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Effects of ethanol on cytokine generation and NFκB activity in human lung epithelial cell

Anne-Sofie M. Johansson [a,*], Johan Lidén [b], Sam Okret [c], Jan E.W. Palmblad [a]

[a] *Center for Inflammation and Hematology Research at Department of Medicine, CIHF pl 7 KFC NOVUM, Huddinge University Hospital, S-141 86 Huddinge, Sweden*
[b] *Bioinformatics Expression Analyzis at Department of Biosciences, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden*
[c] *Department of Medical Nutrition, Karolinska Institutet at Karolinska University Hospital Huddinge, Stockholm, Sweden*

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Abstract

Alcohol abuse is associated with enhanced risk for pulmonary infections, but the mechanisms remain obscure. We assessed whether ethanol reduced generation of cytokines from a human lung epithelial cell line (A549) in vitro and if effects on the NFκB transcription factor were involved. Exposure of A549 to ethanol (0.1–1%) dose-dependently inhibited (by 15–49%) the release of G-CSF and IL-8, but not of M-CSF, triggered by IL1β or TNFα. Ethanol also inhibited by 49% the IL-1β stimulated translocation of the p65 subunit of NFκB from the cytoplasm into the nucleus. Using a κB binding and luciferase coupled construct, transfected into A549 cells, we found that 1% ethanol specifically reduced IL-1β and TNFα induced luciferase activity with 34 and 40%, respectively. Thus, in vitro exposure of lung epithelial cells to ethanol reduced the generation of cytokines, as well as translocation and gene activation by NFκB.

1. Introduction

Ethanol abuse is associated with pulmonary infections, mainly pneumococcal and *Klebsiella pneumoniae* [1–4]. This has been shown not only after chronic alcohol intake but also after an acute alcohol intoxication [5–10]. The mechanisms for this impairment of host defense has traditionally been attributed to effects of ethanol on phagocytic cells, e.g. reduced neutrophil (PMN) adherence, chemotaxis, generation of superoxide ions and bactericidal functions [6,9,11–14]. These reactions have been linked to the ability of ethanol to impair generation of phosphatidic acid and of calcium ion fluxes [15,16] or interaction with signal coupling systems and generation of cytokines in phagocytes [17–21]. Recently, we reported that ethanol also reduced the generation of myeloid growth factors in human endothelial cell and their interactions with neutrophils [22].

The pulmonary epithelial cells represent an important part of local host defense. They are pivotal for emigration of leukocytes into the alveolear and bronchial tissues by means of adhesion and activation molecules. They also represent an important source of various myeloid growth factors and cyto/chemokines (e.g. G-, GM- and M-CSF and IL-8, etc.), which are released upon stimulation with IL-1 and other agonists [23]. Lack of these growth factors, cyto- and chemokines confer undue susceptibility to infectious agents [24–26].

Against this background we hypothesized that lung epithelial cells might be affected by ethanol. Apart from reductions in bronchial ciliary motility, secretion of some chemokines and impaired barrier functions [27–29], very little is known about ethanol and functional responses of epithelial cells. Based on findings in endothelial cells [22], we sought to determine if an acute exposure to ethanol would attenuate generation of cytokines and other recruiting systems for inflammatory and immune cells, focusing on G-CSF and IL-8. This choice was also based on suggestions that administration of G- (or GM-) CSF might ameliorate alcohol related infections [30,31].

Here, we exposed a human epithelial cell line, A549, to ethanol in vitro. This resulted in reduced release of G-CSF and IL-8. Furthermore, we found that ethanol interfered with an important signal transduction step, the translocation

* Corresponding author. Tel.: +46 8 5858 3986; fax: +46 8 6897 730.
E-mail address: anne-sofie.johansson@crc.ki.se (A.S.M. Johansson).

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from the cytosol to the nucleus of the p65 components of the NFκB complex (important for activation of many genes controlling pro-inflammatory systems including G-CSF generation). Comparisons were made with the synthetic glucocorticoid dexamethasone, a well-defined inhibitor of NFκB activity [32].

2. Material and methods

2.1. Materials

Chemicals and antibodies were obtained as follows: TNFα (Sigma Chemical); IL-1β (Boehringer Mannheim); fetal bovine serum, HEPES, penicillin, streptomycin, Dulbecco's modified Eagle's medium, Ham's F-12 medium, PBS, Lipofectamine™ and Hanks' balanced salt solution (HBSS; Life Technologies), ethanol (Kemetyl), acetone, and methanol (Apoteksbolaget), polystyrene plates (96 and 6-well) and other tissue culture plastic material (TPP). All other chemicals were obtained from Sigma.

