

## Article

# Reduction of Cell Proliferation by Acute C<sub>2</sub>H<sub>6</sub>O Exposure

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**Simple Summary:** Alcoholic beverages and acetaldehyde formed during their metabolism are carcinogenic to humans. Alcohol drinking may affect bone marrow stem cell niche, suppressing physiological hematopoiesis and ultimately reducing the organism's capacity to fight against cancer, infections, and to promote tissue regeneration. To elucidate in vivo the cellular mechanisms associated with alcohol intake toxicity, we used a mouse model in which proliferating cells produce the firefly's light-emitting protein. In this animal, alcohol exposure transiently "turns off the light", indicating a negative effect on cell proliferation in the bone marrow and spleen. Pharmacological treatment with substances interfering with ethanol metabolism, reducing acetaldehyde production, partially restores the physiological cell proliferation rate. Over 560 million people worldwide have increased susceptibility to acetaldehyde toxicity and 4% of cancer deaths are attributable to alcohol. Our model might provide a suitable tool to further investigate in vivo the effects of alcohol metabolism and aldehydes production on carcinogenesis.

**Abstract:** Endogenous acetaldehyde production from the metabolism of ingested alcohol exposes hematopoietic progenitor cells to increased genotoxic risk. To develop possible therapeutic strategies to prevent or reverse alcohol abuse effects, it would be critical to determine the temporal progression of acute ethanol toxicity on progenitor cell numbers and proliferative status. We followed the variation of the cell proliferation rate in bone marrow and spleen in response to acute ethanol intoxication in the MITO-Luc mouse, in which NF- $\kappa$ B-dependent cell proliferation can be assessed in vivo by non-invasive bioluminescent imaging. One week after ethanol administration, bioluminescent signals in bone marrow and spleen decreased below the level corresponding to physiological proliferation, and they progressively resumed to pre-treatment values in approximately 4 weeks. Boosting acetaldehyde catabolism by administration of an aldehyde dehydrogenase activity activator or administration of polyphenols with antioxidant activity partially restored bone marrow cells' physiological proliferation. These results indicate that in this mouse model, bioluminescent alteration reflects the reduction of the physiological proliferation rate of bone marrow progenitor cells due to the toxic effect of aldehydes generated by alcohol oxidation. In summary, this study presents a novel view of the impact of acute alcohol intake on bone marrow cell proliferation in vivo.

**Keywords:** cell proliferation; alcohol; bone marrow; binge drinking; in vivo bioluminescence imaging; ethanol; acetaldehyde; alcoholism; animal model; nuclear factor  $\kappa$ B



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## 1. Introduction

Alcoholic beverages contain different amounts of ethanol (ethyl alcohol,  $\text{CH}_3\text{—CH}_2\text{—OH}$  or  $\text{C}_2\text{H}_6\text{O}$ ) and low levels of methanol (methyl alcohol,  $\text{CH}_3\text{—OH}$ , or  $\text{CH}_4\text{O}$ ). Acetaldehyde and formaldehyde are endogenously produced, respectively, during the metabolism of ethanol and methanol ingested via alcoholic drinks. The International Agency for Research on Cancer, part of the World Health Organization, has classified alcoholic beverages, acetaldehyde, and formaldehyde as “carcinogenic to humans” (Group 1 Carcinogen) [1].

Consumption of alcoholic beverages may reduce the generation of inflammatory mediators leading to immunologic alterations, with increased susceptibility to infection and tumor formation and reduced healing after traumatic injury [2–8]. While the fact that chronic alcohol abuse severely damages immune function is well-established, only recently the role of acute ethanol exposure (binge drinking) in deteriorating protective immunity has been investigated [9]. Alterations in the hematologic profile have been observed not only in alcoholics, but also in short-term moderate alcohol drinkers [10]. In addition, maternal ethanol consumption causes immune deficiencies in newborns [11]. Some of these harmful consequences of alcohol exposure on the hematopoietic system [12] may determine an increased risk of some types of cancer [13,14].

