expression and cytokine production), DC from EtOH fed mice show substantial defects in their ability to respond to CpG 1836 or TNF α stimulation. Such defects could result from a lack of receptors for these stimuli, and/or deficiencies in the intracellular signaling pathways triggered by receptor stimulation. To begin to investigate the mechanism for failure of DC to respond to these stimuli, TLR9, TLR4, TNFR1 and TNFR2 expression were measured in/on DC subsets obtained from mice fed EtOH for 16 weeks and water fed controls. Figure 7 demonstrates that expression of each of these receptors was decreased in/on both EtOH cDC and pDC (although the decrease in pDC expression of TNFR2 did not reach statistical significance). In contrast, TLR4 expression was unchanged by EtOH feeding. Thus diminished receptor expression appears to account for at least part of the failure of EtOH DC to respond appropriately to inflammatory mediators.

DISCUSSION

Previous work in our laboratory demonstrated that chronic EtOH feeding decreases DC numbers and delays their migration from skin to regional lymph nodes in a murine model of alcoholism that is free of corticosterone-induced stress effects (Cook et al., 2007; Edsen-Moore et al., 2008; Ness et al., 2008). Both of these characteristics would be expected to contribute to the increased susceptibility to and severity of infection seen in this model (Jerrells et al., 2007; Meyerholz et al., 2008; Ray et al., 2003), as well as in human alcoholics (Cook, 1998; Lau et al., 2009; MacGregor and Louria, 1997; Szabo, 1999; Zhang et al., 2008). A few reports provide evidence that chronic EtOH feeding also leads to defects in certain aspects of individual DC function and decreased ability to stimulate T cell proliferation, even if DC are placed in direct proximity of T cells (i.e., a requirement for migration is removed) (Aloman et al., 2007; Lau et al., 2006; Siggins et al., 2009). However, systematic investigation of the ability of DC from EtOH fed mice to provide Signals 1, 2, and 3 leading to robust naïve T cell proliferation has not previously been described.

EtOH DC were deficient in their ability to promote proliferation of normal T cells, regardless of whether the DC were exposed to the relevant protein or peptide, and whether the interaction took place *in vitro* or *in vivo* (Figures 1, 3, and 4). The ability of EtOH DC to optimally stimulate T cell proliferation in the *in vitro* assays at high but not low concentrations of Ag may reflect the relative lack of signals 2 and 3 on these cells. At low dose Ag (but less so at high dose Ag), accessory molecule ligation is required to promote T cell activation, including proliferation (Cai and Sprent, 1996; Iezzi et al., 1998; Rogers and Croft, 2000; Viola and Lanzavecchia, 1996). In addition to decreased T cell proliferation, alterations in T cell subset differentiation may result (Ruedl et al., 2000; Rogers and Croft, 2000). Finally, the likelihood of adaptive regulatory T cell (Treg) differentiation (which is promoted at low Ag dose even with healthy DC (Turner et al., 2009)) may be further increased by interaction with EtOH DC as a result of lack of signals 2 and 3 (Cools et al., 2007). This possibility is actively under investigation in our laboratory. The observed diminished ability to stimulate T cell proliferation when Ag dose is low would, at a minimum, give pathogens an advantage to establish a serious infection before the Ag dose increases enough to minimize the need for costimulation. A more serious consequence could result from early adaptive Treg induction, which theoretically could limit adequate immune responsiveness even after pathogen load increases.

CFSE dilution studies indicated that EtOH DC were less able to stimulate Tg T cells to undergo their first division by 72 h (i.e., the number of Tg T cells remaining in Generation 0 was significantly greater following EtOH DC than control DC stimulation), and the proliferating T cell population was overall less able to achieve high generation numbers (Figure 3). Possible explanations for the latter observation include longer cell cycle times for T cells stimulated by EtOH DC, or increased apoptosis following completion of one to two cell divisions. The *in vivo* T cell proliferation experiments utilized DC pulsed with 100 nM OVA peptide, a concentration shown to overcome EtOH DC-driven T cell proliferation defects *in vitro* (Figure 3). This apparent discrepancy may simply be due to differing sensitivities of the two assays, or to diminished ability of EtOH DC to make adequate contact with Tg T cells in the more complex splenic microenvironment. The defects in ability to stimulate CD4⁺ and CD8⁺ T cell proliferation appeared similar in magnitude. These findings may partially explain the decreased numbers of Ag-specific CD8⁺ T cells detected in EtOH fed mice vs. controls following *in vivo* stimulation with attenuated *Listeria monocytogenes* (Gurung et al., 2009), and the decreased number of Ag-specific CD4⁺ T cells recovered following Hepatitis C immunization (Geissler et al., 1997).