We used rabbit polyclonal antibodies to human NFκB subunits p65 from Calbiochem (La Jolla, CA), biotinylated goat antibodies to rabbit IgG from Vector Laboratories (Burlingame, CA).

The here chosen ethanol (EtOH) concentrations (0.1–1 vol./vol.%, corresponding to 0.79–7.9 mg ethanol/ml or 17–170 mM) were based on our previous and others' considerations and to enable such comparisons [12,16,21,22,33–36].

2.2. Cell culture and transfections

The human lung adenocarcinoma cell line A549 was a kind gift from Dr. Magnus Nord at Karolinska Institutet and cultured in 1:1 mixture of Dulbecco's modefied Eagle's medium and Ham's F-12 medium supplemented with 10% fetal bovine serum and 1% (v/v) penicillin/streptomycin. All cells were grown in humidified incubator at 37 °C and 5% CO_2. In some experiments A549 cells were transfected with reporter genes (see below) using Lipofectamine™ reagent (Life Technologies Inc.) according to the recommendation of the manufacturer. In each experiment, six 35 mm wells containing 8×10^5 cells were transfected with 2 μg reporter plasmid, as described in [32]. A549 cells were incubated for 24 h with indicated agonist alone or together with 0.1–1% ethanol or 1 μM dexamethasone, added 10 min prior to the agonists. The viability of the A549 cells was >95% after incubation with 1% ethanol and IL-1β for 24 h, as measured by trypan blue exclusion.

2.3. Measurement of cytokine production by A549 cells

After incubation of A549 cells with 0.1–1% ethanol or 1 μM dexamethasone and various agonists for 24 h (a time point found to be optimal based on our previous findings), culture supernatants were harvested. The concentrations of cytokines were determined by use of Quantikine assays (R&D Systems). At the concentrations used in the present study, ethanol had no effect on the cytokine assays. We have previously reported that >90% of G- and M-CSF and IL-8 were secreted to the extracellular space [22].

2.4. Immunostaining of NFκB

The staining of NFκB was based on a method described elsewhere [22]. A549 cells, grown on glass cover slips to confluence, were incubated with ethanol or dexamethasone and stimulated with indicated agonist for 1 h, fixed in methanol, permeabilized with acetone and exposed to 3% H_2O_2. The cells were incubated with 1.5% normal goat serum for 20 min and then with antibodies to NFκB subunit p65. Immune complexes were detected with a Vectastain ABC kit and DAB substrate for peroxidase (Vector). The cells were examined with an Olympus microscope, and micrographs were scanned with a Jandel SigmaScan Pro™ instrument for densitometric assessment of the ratio of staining between nucleus and cytoplasm. We analyzed ≥5 cells on each micrograph, i.e. a total of >40 cells.

2.5. Reporter plasmids and expression vector

The plasmid 3×NF-κB(IC)tk-LUC [32] contains three copies of the NF-κB binding site from the human intercellular adhesion molecule-1 promoter in front of a minimal thymidine kinase promoter (−105 to + 52) fused to the luciferase (LUC) reporter gene. The RSV-LUC reporter drives LUC expression under the control of the Rous sarcoma virus promoter [32].

2.6. Luciferace assay

For measurement of LUC activity, 100 μL cell extract per sample was used together with the Geneglow™ kit from Bio-Orbit (Turku, Finland). Luciferace and ATP were added to the cellular extracts after transfection and production of light was measured by luminometer, Lucy 1 from Anthos Labtec Instruments (Salzburg, Austria).

2.7. Statistical analyses

Data are presented as mean ± S.E.M. for the indicated number of separate (>5) experiments. Each experiment was done at least in duplicate and each assay at least in triplicate. Differences between the groups were assessed by analysis of variance (ANOVA). When the ANOVA test was significant, a Dunn test was performed between the groups. All statistical analysis was performed by STATISTICA (data analysis software system), version 7. www.statsoft.com.