Alcohol bone marrow toxicity leads to a reduction in blood cells precursors, thus interfering with blood cell production and functionality [15]. As a result, alcoholic patients may experience anemia, bleeding disorders, and, as mentioned above, compromised immune response [16]. Hematopoiesis is a highly regulated process by which blood and immune cells are replaced and produced by a small population of hematopoietic stem cells (HSCs). Some of alcohol’s adverse effects are indirect, due to nutritional deficiencies that impair the production and function of various blood cells. Direct effects of alcohol toxicity target the bone marrow (BM), possibly lowering the number of blood cell precursors [15]. In fact, the reduction of red blood cells (RBCs), white blood cells (WBCs), and platelets suggests that ethanol toxicity may target BM precursor cells [17]. In line with this hypothesis, the formation of vacuoles, which interfere with cells’ functionality, has been observed in RBC and WBC precursors. Hematopoiesis also involves the interaction of the developing cells with BM stroma, which provides the appropriate microenvironment for differentiation [18]. Ethanol could therefore affect the BM progenitor cells directly and/or act indirectly by altering the HSC microenvironment. As a matter of fact, alcohol has a harmful effect on BM stromal cells, which has been associated with the reduction of bone mass and decreased bone formation observed in alcoholics [19]. Moreover, several studies have suggested a direct toxic effect of ethanol and/or its metabolites on both neuronal [20] and hematopoietic progenitor cells [21]; however, the exact mechanisms need to be further characterized and probably depend on the target organ [22].

Some studies on the toxicity of ethanol and its derivatives have been performed on cultured precursor cells of different origin (bone marrow- and adipose tissue-derived stromal cells, neuronal precursors, hematopoietic cells) [20,23,24]. These studies are only partially informative since *in vitro* cells are exposed to rather elevated and constant doses of ethanol. Therefore, *in vivo* studies are needed to more precisely define the physiological and pathological processes related to alcohol intoxication. Performing studies of acute ethanol exposure in humans raises serious ethical concerns. Recently, studies on non-human primates have been performed, demonstrating that alcohol intake may result in impairment of the bone marrow niche and hematopoietic stem/progenitor function [25]. Notwithstanding the complexity and limitations of alcohol drinking studies in rodents, the existence of transgenic models facilitates mechanistic studies [26].

In the current work, we explored *in vivo* the effects of experimental acute ethanol intoxication in the transgenic MITO-Luc mouse [27]. In this animal model, luciferase activity is under the control of the nuclear factor- $\kappa$ B-dependent cyclin B2 (NF- $\kappa$ B) promoter and consequently restricted to proliferating cells. Consequently, in this transgenic mouse, it is possible to visualize and map by *in vivo* bioluminescence imaging (BLI) the regions of active cell proliferation. In particular, in MITO-Luc mice, high luciferase activity can

be detected in spleen, testis, and bone marrow (vertebral column, sternum, femur), while non-proliferating tissues, such as lung, brain, heart, aorta, skeletal muscle, liver, and kidney, do not emit light under physiological conditions [27]. This experimental model has been instrumental in studies analyzing cell proliferation in response to hyperbilirubinemia [28], toxic insult [29], and ischemic [30] and spinal cord [31] injuries.

## 2. Materials and Methods

### 2.1. Experimental Animal Procedures

Animals used in the study were 6–8 weeks old male and female albino MITO-Luc mice [27] gathered in groups of 4 to 8 animals each. Gavage administration of a solution of ethanol (dose range 3–6 g/kg body weight) in 0.9% saline in a total volume of 150  $\mu$ L -solution per 10 g of body weight was performed. According to previous reports, this dose range corresponds to a binge-like drinking pattern in humans [32]. Randomly selected animals received intra-peritoneal (i.p.) administration of the ALDH activator ALDA-1 (5 mg/kg) [33] or the ALDH inhibitor cyanamide (25 mg/kg) [34] (Sigma Aldrich, St. Louis, MO, USA) 30 min before ethanol treatment. Similarly, a group of animals received i.p. a solution containing polyphenols (50 mg/kg) (Phenolea Active Complex, kindly provided by Phenofarm, Rieti, Italy) [35] 2 h before alcohol administration. Methanol was administered (1.5 g/kg body weight) in 0.9% saline solution in a total volume of 150  $\mu$ L per 10 g of body weight [36]. Experimental procedures for assessment of cell proliferation in the MITO-Luc mouse model conformed to Animal Care guidelines (D.lgs 26/2014, 4 March 2014).

