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Ethanol exhibits specificity in its effects on differentiation of hematopoietic progenitors¹

Hao Wang^{*,†}, Huijuan Zhou^{*}, Robert Chervenak^{*,§}, Kim M. Moscatello[‡], Lee Ellen Brunson^{*}, Deborah C. Chervenak[¶], and R. Michael Wolcott^{*,§,2}

^{*}Department of Microbiology and Immunology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

[†]Department of Emergency Medicine, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

[§]Center for Excellence in Cancer Research, and the Center for Excellence in Arthritis and Rheumatology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

[¶]The Research Core Facility, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

[‡]Department of Microbiology, Lake Erie College of Osteopathic Medicine Erie, PA 16509, USA

Abstract

Ethanol is a known teratogen but the mechanisms by which this simple compound affects fetal development remain unresolved. The goal of the current study was to determine the mechanism by which ethanol affects lymphoid differentiation using an in vitro model of ethanol exposure. Primitive hematopoietic oligoclonal neonatal progenitor cells (ONP), with the phenotype Lin[−]HSA^{lo}CD43^{lo}Sca-1[−]c-Kit⁺ that are present in neonatal but not adult bone marrow were sorted from the bone marrow of 2-week-old C57BL/6J mice and cultured under conditions that favor either B cell or myeloid cell differentiation with or without addition of ethanol. The overall growth of the ONP cells was not significantly affected by inclusion of up to 100mM ethanol in the culture medium. However, the differentiation of the progenitor cells along the B-cell pathway was significantly impaired by ethanol in a dose dependent manner. Exposure of ONP cells to 100mM ethanol resulted in greater than 95% inhibition of B cell differentiation. Conversely, ethanol concentrations up to and including 100mM had no significant effect on differentiation along the myeloid pathway. The effect of ethanol on transcription factor expression was consistent with the effects on differentiation. ONP cells grown in 100mM ethanol failed to up-regulate Pax5 and EBF, transcriptional regulators that are necessary for B cell development. However, ethanol had no significant effect on the up-regulation of PU.1, a transcription factor that, when expressed in high concentration, favors myeloid cell development. Taken together, these results suggest that ethanol has specificity in its effects on differentiation of hematopoietic progenitors.

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2Address correspondence and reprint request to Dr. R. Michael Wolcott, Department of Microbiology and Immunology, Louisiana State University Health Sciences Center in Shreveport, P.O.Box 33932, Shreveport, LA 71130-3932. Tel: (318)-675-5763, Fax: (318)-675-5764, Email address: E-mail: mwolco@lsuhsc.edu.

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Keywords

Rodent; B cells; Monocytes/macrophages; Cell differentiation; Hematopoiesis

INTRODUCTION

Chronic abuse of alcohol has a tremendous impact on human health and has been shown to be a risk factor for liver and breast cancer, liver cirrhosis, cardiovascular disease and hemorrhagic stroke, and has been associated with an increased incidence and severity of infections [1–3]. Alcohol abuse can also result in bone loss and in deficient bone repair [4]. These sequelae of alcohol abuse suggest that ethanol interferes with cellular differentiation and homeostasis. Fetal alcohol syndrome (FAS)³ provides irrefutable evidence that ethanol disrupts developmental processes and results in malformations during fetal development. A diagnosis of FAS is specified for those children who show fetal and neonatal growth retardation, a characteristic constellation of craniofacial malformations, abnormal brain and neural development, and developmental defects in major organ systems including cardiac and musculoskeletal systems [5–10]. Ethanol teratogenicity shows that this compound can interrupt regulatory processes that control normal patterns of differentiation; however, the broad spectrum of ethanol effects makes it difficult to determine its mechanism(s) of action.

The mechanisms of ethanol effects on normal cellular processes appear to vary depending on the tissue origin and in the case of fetal development on the timing of ethanol exposure. Developing brain tissue is particularly sensitive to ethanol and several mechanisms may be involved. One mechanism appears to involve increased apoptotic neurodegeneration [11] and other mechanisms involve the interference with expression of cell adhesion molecules and neuronal migration [12–15].