3. Results

3.1. Cytokine production

Quiescent A549 cells secreted only relative small amounts of G-, M-CSF and IL-8 (3 ± 1, 268 ± 57 and 2840 ± 730 pg/ml, respectively, $n = 4–10$) into the culture supernatant. Exposure of cells to 0.01–1% ethanol alone did not affect the release of these cytokines (data not shown).

3.1.1. G-CSF secretion

It has previously been reported that human umbilical vein endothelial cells reacted with substantial G-CSF production when stimulated with LPS and IL-1β, but that TNF was a poor agonist [22,37]. Now, when A549 cells were stimulated to produce G-CSF, 5 U IL-1β/ml caused a massive, i.e. a 290-fold, increment of G-CSF secretion (870 ± 113 pg/ml, Fig. 1A). 200 U TNFα/ml induced considerably lower G-CSF secretion: a 36-fold increase of G-CSF release into the supernatants was observed after 24 h of incubation of A549 cells (109 ± 38; Fig. 1B).

Exposure of A549 cells to ethanol before incubation with IL-1β resulted in a dose-dependent inhibition of stimulated G-CSF release, where the most pronounced inhibition was noted for 1% ethanol (being 48% of controls). Also, 0.5% ethanol induced a reduction in all experiments, with 35% (Fig. 1A). In contrast, the observed TNFα-induced G-CSF release was not consistently inhibited by ethanol (Fig. 1B).

Incubation of A549 cells with 1 μM dexamethasone together with IL-1β or TNFα resulted in an enhanced G-CSF release (1655 ± 129 and 438 ± 225 pg/ml, respectively) (Fig. 1A and B); this agrees with previous reports [38,39] that dexamethasone and IL-1β or TNFα-treatments potently synergize to stimulate the production of G-CSF in differentiated THP-1 cells or in BEAS-2B cells.

3.1.2. M-CSF secretion

M-CSF secretion was induced 2.5-fold by IL-1β and 2.3-fold by TNFα (662 ± 138 and 610 ± 180 pg/ml, respectively). Ethanol caused no reductions of these responses (Fig. 2A and B). However, 1 μM dexamethasone conferred a 45% reduction following IL-1β activation (Fig. 2A).

3.1.3. IL-8 secretion

IL-1β as well as TNFα (at the same concentrations as used above) caused an 8.9- and a 12-fold increase of the release of this chemokine (24.9 ± 4.7 and 33.8 ± 8.9 ng/ml, respectively) (Fig. 3A and B). One percent ethanol impaired the IL-1β-stimulated IL-8 secretion with 53% and inhibited TNFα-induced IL-8 release by 59% (Fig. 3). As previously observed in HUVEC [22], 0.5% ethanol induced a 28% decrease of IL-8 release in IL-1α-stimulated and in TNFα-stimulated A549 cells (Fig. 3B).

Dexamethasone (1 μM) caused a 49% and a 59% reduction for IL-1β and TNFα activation responses, respectively (Fig. 3).

3.2. NFκB activity

Previous experiments on HUVEC [22] suggested that ethanol modulated secretion of cytokines that are dependent of NFκB activation for gene expression (e.g. G-CSF and IL-8, but not M-CSF) [40,41] and that NFκB translocation from the cytoplasm into the nucleus was hampered by ethanol. Given the present results of how ethanol affected cytokine secretion in A549 cells, we asked whether EtOH influenced the transcriptional activity of NFκB. For this purpose, we used a luciferase reporter gene under the control of three defined NFκB binding sites. When A549 cells were treated with IL-1β or TNFα for 24 h we observed that both agonists caused activation of the gene. The IL-1β as well as the TNFα responses mounted to 18- and 24-fold increases of luc-units, respectively. When A549 cells had been exposed to ethanol prior to the agonist, these responses were reduced in a dose dependent way (Fig. 4). Maximal inhibition (40%) was apparent at an ethanol concentration of 1%; significant inhibitory effects (15 and 28%) were also apparent at 0.1 and 0.5% ethanol, respectively. In agreement, the observed