### 2.2. In Vivo and Ex Vivo Optical Bioluminescence Imaging

BLI analysis was performed using the IVIS Lumina II equipped with the Living Image 4.7.3 software for data quantification (PerkinElmer, Waltham, MA, USA), as previously described [37]. For in vivo imaging, mice were anesthetized, and D-luciferin (150 mg/kg body weight) (PerkinElmer) dissolved in phosphate-buffered saline (PBS) was administered i.p. 10 min before analysis. For ex vivo imaging, animals were euthanized, organs excised, placed into tissue culture dishes, and incubated for 10 min in PBS containing D-luciferin (150  $\mu$ g/mL) before analysis [38].

### 2.3. Complete Blood Counts

Blood was collected from the jugular vein and transferred into heparin-containing vials. Complete blood counts were performed automatically on a hematology analyzer.

### 2.4. Proteome Analysis

A specific Proteome Profiler™ Mouse Cytokine Array (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's specifications to determine plasmatic levels of a panel of 40 soluble factors including G-CSF, M-CSF, GM-CSF, SCF, IL-3, IL-6, TNF $\alpha$ , and ICAM-1 in response to acute alcohol exposure compared to control samples.

### 2.5. Immunoblot Analysis

Spleen homogenates (100  $\mu$ g) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and transferred into nitrocellulose membranes. Immunoblotting was performed following standard protocols using primary antibodies against SDF-1 (Santa Cruz Biotechnology, Dallas, TX, USA), SOD2 (Upstate Biotechnology–Millipore, Temecula, CA, USA) [37], BAX (Santa Cruz Biotechnology), and I $\kappa$ B $\alpha$  (Cell Signaling Technology, Danvers, MA, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a protein loading control. Immunoblot densitometry band quantification was performed using the ImageJ software (<https://imagej.nih.gov/ij/>) (accessed on 4 October 2021). All uncropped WB was shown in Figure S1.

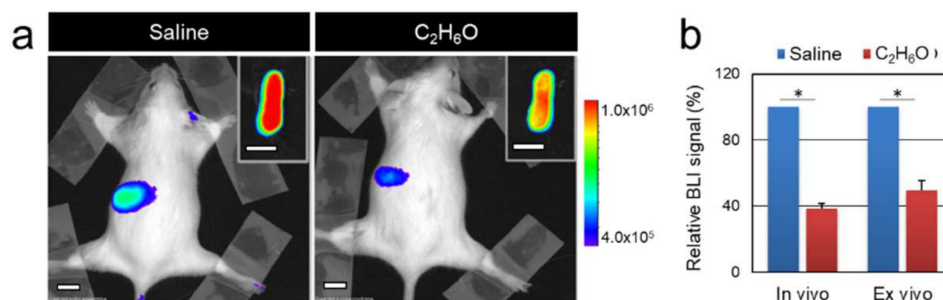
## 2.6. Statistical Analysis

Results are expressed as means  $\pm$  standard error of the mean (SEM). We used the INSTAT software (GraphPad, San Diego, CA, USA) for data analysis and comparisons between groups. The significance of differences was assessed with a two-tailed Student *t*-test for unpaired data; a *p*-value below 0.05 was considered statistically significant.