Previous studies from this laboratory suggest that in utero ethanol exposure can interfere with lymphopoiesis during fetal development and extending through neonatal life. In these studies we showed that neonatal animals that had been exposed to ethanol in utero had a profound decrease in B lymphocytes throughout the first few weeks of life [16,17]. B cell development occurs in discrete stages that are definable by cell morphology, molecular markers and differential expression of a number of cell surface molecules [18]. Analysis of the bone marrow of the ethanol exposed neonatal mice showed a decrease in all of the intermediates that have been ascribed to the B-lineage, suggesting that the effects of ethanol exposure were focused on progenitor cells prior to commitment to the B lineage. Recently we demonstrated that the differentiation potential of primitive hematopoietic oligoclonal neonatal progenitor cells, with the phenotype Lin[−]HSA^{lo}CD43^{lo}Sca-1[−]c-Kit⁺, were altered by in utero exposure to ethanol. Liquid cultures of these progenitor cells isolated from normal neonatal animals yield both myeloid and B lineage cells depending on the cytokines present in the culture medium. Further characterization of these progenitor cells has shown that the capacity to produce B lymphocytes is maximal when isolated from neonatal bone marrow and decreases sharply when isolated from animals after weaning. By 7 weeks of age the B cell potential has decreased by greater than 90%. Based on these characteristics we have termed this cell an oligoclonal-neonatal-progenitor (hereafter referred to as ONP³). Liquid cultures of ONP cells sorted from the bone marrow of neonatal mice exposed in utero to ethanol showed a significant decrease in the ability of these cells to differentiate along the B cell but not the myeloid pathway. Thus, the effects of ethanol on lymphoid development appear not to involve direct cytotoxicity but instead work specifically on progenitor cells, altering the fate of these developing cells. Although, the ability

³Abbreviations: FAS- fetal alcohol syndrome; ONP- oligoclonal-neonatalprogenitor

of ethanol to alter the differentiative potential of the ONP cells suggests some degree of specificity, the mechanism(s) governing this differential effect remains undefined.

A fundamental step in elucidating the mechanism by which ethanol alters the differentiation potential of ONP cells is to determine if ethanol works directly on these progenitor cells or alters the differentiation of some earlier hematopoietic precursor that gives rise to the ONP cells. As an approach to studying the mechanisms by which ethanol interferes with normal patterns of differentiation we have developed an in vitro model for studying ethanol effects on the differentiation of hematopoietic progenitors. Here we report that inclusion of ethanol in liquid cultures of ONP cells, sorted from the BM of normal neonatal animals, inhibits the differentiation to the B but not to the myeloid lineage, which is in agreement with the effects observed following in utero exposure to ethanol [17]. We further show that in vitro ethanol exposure affects the expression of transcriptional regulators (EBF, Pax5) and the IL-7R α in a manner consistent with the observed effects on differentiation.

MATERIALS AND METHODS

Mice

Six- to 8-wk-old male and female C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate for one week prior to experimental use. Mice were bred in house and two-week-old neonatal mice were used in all experimental protocols. All procedures utilizing animals have been reviewed and approved by the LSUHSC-S Animal Care and Use Committee.