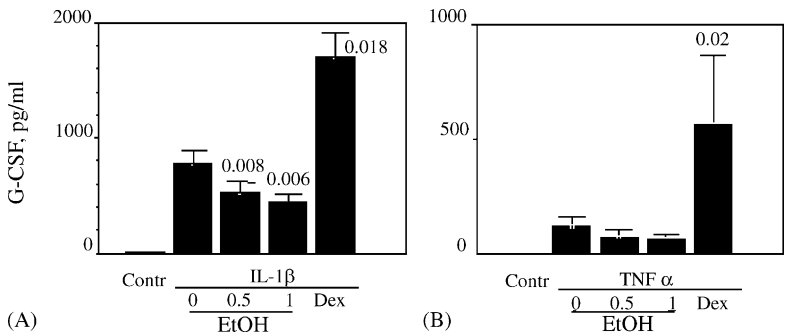

Fig. 1. Effect of ethanol or dexamethasone on G-CSF secretion by A549 cells, induced by IL-1β (panel A) or TNFα (panel B). Cells were treated with 0, 0.5 or 1% ethanol or 1 μM dexamethasone (as indicated below the *x*-axis) and then stimulated with 5 U IL-1β/ml or 200 U TNFα/ml (in continued absence or presence of ethanol or dexamethasone). After 24 h of subsequent incubation, supernatants were analyzed for G-CSF. Data are mean ± S.E.M. ($n = 5–10$). Figure above columns are *P*-values for comparison with cells stimulated with agonists only.

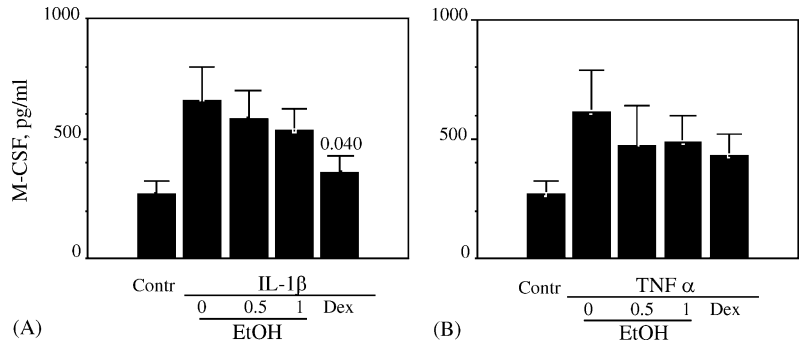

Fig. 2. Effect of ethanol or dexamethasone on M-CSF secretion by A549 cells, induced by IL-1β (panel A) or TNFα (panel B). Cells were treated with 0, 0.5 or 1% ethanol or 1 μM dexamethasone (as indicated below the *x*-axis) and then stimulated with 5 U IL-1β/ml or 200 U TNFα/ml (in continued absence or presence of ethanol or dexamethasone). After 24 h of subsequent incubation, the concentration of M-CSF in the culture supernatants was then measured. Data are mean ± S.E.M. ($n = 4$). Figures above columns are *P*-values for comparisons with cells stimulated with agonists only.

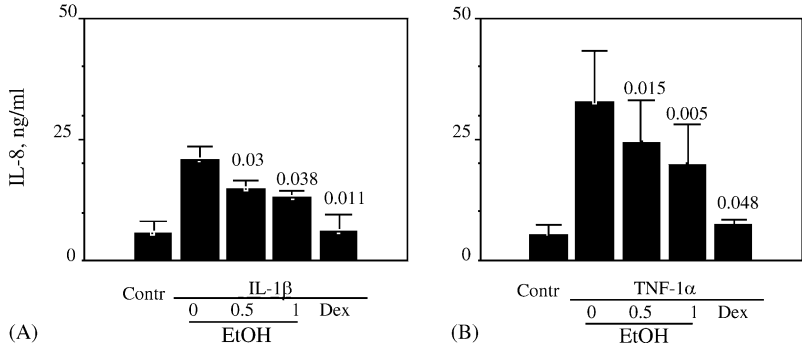

Fig. 3. Effect of ethanol or dexamethasone on IL-8 secretion by A549 cells, induced by IL-1β (panel A) or TNFα (panel B) Cells were treated with 0, 0.5 or 1% ethanol or 1 μM dexamethasone (as indicated below the *x*-axis) and then stimulated with 5 U IL-1β/ml or 200 U TNFα/ml (in continued absence or presence of ethanol or dexamethasone). After 24 h of subsequent incubation, supernatants were analyzed for IL-8. The data are mean ± S.E.M. ($n = 6$-8). Figures above columns are *P*-values for comparisons with cells stimulated with agonists only.

luc-units response to IL-1β was reduced with 15 by 0.5% ethanol and with 33 by 1% ethanol.