The failure of protein-pulsed EtOH DC to adequately stimulate T cell proliferation (Figure 1) suggested no defect in Ag acquisition and processing/presentation (Signal 1), and specific investigation of these processes confirmed this hypothesis (Figure 2). These results confirm and extend the report of adequate Ag acquisition by DC from EtOH fed mice, using a different means of EtOH administration (Aloman et al., 2007).

T cell functions other than proliferation may also be diminished when EtOH DC activate naïve T cells, as T cell proliferation can occur without gain of effector function and vice versa (Brinster and Shevach, 2005; Curtsinger et al., 1998; Hernández et al., 2002; Oehen and Brduscha-Riem, 1998; Sporri and Reis e Sousa, 2005; Zajac et al., 1998). Studies evaluating the effect of altered EtOH DC function on other T cell functions such as memory cell generation, cytokine production and cytolytic activity are ongoing in our laboratory.

Further investigation into the mechanism by which peptide-pulsed DC from EtOH fed mice suboptimally stimulate Ag-specific T cells revealed that Signal 2 is deficient following exposure of EtOH DC to certain inflammatory stimuli (Figure 5). The consequences of this deficiency in the initiation of the immune response are substantial. Lack of CD80/86 expression results in failure of the DC to signal T cells through CD28, that is required for IL-2 production and autocrine stimulation of further T cell proliferation (McKnight et al., 1994). In addition CD28 signaling is important for the continued expression of CD154, to allow the T cells to interact with CD40 on DC (de Boer et al., 1993). This in turn promotes further maturation and production of IL-12 by the DC, in a positive feedback loop leading to robust adaptive immunity (Kelsall et al., 1996). Finally, CD28 directly stimulates further DC activation by reverse signaling through CD80 and CD86 (Orabona et al., 2004). Thus EtOH-induced costimulatory molecule deficiency on maturing DC provides multiple mechanisms that may contribute to the failure of these cells to promote optimal T cell proliferation.

Using these same inflammatory stimuli, EtOH cDC populations were found to be deficient in their production of IL-12 p40 and TNF α . (Because our studies demonstrate specifically a deficiency in the fraction of DC producing the p40 subunit, which is common to both IL-12 and IL-23, deficiency of both of these cytokines would be predicted.) EtOH pDC populations were also deficient in IFN α production, in addition to IL-12 p40 and TNF α (depending on the nature of the inflammatory stimulus; Figure 6). Conventional DC are the major source of IL-12 leading to Th1 differentiation of naïve T cells (Macatonia et al., 1995), as well as IL-23 leading to Th17 differentiation (Aggarwal et al., 2003). Furthermore both of these cytokines have autocrine effects on DC, leading to additional production of IL-12 as well as IFN γ (Belladonna et al., 2002; Ohteki et al., 1999). TNF α deficiency results in decreased CD4⁺ and CD8⁺ T cell proliferation, poor survival at early time points after Ag stimulation, and decreased production of IL-2, IFN γ , and cytolytic effector function (Hill et al., 2000; Kim and Teh, 2001; Kim and Teh, 2004). Finally, IFN α promotes IFN γ secretion and cytolytic function of T cells, as well as increasing survival of activated and memory T cells (Curtsinger et al., 2003; Marrack et al., 1999; Tough et al., 1996). Thus the failure of EtOH DC to provide an optimal signal 3 in the form of TNF α is another mechanism that likely specifically contributes to the observed deficiency of these DC to stimulate T cell proliferation. Additionally, deficient IL-12 p40, TNF α and IFN α production would be expected to lead to defects in multiple additional aspects of T cell activation by these DC including cytolytic function, cytokine production, and memory T cell survival. For example, failure of activated DC to produce IL-12 leads to loss of IFN γ production by CD4 T cells despite the ability of such DC to promote T cell proliferation (Sporri and Reis e Sousa, 2005). The degree to which these aspects of T cell function are impacted following interaction with EtOH DC, potentially compounding the demonstrated defects in EtOH DC driven T cell proliferation (Figures 1, 3, 4) is currently under investigation.