Antibodies

The following monoclonal antibodies (mAbs) were purchased from either eBioScience (San Diego, CA) or BD-PharMingen (San Diego, CA): APC-conjugated anti-B220, PE-conjugated anti-B220 (Clone RA3-6B2), APC-conjugated anti-Gr-1 (Clone RB6-8C5), APC-conjugated anti-CD19 (Clone 6D5), APC-conjugated anti-CD3 (Clone 17A2), APC-conjugated anti-CD4 (Clone GK1.5), APC-conjugated anti-CD8 (Clone 53-6.7), APC-conjugated anti-Mac-1, FITC-conjugated anti-Mac-1 (Clone M1/70), APC-conjugated anti-Ter-119 (clone Ter-119), APC-conjugated anti-Sca-1 (Ly-6A/E, Clone D7), APC-Cy7-conjugated anti-CD117 (c-Kit, Clone 2B8), and FITC-conjugated anti-CD24 (HSA, clone M1/69). Two isotype controls were purchased from eBioScience: FITC-conjugated Rat IgG2b κ , control for rat IgG2b antibodies, and PE-conjugated Rat IgG2a κ , control for rat IgG2a antibodies. Two other isotype controls, APC-Cy7-conjugated Rat IgG2a κ and APC-conjugated Rat IgG 2b κ , were purchased from CalTag (Burlingame, CA). PE-conjugated anti-CD43 (Clone S7) and APC-Cy7-conjugated anti-CD19 (Clone 1D3) were purchased from PharMingen (San Diego, CA). Anti-Fc receptor mAb (anti-FcR, clone 2.4G2) was produced in house from cells purchased from American Type Culture Collection (Rockville, MD).

Cytokines

Recombinant mouse Stem Cell Factor (SCF), mouse Flt-3/Flk-2 Ligand (FL), mouse interleukin-3 (IL-3), mouse granulocyte macrophage colony stimulating factor (GM-CSF), and mouse interleukin-7 (IL-7) were purchased from R&D Systems (Minneapolis, MN).

Flow Cytometry and Cell Sorting Strategy

ONP cells were sorted from bone marrow of two week-old C57BL/6J mice as previously described [17]. Cell viability was measured by staining with Trypan Blue (Sigma) and cell samples with viability of more than 98% were used for cell sorting. Dead cells were excluded from analysis by forward angle and 90 degree light scatter gating. Analyses of cells were

performed on a FACS Vantage SE Turbo Sorter flow cytometer/sorter (Becton Dickinson, San Jose, CA) or a FACS Calibur cytometer (Becton Dickinson), made available through the University Research Core Facility at LSU Health Sciences Center (Shreveport, LA). A lineage cocktail was prepared from a mixture of APC-conjugated antibodies specific for Mac-1 (CD-11b), Gr-1, Ter-119, B220, CD19, CD3, CD4, CD8, and Sca-1.

Lin⁻HSA^{lo}CD43^{lo}Sca-1⁻c-Kit⁺ stained cells were sorted on FACS Vantage SE Turbo Sorter. After ONP cells were sorted, post-sort analysis was performed and showed the average of over 95% of purity (Figure 1). Cultured cells were stained using the appropriate mAbs and isotype-matched antibodies were used as negative controls. All samples were treated with an unlabeled anti-FcR to prevent inappropriate binding of antibodies. Data analysis was accomplished using FlowJo software (TreeStar, San Carlos, CA).

Cell Culture

A two-step culture strategy was used to grow the hematopoietic progenitors. Sorted cells were placed into 24 well culture plates (Costar, Cambridge, MA) containing complete IMDM supplemented with 10% fetal bovine serum, 10ng/ml recombinant mouse SCF, 10ng/ml recombinant mouse FL, and 10ng/ml recombinant mouse IL-3. After 3 days, cell cultures were washed and divided into two wells under two different culture conditions. Media in the first set of cultures contained SCF, FL, IL-3, and IL-7 (10ng/ml) to facilitate cell differentiation to B-lineage [19] and the second set of cultures contained SCF, FL, IL-3, and GM-CSF (10ng/ml) to facilitate cell differentiation along the myeloid pathway [20]. Cells were then grown for an additional 9 days and were fed every 2–3 days.

To determine the effects of ethanol on the growth and differentiation of the ONP cells, various concentrations of ethanol were added to the growth medium on Day 0. At the time of feeding, ethanol at the appropriate concentration was added to the culture medium used for feeding. Initial analyses were done to determine the stability of the ethanol concentration in the culture medium using a NAD-alcohol-dehydrogenase assay. The concentration of ethanol in the culture medium did not change significantly over a 14 day period (data not shown), thus, with periodic feeding (culture medium changed every 48 hours) the cells were exposed to a constant concentration of ethanol.