## 3. Results

### 3.1. Alcohol Intake Transiently Modulates Bioluminescence Signals Associated with Cell Proliferation in the MITO-Luc Mouse

Luciferase (Luc) expression in the MITO-Luc mouse is restricted to proliferating cells, being under the control of the NF- $\kappa$ B transcription factor. Therefore, luciferase-mediated light emission can represent an easily detectable surrogate for assessing *in vivo* cell proliferation. We determined the systemic effect of acute alcohol administration on cell proliferation in MITO-Luc mice subjected to a single administration of ethanol solution (6 g/kg body weight), described as the dose mimicking binge drinking in humans [9,32]. Longitudinal BLI imaging was performed, beginning before treatment (baseline) and then at different time points after alcohol administration. Five days after acute ethanol administration, BLI signals in the spleen were below the level observed before ethanol administration and in control animals receiving saline solution, corresponding to physiological proliferation (Figure 1). Luciferase levels in liver samples remain unaltered, as assessed by *in vivo* and *ex vivo* (not shown) BLI analysis, confirming that acute alcohol exposure does not induce hepatic cell proliferation [39].

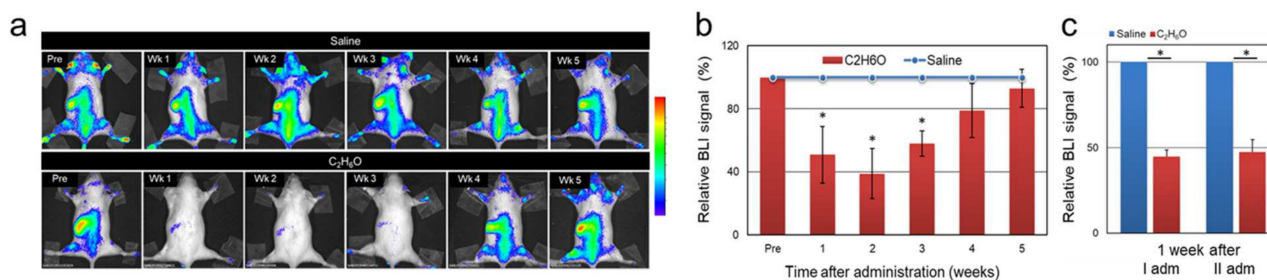


**Figure 1.** Response of NF- $\kappa$ B-dependent cell proliferation to acute ethanol administration in the MITO-Luc mouse model. (a) Bioluminescence imaging analysis performed on a representative animal 5 days after administration of saline or C<sub>2</sub>H<sub>6</sub>O solution. The insets show *ex vivo* imaging of excised spleens. (b) Regions of interests were drawn around spleens and quantified BLI intensities relative to saline control levels are shown in the graph. White scale bars: 1 cm. The pseudo-color scale bar represents the relative bioluminescent signal intensities from the lowest (blue) to the highest (red); radiance is expressed in photons per second per square centimeter per steradian (photons/s/cm<sup>2</sup>/sr). The asterisk (\*) indicates a statistically significant difference between the indicated groups (*p*  $\leq$  0.05).

One week after the administration, a residual BLI signal was detectable in the spleen, while bioluminescence in the BM was below the detection limit. Suppression of BM and spleen cell proliferation was transient and progressively resumed to control values in approximately 4–5 weeks (Figure 2a,b). These results are consistent with previous reports describing hematopoietic impairment associated with alcohol-dependent direct and/or indirect damage of hematopoietic stem cells [40], recovered in approximately a month [41].

In addition, we performed complete blood counts 1 week after acute administration, in correspondence with the reduction of the BLI signal. We did not detect any significant difference in the total number of erythrocytes, leukocytes, and platelets between the saline and the ethanol-administered group.