The consequences of deficient IL-12 p40, TNF α and IFN α production following inflammatory stimulation of EtOH DC populations is predicted to affect the host response to pathogens beyond their effect on T cell function. For example, IFN α has direct antiviral effects, promotes NK cell cytotoxicity and synergizes with TNF α to induce inducible nitric oxide synthase (iNOS) mediated pathogen elimination by macrophages (MacMicking et al., 1997; Nguyen et al., 2000; Nguyen et al., 2002). IL-12 is required for optimal NK cell IFN γ production (Orange and Biron, 1997). Cumulatively, these downstream effects of deficient EtOH DC cytokine production could substantially contribute to the observed increased susceptibility of EtOH fed mice to a wide variety of infectious agents (Geissler et al., 1997; Gurung et al., 2009; Meyerholz et al., 2008).

T cells that receive Signal 1 in the absence of Signals 2 and 3 have a high likelihood of becoming refractory to activation upon future Ag encounters, in addition to failing to participate in the adaptive immune response to the current pathogenic challenge (Schwartz, 2006). The degree to which T cell anergy is present in chronic EtOH-fed mice remains largely unexplored, but clearly this is another means by which chronic alcoholism could lead to increased infectious morbidity and mortality.

Finally, Figure 7 reveals that deficient receptor expression is a mechanism by which EtOH DC are rendered relatively unresponsive to CpG and TNF α stimulation. Thus the intracellular pathways leading to DC NF- κ B activation are poorly initiated. Whether additional, independent alterations in assembly of TLR complexes in lipid rafts (previously demonstrated for TLR4 following acute EtOH exposure (Szabo et al., 2007)) or intracellular elements of TLR signaling pathways are also present in DC exposed to chronic EtOH and/or its metabolites *in vivo* remains to be determined.

It is of interest that TLR4 expression is unchanged in our model of chronic EtOH feeding, whereas TLR9 and TNFRI/II are decreased. We have previously demonstrated that the major gut flora in our mouse colonies is *Lactobacillus*, Gram positive bacteria that would be expected to release CpG but not LPS into the circulation following the increased gut permeability that has been demonstrated in human alcoholics and in rodent models of alcoholism (Bode et al., 1987; Cook et al., 2007; Keshavarzian et al., 2009; Tabata et al., 2002). Chronic stimulation of TLR9 by CpG may ultimately lead to downregulation of this receptor. Similarly, chronic presence of the endogenous inflammatory mediator TNF α (Gobejishvili et al., 2006; Sztrymf et al., 2005) may lead to the observed downregulation of its receptors, while TLR4 expression remains relatively unaffected. Regardless of the relative preservation of TLR4 expression in our model, the demonstrated loss of multiple receptors for inflammatory mediators, which are required for optimal host response to a wide range of infectious agents, supports the observed DC defects as contributors to the increased incidence and severity of infections in chronic alcoholics.

In summary, decreased proliferation of T cells from non-EtOH fed mice when stimulated with EtOH DC is likely a result of decreased costimulatory molecule expression and inflammatory cytokine production, but not altered Ag uptake and processing. Less naïve Ag-specific T cell proliferation contributes to a poor adaptive immune response to pathogens and increased susceptibility to infection seen in alcoholism. In addition, marginal DC inflammatory cytokine production may also partially explain observed deficiencies in innate immunity following EtOH feeding as well (Boyadjeva et al., 2001; Chen et al., 2003; Frank et al., 2004; Laso et al., 1997; Laso et al., 2007b; Morio et al., 2000; Pan et al., 2006; Silvain et al., 1995).

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Figure 1.

Chronic EtOH feeding inhibits the ability of DC to promote Ag-specific T cell proliferation in response to OVA protein *in vitro*. **(A)** DC from mice fed EtOH for 4 or 16 weeks or control mice were pulsed with 10 $\mu\text{g/ml}$ OVA protein and cultured with T cells from OTI or OTII mice at various ratios of DC:T cells (2×10^5 T cells/well). T cell proliferation was measured by ^3H -thymidine incorporation. Data shown are representative of 3 independent experiments per Tg T cell type; error bars represent standard deviation (SD) between the replicates in the experiment. OTII curves are statistically different ($p=0.02$ at 4 wk and $p=0.03$ at 16 wk), and OTI curves trend similarly at both time points. **(B)** 2×10^4 DCs from mice fed EtOH for 4 or 16 weeks or control mice were pulsed with various concentrations of OVA protein and cultured with 2×10^5 T cells from OTI or OTII mice. T cell proliferation was measured as in (A). $N=3$ mice/group; error bars represent SD.