Cell Proliferation Assay

To measure the cell viability and proliferation in the presence of ethanol, ONP cells were sorted and cultured with varying concentrations of ethanol (0mM, 25mM, 50mM, 100mM, and 200mM). Cells were cultured as described above. During the culture, cells were harvested at the different time points and prepared for the proliferation assay using CellTiter 96 Aqueous Non-radioactive cell Proliferation Assay kit (Promega, Madison, WI).

Quantitative Real-time RT-PCR Analysis of Transcription Factors (Pax5, EBF, and PU.1), and the Cytokine Receptor

RNA was prepared from ONP cells using an RNA extraction kit (Qiagen Inc., Valencia, CA). cDNA was generated with the ThermoScript RT-PCR kit (GibcoBRL). Quantitative real-time PCR was performed using the Applied Biosystems Model 7700 sequence detection system (Foster City, CA). Primers and probes were designed using Primer Express Software (Applied Biosystems) and were as previously described [17]. mRNA for the transcription factors Pax5, EBF, and PU.1 and cytokine receptor IL-7R α were quantitatively measured using GAPDH as an internal control. The relative expression of EBF, Pax5, PU.1, and IL-7R α versus GAPDH in ONP cells was calculated by determining the Δ Ct value for each sample. The Δ Ct value = (threshold cycle for the transcriptional factor - the threshold cycle for GAPDH); i.e. the number of additional cycles at which the sample reached the threshold value after the GAPDH threshold

value. Therefore, the amount of input RNA for a given sample relative to GAPDH is given by $2^{-\Delta Ct}$.

Statistics

Each experiment was repeated at least three times and Student *t* test and ANOVA of mean values from each experiment were used in comparison of differences in cell populations between the ethanol and normal control groups and analyzed by a statistical software package (Instat, Graphpad Software, San Diego, CA). Differences between means were considered significant when $p < 0.05$.

RESULTS

100mM ethanol does not affect Lin⁻HSA^{lo}CD43^{lo}Sca-1⁻c-Kit⁺ cell proliferation

In order to establish an in vitro model that mimics the effects of ethanol noted in vivo on the ONP cells, an initial dose-response analysis was conducted to determine a concentration of ethanol that was not directly toxic and allowed normal growth of the cultured ONP cells. Bone marrow from 2 week-old mice served as the starting population for purification of the ONP cells. Figure 1 shows the sort strategy for isolation of the ONP cells.

The results of the dose-response analysis are shown in Figure 2A. ONP cells were grown in two-step liquid cultures (as described in Materials and Methods) in the presence of 0–200 mM ethanol. The cells were grown for 3 days in base medium and then GM-CSF was added to one set of cultures and IL-7 to the other set and the plates were incubated for an additional 9 days. After 12 days in culture, cells were harvested and cell proliferation assays were performed. The results show that concentrations of ethanol up to and including 100mM had no significant effect on the number of cells derived from either set of cultures. However, viable cell numbers were significantly decreased when the ethanol concentration was increased to 200mM. The above results show that 100mM ethanol in the cell culture medium had no significant effect on cell yield at the end of 12 days culture.

We next determined the kinetics of cell growth in medium containing 100mM ethanol. Cells were harvested at different time-points and cell proliferation was determined. The cells grown for 3 days with/without the presence of 100mM ethanol in base medium, then GM-CSF and IL-7 were added separately into the culture plates and cells were incubated for an additional 3, 6, and 9 days. The results in Figure 2B show that the viable cell numbers in samples harvested at different time-points during the culture had no statistically significant difference between the ethanol and normal control groups. These results indicated that there were no direct inhibitory effects of ethanol on cell proliferation during each stage of cell culture. For subsequent experiments a maximum concentration of 100 mM ethanol was used.