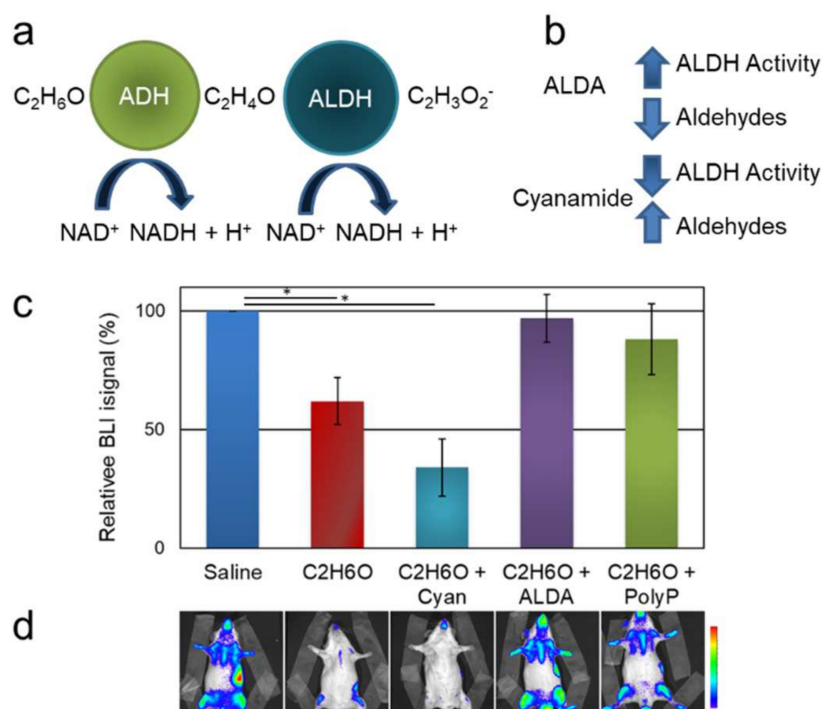
**Figure 2.** Kinetics of the response of NF- $\Upsilon$ -dependent cell proliferation to acute ethanol administration in the MITO-Luc mouse model. Longitudinal BLI imaging performed at different time points after administration on MITO-Luc mice administered with either vehicle (saline) or ethanol solution. Luciferase emission was determined by BLI in (a) a representative animal and (b) in each acquisition, regions of interests countering the whole animal were delineated and BLI emission quantified using the Living Image software. The graph illustrates BLI signal intensities relative to saline control levels set as 100%. (c) MITO-Luc mice were administered with either vehicle or C<sub>2</sub>H<sub>6</sub>O solution; after 8 weeks of recovery, the administration was repeated at the same dose. Quantification of the BLI signals was performed 1 week after each administration. The pseudo-color scale bar indicates the relative signal intensity from the lowest (blue) to the highest (red) (range  $1 \times 10^4$ – $1 \times 10^5$  photons/s/cm<sup>2</sup>/sr). The asterisk (\*) indicates a statistically significant difference compared with the relative saline group.

Furthermore, in a similar fashion, we examined the effects of multiple binge-like episodes on cell proliferation. To this end, a group of 4 MITO-Luc mice was administered with an ethanol solution by gavage administration and BLI analysis was performed at different time-points throughout the experiment. Animals were then allowed 8 weeks to recover, then administration was repeated at the same dose. After the second episode of alcohol administration, we observed a transient reduction of bioluminescence similar in magnitude to that observed after the first alcohol intoxication episode (Figure 2c). This indicates that repeated cycles of binge-like ethanol drinking have a similar impact on NF- $\Upsilon$ -dependent cell proliferation in hematopoietic tissues in the MITO-Luc mouse.

### 3.1.1. Modulation of Aldehyde Dehydrogenases Activity Affects Ethanol-Induced Alteration of Bioluminescence

Ethanol is removed from the body through oxidation. In particular, alcohol dehydrogenases (ADH) catalyze the oxidation of ethanol to acetaldehyde, known as a carcinogen and a key generator of free radicals [42], which is then converted to non-toxic acetate by aldehyde dehydrogenases (ALDH) (Figure 3a). In particular, ALDH2 is the most efficient of the 19 human ALDH isoforms in detoxifying ethanol-derived acetaldehyde. It has been unambiguously demonstrated that endogenous aldehydes, such as the ones produced during ethanol metabolism, are genotoxic in hematopoietic cells [43–45].