Dexamethasone (1 μM) inhibited the IL-1β and the TNFα induced luciferase activity by 40 and 37%, respectively (Fig. 4), which is in line with results obtained by others [32,42,43].

Since ethanol exerted inhibitory effects on the reporter gene activity, it might be argued that this can reflect unspecific effects of the alcohol. We, then, sought to resolve this possibility by using a constitutively activated reporter gene system, the RSV-LUC construct. When 1% ethanol was added to this system no change in the luciferase activity was observed after 24 h, suggesting that ethanol did not exert a general negative effect on transcriptional reporter gene activity (18.3 ± 1.2 and 17.7 ± 3.1, respectively).

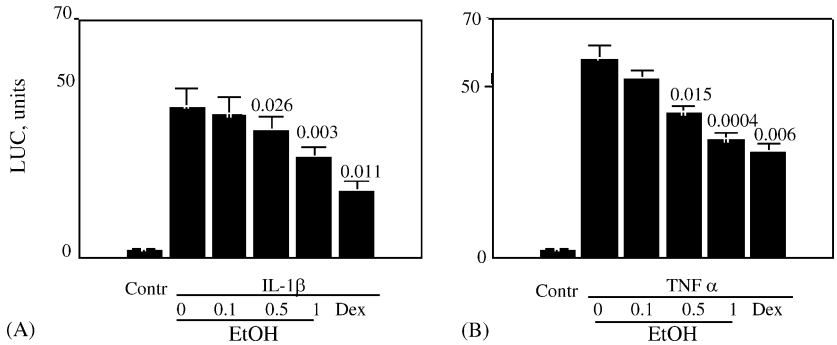

Fig. 4. The effect of ethanol or dexamethasone on the activity of A549 cells transiently transfected with 3×NFκB(IC)tk-LUC. Cells were treated with 0, 0.1, 0.5 or 1% ethanol or 1 μM dexamethasone (as indicated below the *x*-axis) and then stimulated for 24 h (in continued absence or presence of ethanol or dexamethasone) with 5 U IL-1β/ml (panel A) or 200 U TNFα/ml (panel B). Results are presented as mean ± S.E.M. of luciferase activity in cell lysates ($n = 16$). Figures above columns are *P*-values for comparisons with cells stimulated with IL-1β or TNFα, respectively.

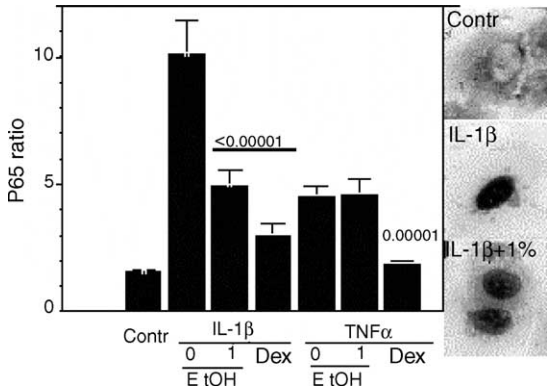

Fig. 5. Densitometric analysis of p65 relocation in A549 cells. Cells were incubated with 1% ethanol or 1 μM dexamethasone and then for 1 h (in continued absence or presence of ethanol or dexamethasone) with 5 U IL-1β/ml or 200 U TNFα/ml, after which they were subjected to immunostaining with antibodies to p65 and densitometric analysis. The nuclear/cytoplasmic ratio of p65 staining was determined in a total of 32 individual cells for each condition. Data are mean ± S.E.M. The indicated *P*-values (above the bars) are for comparisons with cells exposed to agonist only. The insert shows micrographs of representative cells treated with medium alone (top), IL-1β (middle) or IL-1β and 1% ethanol (bottom).

Next, we compared the ratios of cytoplasm to nuclei staining for the endogenous NFκB component p65 in IL-1β or TNFα-stimulated A549 cells and analyzed whether treatment with ethanol or dexamethasone changed the ratios. The results, given in Fig. 5, show that IL-1β and TNFα caused a six- and three-fold relocation of p65 from the cytosol to the nucleus after 1 h, respectively, and that treatment with 1% ethanol impaired this translocation with 52% when IL-1β was used as stimuli. In contrast, ethanol did not affect translocation induced by TNFα. IL-1β and TNFα-induced relocation of p65 was inhibited by dexamethasone with 70 and 59%, respectively.