Ethanol alters the differentiation of ONP to the B- but not myeloid-lineage in a dose dependent manner

ONP cells were sorted and cultured in the presence of 0–100 mM ethanol with the addition of either IL-7 or GMCSF as described in Materials and Methods. The cells were grown for 12 days and were harvested and stained with antibodies specific for B220, CD11b, and CD19. B cell differentiation was determined by analyzing CD11b⁻B220⁺CD19⁺ cells, while myeloid differentiation was determined by analyzing CD11b⁺B220⁻ cells. Figure 3A shows the dose response effect of ethanol on the phenotype of ONP cells grown in cultures supplemented with IL-7 (conditions favoring differentiation to the B lineage). Figure 3B shows the mean expression of CD19⁺ cells in three separate experiments. Inclusion of ethanol in the growth medium significantly inhibited the differentiation of ONP cells to the B lineage. The percentage of cells that were CD19⁺ was significantly decreased by both 50 and 100 mM ethanol. The

level of CD19⁺ cells decreased by approximately 50 % in the cultures containing 50mM ethanol and 100 mM ethanol resulted in greater than 95% reduction in the cells that committed to the B lineage. These data indicate that exposure of ONP cells to 100mM ethanol results in greater than 95% inhibition of B lineage differentiation. In cultures supplemented with GM-CSF, approximately 80–85% of control cells stained positive for CD11b (Figures 4A and 4B), an indication of myeloid differentiation. Addition of ethanol up to 100mM had no significant effect on the percentage of CD11b⁺ cells (Fig 4B). These data indicate that in vitro ethanol exposure impairs the ability of ONP cells to commit to the B lineage without affecting their ability to differentiate along the myeloid pathway.

From the data reported here we conclude that ethanol shows a remarkable degree of specificity in affecting the cell fate decisions of the ONP cells. We base the conclusion on the observation that ethanol up to and including 100mM had no significant effect on the proliferation of the ONP cells regardless of the culture conditions. Furthermore, ethanol up to and including 100mM had no significant effect on the differentiation of the ONP cells to the myeloid lineage. However, the effects of ethanol on differentiation to the B lineage were profound.

Ethanol inhibits the expression of transcription factors EBF and Pax5 genes but not PU.1 in ONP cells grown in liquid cultures containing IL-7

Specificity of a B cell fate of progenitor cells is regulated by the sequential expression of transcription factors and the coordinate signaling through cytokine receptors. The expression of the transcription factors EBF, Pax5 and cytokine receptor IL-7R α are among the essential steps in the differentiation of progenitor cells to the B lineage. An important function of EBF is to directly bind and activate the Pax5 promoter, suggesting a role in regulating the expression of Pax5, the expression of which is necessary for the irrevocable commitment of progenitor to the B cell lineage [21–23]. Therefore, we determined the effects of 100mM ethanol on the expression of these transcriptional regulators. At selected time-points, cells were harvested from the liquid cultures and analyzed for changes in the expression of specific mRNAs using quantitative-real-time RT-PCR. The data in Figure 5 show that the level of expression of Pax5 and EBF mRNA in ONP cells exposed to ethanol were significantly less than the level of expression in the cells not exposed to ethanol. It is also important to note that inclusion of ethanol in the growth medium blocked the expression of Pax5 by the ONP cells. The expression of PU.1 mRNA was not inhibited by ethanol. In fact, the results indicate a lower level of PU.1 expression in the normal control group compared to the ethanol exposed cells. These results are consistent with a model in which more cells enter the myeloid pathway at the expense of cells entering the B lineage pathway (Figure 3A).

ONP cells grown in 100mmol ethanol failed to up-regulate IL-7R α expression when cultured in the presence of IL-7

In the hierarchy of transcription factor expression, signaling through the IL-7R α has been shown to play a critical role. A greatly decreased B cell generation was found in the bone marrow of IL-7^{-/-} mice and IL-7^{-/-} CLPs also expressed considerably lower levels of EBF and Pax5 transcripts when compared with wild type CLPs [24,25]. PU.1 is expressed in the absence of IL-7R α signaling but EBF and subsequently Pax5 require a signal from IL-7. Therefore, we determine the effects of ethanol on the expression of the IL-7R α mRNA. ONP cells grown in the presence of IL-7 proliferated and upregulated the expression of IL-7R α (Figure 6). However, inclusion of 100mM ethanol in the cultures prevented the upregulation of IL-7R α . These data suggest that ethanol impairs B cell differentiation by preventing the upregulation of IL-7R α and subsequently EBF and Pax5.