In wild-type C57B6 mice, traces of acetaldehyde in blood and brain can be assessed within minutes upon ethanol administration [46]. We evaluated whether pharmacological treatment with ALDH modulators [the inhibitor cyanamide (25–50 mg/kg) and the activator ALDA-1 (8.5 mg/kg) (Figure 3b)] might determine any possible modification in the cell proliferation pattern observed in the ethanol-treated MITO-Luc mice (Figure 3c). Indeed, upon alcohol administration in the MITO-Luc mice, treatment with the ALDH activity inhibitor determined a severe toxic effect resulting in 50% mortality within 2 days upon administration, while the surviving animals displayed a more severe modulation of BLI compared with the animal receiving alcohol solution only. These data suggest that increased levels of acetaldehyde subsequent to inhibition of ALDH activity may result in increased toxicity on BM cells. No deaths occurred in control animals administered with either ethanol or cyanamide alone. Conversely, administration of the ALDH activity activator ALDA-1 restored, at least in part, BM cells' physiological proliferative profile as assessed by BLI, possibly by reducing toxicity due to boosted acetaldehyde catabolism (Figure 3c). Taken together, these data point to acetaldehyde as a possible mediator of an alcohol-driven reduction of BM cells' proliferation and to ALDH2 as an actionable therapeutic target.



**Figure 3.** Effects of pharmacological treatments on NF-Y-dependent cell proliferation in MITO-Luc mice administered  $C_2H_6O$ . (a) Illustration of the metabolism of ethanol into acetaldehyde and acetate. ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase. (b) Schematic representation of the effects of pharmacological treatment with the ALDH inhibitor cyanamide or the activator ALDA. (c) Quantification of the BLI signals relative to the control (saline) group in animals administered  $C_2H_6O$  treated either with cyanamide, ALDA, or the antioxidant compound Phenolea Active Complex (PolyP). (d) Representative BLI analysis in one mouse per group at approximately 10 days after treatment. The pseudo-color scale bar indicates the relative signal intensity from the lowest (blue) to the highest (red) (range  $1 \times 10^4$ – $1 \times 10^5$  photons/s/cm<sup>2</sup>/sr). The asterisk (\*) indicates a statistically significant difference between the indicated groups ( $p \leq 0.05$ ).

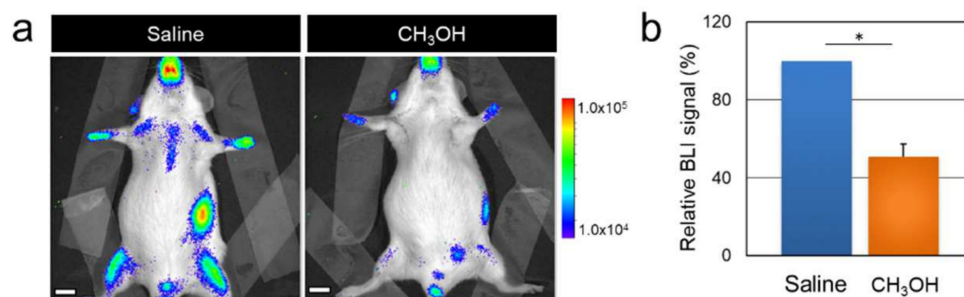
### 3.1.2. Polyphenol Administration Partially Restores Bioluminescence upon Ethanol Exposure

Ethanol metabolism generates free radicals, altering the cellular redox status and leading to cellular damage [47–49]. Fetal tissues are particularly vulnerable to oxidative damage since the levels of enzymatic and non-enzymatic antioxidants are lower than in adults [50]. Accordingly, antioxidant strategies have been proposed to counteract the toxic actions of alcohol-mediated oxidative stress [51,52]. Recently, the administration of polyphenols derived from olive leaves has been described to attenuate damages associated with chronic alcohol abuse by reduction of reactive oxygen species (ROS) in the serum [35]. We therefore evaluated whether the administration of antioxidants may affect the possible perturbation of BLI in mice receiving acute alcohol intoxication. In this study, we used a polyphenol blend derived from a standardized olive pulp (*Olea europaea* L.). This product, named Phenolea Active Complex, has been extensively characterized before [35,53]. Indeed, the delivery of olive-derived polyphenols before alcohol intoxication attenuates the reduction of BM cell proliferation (Figure 3c,d), while the administration of polyphenols alone did not produce any significant effect (not shown). This result further supports the role of olive polyphenols in conferring protection against ethanol-induced oxidative stress [35,54].