4. Discussion

It is obvious that epithelial cells are not a passive barrier, but plays a highly active role in the inflammatory process by means of production of various mediators (such as leukotrienes, cytokines/chemokines) and expression of adhesion molecules. Thus, G-CSF and IL-8, present systemically or locally, are not only stimuli of neutrophil delivery systems from the bone marrow, of functional responses of mature cells, but they also promote emigration of inflammatory cells to the alveolar and bronchial tissues. Neutrophils and monocyte interaction with epithelial cells may partake in an amplification process, where IL-8 and other cytokines are generated, which in turn recruit more inflammatory cells, prolong their life-span and in the maturation of monocytes into macrophages [44,45].

Apart from reductions in bronchial ciliary motility, a few chemokines and barrier functions, little has been published about effects of ethanol on lung epithelial cells [27–29]. This paucity of reports is in contrast to the vast interest over the years in effects of ethanol on gastrointestinal epithelial cells and inflammatory cells. Our paper points to novel immunosuppressive aspects of ethanol.

M-CSF production was not as susceptible to ethanol treatment, as was G-CSF and IL-8. This discrepancy was also observed in endothelial cells [22] and points to different signal transduction systems for IL-1β and TNFα for these myeloid growth factors; one that is affected by ethanol and essential for G-CSF and IL-8 generation, and one that is less sensitive to ethanol and employed for M-CSF generation. Since NFκB is a (but most probably not the only) transcription factor for G-CSF and IL-8 genes and other data suggests that TNFα induced M-CSF secretion is not or only partly dependent on NFκB activation [40,41], we sought to examine the NFκB pathway in relation to ethanol. It should be observed that TNFα is a poor agonist of G-CSF generation (Fig. 1B) [37] so that effects of EtOH cannot be clearly discerned and discussed further.

Our study showed an increase in the NFκB p65 components in the nucleus after stimulation with IL-1β and a decrease in this translocation after treatment with ethanol. This is in agreement with studies on monocytes/macrophages and endothelial cells where ethanol also inhibited translocation of NFκB subunits to the nucleus and subsequent cytokine production [19,20,22,46]. These findings might be relevant for the dependence of the myeloid growth factors for the NFκB pathway for production. Also, the data generated with the NFκB dependent-reporter gene system suggests that κB-dependent transcription process was impaired by ethanol, which was to be expected if NFκB translocation is reduced. One alternative, i.e. that ethanol negatively interacts with the binding of transcription factors to the promotor region or with luciferase signal, is less likely since ethanol did not affect the constitutively active RSV promoter controlled reporter gene system. However, at this time it is not known if ethanol directly interacts with the IκB/NFκB complex or with proximal or distal signaling components, making less p65 available for binding to the κB site.

Although peripheral venous blood ethanol concentrations exceeding 0.5% ethanol only rarely are seen in clinical and legal practice, it is very probable that regional epithelial (and endothelial) cells encounter much higher ethanol concentrations. For example, during ingestion of strong liquor (e.g. week-long bouts in alcoholics), oral, esophageal, gastric and enteral epithelial (and mucosal endothelial) cells are exposed to 20–50% ethanol. Likewise, the ethanol concentration in portal blood vessels, prior to metabolism of hepatic alcohol dehydrogenases, is likely to be manifold higher than in any conventional peripheral blood tests for drunk driving.

Thus, this study shows that ethanol impairs various parts of the first line of host defense to inflammatory stimuli, represented by the reactivity of epithelial cells. The findings of reduced secretion of G-CSF and IL-8 by ethanol may partly explain why alcohol abuse is associated with a

higher incidence and worse outcome of bacterial diseases [1,3,16,19–22], particularly in the respiratory system. Also, local generation of dendritic cells of significance for the antigen presenting might be impeded by reduced G-CSF. Although ethanol conferred reductions of G- and IL-8 was modest, and only observed at high in vitro concentrations of ethanol, the accumulated effect on the two (or more) myeloid growth factors might be of clinical significance.

The reduced G-CSF production from one of the major myeloid growth factor-producing cells in lung, the epithelial cell, might also be relevant for findings of beneficial effects of exogenous G-CSF. Reports have shown that administration of G-CSF to ethanol intoxicated animals led to reduced mortality in LPS induced inflammation or bacterial infections [30] and GM-CSF administration ameliorated epithelial injury in ethanol-treated rats [31].

Acknowledgments

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