DISCUSSION

Hematopoiesis is a complicated process that involves gene programming in the progenitor cells and the intimate interaction of the developing cells with stroma of the bone marrow. The developing cells interact with the bone marrow microenvironment via adhesion molecules and provide growth factors and cytokines that nurture the progenitor and progeny cells, and chemokines that shepherd them through their bone marrow migration. The ultimate cell fate of the developing cells is dictated by all of these interacting factors. Understanding the mechanism by which ethanol alters lymphopoiesis is correspondingly complicated. Ethanol could affect the progenitor cells directly, or act indirectly by altering the microenvironment.

The experiments reported here focused on the effects of ethanol on the differentiation of oligopotential-neonatal-progenitor (ONP) cells in the absence of the contribution of the stromal environment of the bone marrow. As stated above, ONP cells are hematopoietic progenitors that have the capacity in vitro to differentiate along myeloid or lymphoid pathways, depending on the growth factors present in the culture medium. IL-7 was added to promote differentiation along the B cell pathway and GM-CSF was used to promote myeloid commitment. In previous studies [17,26] we reported that ONP cells isolated from pups born of ethanol fed dams showed a significant decrease in the ability to differentiate along the B-lineage while maintaining normal potential to differentiate along the myeloid lineage. Since the ONP cells were purified from bone marrow after exposure to ethanol the question remained as to whether ethanol had a direct effect on the ONP cells.

In the present study we added ethanol to liquid cultures of purified ONP cells in the absence of stromal cell feeder layers to determine if ethanol directly affected the ONP cell's pattern of differentiation. It was necessary to carry out these studies in the absence of stromal cell feeder layers since we have observed that ethanol can effect the growth promoting properties of the stromal cells (unpublished results).

The concentrations of ethanol, up to 100mM (0.46%), used here are blood alcohol levels that can be achieved in mice fed ethanol [27,28]. Furthermore, blood alcohol levels of 0.46% can be lethal to unadapted humans but these and higher concentrations are often seen in emergency rooms [29]. In our study, concentration of ethanol up to and including 100mM had no significant effect on the growth of the ONP cells in conditions that favored either myeloid or B lineage differentiation.

The addition of ethanol to the cultures of ONP cells supplemented with IL-7 affected the differentiation to the B lineage in a dose dependent manner in the range of 25mM to 100mM. In cultures without ethanol there was a significant upregulation of the IL-7R α as well as the expression of the EBF and Pax-5 transcription factors, expected events for cells committing to the B lineage. Addition of ethanol to the liquid cultures prevented the upregulation of IL-7R α , EBF, and Pax-5, which is consistent with the failure to commit to the B lineage. PU.1 is a transcription factor that is required for the differentiation of hematopoietic progenitors to both the myeloid and lymphoid lineages. Inclusion of ethanol in the growth medium did not prevent the expression of PU.1. Furthermore, ethanol had no significant effect on the growth or the myeloid differentiation of ONP cells in cultures supplemented with GM-CSF. In the hierarchy of transcription factor expression, PU.1 is one of the upstream of IL-7R α and the effect of IL-7R α can subsequently after the regulation of EBF, and Pax5 [23,30,31]. From these results it appears that the effects of ethanol on the differentiation of ONP cells occurs at the decision point, downstream of PU.1 and upstream of the IL-7R α , specifying the cell fate decision of myeloid versus lymphoid commitment. Taken together these results suggest that ethanol shows specificity in its effects on the differentiation of ONP cells. However, the mechanism of this specificity warrants additional study.