### 3.2. Acute Methanol Administration Transiently Modulates Cell Proliferation in the MITO-Luc Mice

In the same way as acetaldehyde [43], formaldehyde also exerts cytotoxic effects on HSCs [55,56]. Formaldehyde exposure can occur from a variety of exogenous sources, such as tobacco and e-cigarette smoke, aspartame and furniture, textiles, and cosmetics containing formaldehyde-based products, such as glue, paint, and resins. Formaldehyde is endogenously produced by enzymatic oxidative demethylation reactions and during catalase- and alcohol dehydrogenase-mediated oxidation of methanol contaminations ingested in alcoholic beverages [57].

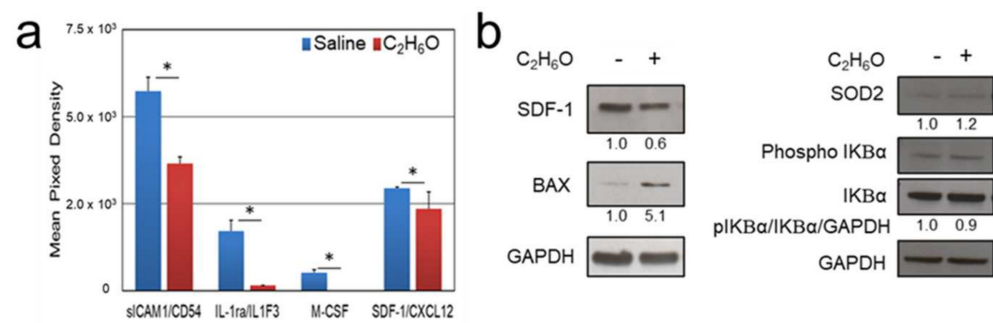
We tested the effects of methanol administration (1.5 g/kg i.p.) on cell proliferation in MITO-Luc mice. BLI imaging signals associated with cell proliferation decreased in MITO-Luc mice administered methanol in a similar fashion as observed upon ethanol administration, with an approximately 50% reduction 10 days after administration (Figure 4). This result further supports the suggestion that endogenously produced aldehydes have a detrimental effect on BM cell proliferation, which can be assessed as a decrement of bioluminescence in the MITO-Luc mouse model.



**Figure 4.** Effect of methanol administration on bioluminescent signal in MITO-Luc mice. Animals were administered either vehicle (saline) or methanol solution (CH<sub>3</sub>OH) and BLI was performed 10 days after administration. Panel (a) shows representative images of an animal per group. The color bar indicates the relative bioluminescent signal intensities in photons/s/cm<sup>2</sup>/sr. (b) Quantification of the BLI signal expressed as the relative percentage of the saline group. The asterisk (\*) indicates a significant difference as assessed by a two-tailed Student *t*-test for unpaired data ( $p < 0.05$ ).