Figure 2.

Chronic EtOH feeding has no effect on multiple aspects of DC Ag acquisition and processing. **(A)** Splenic DC from 16 wk EtOH fed mice or controls were incubated with FITC-OVA, FITC-dextran or DQ-OVA for 0-30 min. FITC fluorescence was identified by flow cytometry. Data shown are representative of 3 independent experiments per Ag type. **(B)** Splenic DC from mice fed EtOH for 16 weeks or control mice were stained to identify cDC and pDC (CD11c^{hi}B220⁺, and CD11c^{mod}B220⁺ respectively; 2% probability contour plot), as well as MHC Class I or Class II. N=6-8 mice/group; error bars represent SD.

Figure 3.

Chronic EtOH feeding inhibits the ability of DC to promote Ag-specific T cell proliferation in response to OVA peptide *in vitro*. (A) DC from mice fed EtOH for 4 or 16 weeks or control mice were pulsed with 10 nM OVA peptide 258-264 or 323-339 and cultured with T cells from OTI or OTII mice respectively at various ratios of DC:T cells (2×10^5 T cells/well). T cell proliferation was measured by ^3H -thymidine incorporation. Data shown are representative of 3 independent experiments per transgenic T cell type; error bars represent SD between the replicates in the experiment. OTI and OTII curves are statistically different ($p=0.01$ for both OTI and OTII at 4 wk, $p=0.003$ for OTI and 0.0003 for OTII at 16 wk). (B) 2×10^4 DCs from mice fed EtOH for 4 or 16 weeks or control mice were pulsed with various concentrations of OVA peptide 258-264 or 323-339 and cultured with 2×10^5 T cells from OTI or OTII mice. T cell proliferation was measured as in (A). $N=3$ mice/group; error bars represent SD.

Figure 4.

Chronic EtOH feeding inhibits the ability of DC to promote Ag-specific T cell proliferation in response to OVA peptide *in vivo*. Splenocytes from OTI or OTII mice were labeled with CFSE and transferred into C57Bl/6 mice. Twenty-four hours later, DC from mice fed EtOH for 16 weeks or control mice were pulsed with 100 nM OVA peptide 258-264 or 323-339 and transferred into the same recipients. Four days following T cell transfer, residual CFSE labeling in splenic Tg CD8⁺ or CD4⁺ T cells was studied. (A) Representative CFSE staining on OTI T cells from an EtOH mouse. (Generation numbers correspond to number of times a cell has divided since CFSE labeling.) (B) Number of Tg T cells recovered in each generation. N=3 mice/group; error bars represent SD.

Figure 5.

Chronic EtOH feeding results in decreased upregulation of certain costimulatory molecules on DC following inflammatory stimulation *in vivo*. Costimulatory molecule expression was measured on DC from mice fed EtOH for 16 weeks or control mice, following *in vivo* treatment with CpG 1826, TNF α , or vehicle alone. Mean fluorescence intensity of costimulatory molecule expression on cDC and pDC at baseline or 24 h following stimulation. DC subsets were identified as in Figure 2B. N=4 mice/group; error bars represent SD.

Figure 6.

Chronic EtOH feeding results in decreased production of certain cytokines by DC relative to water controls following inflammatory stimulation *in vitro*. Enriched splenic DC preparations from mice fed EtOH for 4-16 weeks (changes consistent over this interval) or control mice were stimulated *in vitro* with CpG 1826 or TNF α in the presence of brefeldin followed by intracellular staining to identify cDC and pDC as well as cytokine production. (A) Representative identification of IL-12 p40 producing cDC (left column) and pDC (right column) in a control mouse. CD11c^{hi}Class II^{hi} cells were B220⁻, and CD11c⁺Class II⁺ cells were verified to be B220⁺ (data not shown). (B) Percentages of cDC and pDC populations from EtOH fed mice that produced the indicated cytokines without stimulation, or following stimulation with CpG 1826 or TNF α normalized to those from water controls (represented as 1). N=5 mice/group for IL-12p40, TNF α and IFN α , and N=8 for IL-6. Error bars represent SD.

Figure 7. Chronic EtOH feeding downregulates TLR9 and TNFR expression in/on DC. Splenic DC from mice fed EtOH for 16 weeks or control mice were permeabilized and stained to identify cDC and pDC (similar to Figure 2B), as well as TLR9, TLR4, TNFR I, and/or TNFR II expression. N=4 mice/group; error bars represent SD.