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Figure 1. Sort strategy for isolation of the Lin⁻HSA^{lo}CD43^{lo}c-Kit⁺ (ONP) cells from bone marrow of 14-day-old mice

The lineage cocktail was prepared from APC labeled antibodies specific for B220, Mac-1 (CD 11b), Gr-1, CD19, CD3, CD4, CD8, Ter119, and Sca-1. Lineage⁻ cells (panel A) were analyzed for CD43, HSA, and sub-gated Lin⁻HSA^{lo}CD43^{lo} as indicated by the box in Panel B. The Lin⁻HSA^{lo}CD43^{lo} cells were finally analyzed c-kit expression. The c-kit⁺ cells, gated as shown in Panel C, were sorted. Post-sort analysis (Figure 1D) showed more than 95% purity. Using this sorting strategy, 20,000 to 30,000 ONP cells per mouse were typically obtained. Data are representative of more than 10 separate experiments.

Figure 2. The growth of $\text{Lin}^- \text{HSA}^{\text{lo}} \text{CD43}^{\text{lo}} \text{Sca-1}^- \text{c-Kit}^+$ cells for 12 days in the presence of different concentration of EtOH

ONP cells were sorted from the marrow of two week old mice and grown using the two step culture conditions described in Materials and Methods. Panel A: The sorted cells were cultured under conditions that favored either B-lineage commitment or myeloid commitment in the presence of 0–200 mM ethanol. After 12 days the number of cells in each culture was measured using a non-radioactive cell proliferation assay. Data are presented as the mean of three separate experiments $\pm$ the standard deviation. Panel B: The effects of 100 mM ethanol on the rate of cell proliferation were determined. Samples were taken every 3 days for 12 days from cultures containing 100 mM ethanol or medium only as the control and cell number was assessed. The results are presented as the expression of cells from the ethanol containing cultures relative to the control cultures. Data are the mean of three separate experiments $\pm$ the standard deviation.

Figure 3. Dose-response effect of ethanol on the differentiation of Lin⁻HSA^{lo}CD43^{lo}Sca-1⁻c-Kit⁺ (ONP) cells to the B-lineage

ONP cells were sorted from the bone marrow of 2 week old mice and grown in two-step cultures as described in Materials and Methods. Ethanol was added at day 0 of culture and IL-7 was added after three days. Nine days later the cells were harvested and stained with APC-anti-B220, PerCp-Cy5.5-anti-Mac-1(CD11b), PE-anti-CD19, and APC-CY7-anti-ckit and the phenotype was determined. A: Histograms showing the dose response effect of ethanol on the differentiation of ONP cells to the B-lineage. Cells were gated on B220⁺CD11b⁻ cell and analyzed for CD19 expression to confirm B-lineage (right-hand histograms in 3A). B: Mean expression of CD19 in three separate experiments. *p<0.04, **p<0.001

Figure 4. Dose-response effect of ethanol on the differentiation of Lin⁻HSA^{lo}CD43^{lo}Sca-1⁻c-Kit⁺ (ONP) cells to the myeloid lineage

ONP cells were sorted, grown in culture, and analyzed as described in the legend to Figure 3 except that GM-CSF was substituted for IL-7. A. Histograms showing the dose response effect of ethanol on the differentiation of ONP cells to the myeloid lineage. B: Mean expression of CD11b positive cells in three separate experiments.

Figure 5. Effects of ethanol on the expression of transcription factors by $\text{Lin}^{-}\text{HSA}^{\text{lo}}\text{CD43}^{\text{lo}}\text{Sca-1}^{-}\text{c-Kit}^{+}$ (ONP) cells

ONP cells were sorted from the bone marrow of 2 week old mice and grown in two-step cultures as described in Materials and Methods. Ethanol was added at day 0 of culture and IL-7 was added after three days. Nine days later the cells were harvested, and the RNA was prepared and analyzed by quantitative real time RT-PCR for expression of EBF, Pax5 and PU.1. The results are expressed relative to GAPDH. Relative expression of transcriptional factor = $\log_2$ (the amount of RNA for a given group relative to GAPDH/ the amount of RNA for the normal control group relative to GAPDH) X 100%. n.d. = not detected.

Figure 6. Effects of in vitro ethanol exposure on the expression of IL-7R α by Lin⁻HSA^{lo}CD43^{lo}Sca-1⁻c-Kit⁺ cells

Conditions were as described in Figure 5 and the RNA was analyzed for the relative expression of the IL-7R mRNA.