### 3.3. Proteome Analysis after Ethanol Administration Indicate Serum and Splenic Reduction of CXCL12 Levels

Alcohol intoxication alters the plasma levels of a large panel of cytokines and growth factors [58,59], possibly affecting cell proliferation. In line with this evidence, using the Mouse Cytokine Array Panel, Proteome Profiler™ array (R&D Systems) we evaluated the serum levels of 40 cytokines and chemokines. Ten days after acute ethanol intake, in correspondence with the severe reduction of BLI emission in the spleen and the BM, we also determined a significant alteration of the circulating levels of soluble intercellular adhesion molecule-1 (sICAM-1), interleukin-1 receptor antagonist (IL-1ra/IL-1F3), macrophage colony-stimulating factor (M-CSF), and stromal cell-derived factor-1 (SDF-1/CXCL12) compared to controls (Figure 5a). SDF-1 is a CXC chemokine family member constitutively expressed by BM stromal cells. Plasmatic reduction of the levels of SDF-1/CXCL12 has been described in patients with alcohol use disorders [60]. Considering its role in regulating HSC homeostasis and trafficking [61], we focused our attention on SDF-1/CXCL12, further confirming by immunoblot analysis the reduction of its expression in spleen homogenates collected from animals subjected to acute alcohol intoxication compared to controls (Figure 5b).



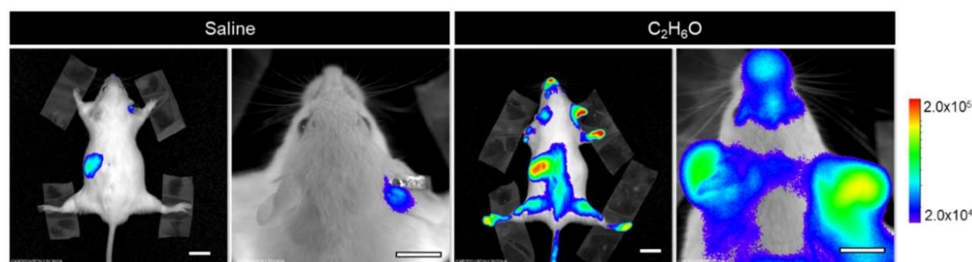
**Figure 5.** Effects of acute ethanol administration on cytokine/chemokine levels. **(a)** Proteome Profiler Mouse Cytokine Array (R&D Systems) analysis performed on pooled sera from MITO-Luc mice (N = 4 per group) 10 days after administration of either vehicle (saline) or ethanol solution. According to the manufacturer's instructions, array data were quantified as mean pixel density, normalized to the density of the positive controls. **(b)** Immunoblot analysis performed on tissue homogenates from spleens collected from animals in the groups described above. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a protein loading control. Band intensities were quantified using the ImageJ software and numbers indicate the normalized ratio of the indicated proteins to relative GAPDH signals.

### 3.4. Immunoblot Analysis after Ethanol Administration Indicate Increased Splenic Expression of Oxidative Stress and Apoptosis Markers

To further examine the cellular mechanism that could underlie the relationship between alcohol exposure and reduced cell proliferation, we performed immunoblot analysis of the splenic tissue lysate obtained from animals sacrificed 10 days after administration of either saline or ethanol solution (Figure 5b). Acute ethanol-induced toxicity is associated with increased mitochondrial dysfunction and oxidative stress, triggering apoptosis. In response to alcohol intake-induced oxidative stress, mitochondrial manganese superoxide dismutase (MnSOD/SOD2) upregulation has been observed [62]. BCL2-associated X protein (BAX), a member of the pro-apoptotic B-cell lymphoma 2 family proteins, plays a pivotal role in the onset of apoptosis induced by ethanol in splenic T and B lymphocytes [63]. Acute and chronic alcohol intake may also induce changes in the levels of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB), which regulate the immune response [64]. In ethanol-treated animals, we observed a moderate albeit significant increase of SOD2, consistent with its induction under oxidative stress conditions [48], and an increment of BAX expression, suggesting activation of the apoptotic signaling pathway [65]. These results are in agreement with previous reports describing apoptosis in splenocytes in the white pulp [66], splenic T and B cells [63,67], and macrophages [68] induced by alcohol administration. Conversely, in our experimental setting, NF-κB is not dysregulated as both the levels of total and phosphorylated inhibitory proteins of κB family (IκB) remained unaltered.

### 3.5. Short-Term Activation of NF-Υ Driven Luciferase after Acute Ethanol Administration

Recently, NF-Υ has been proven to participate in the early stages of alcoholic liver disease [69]; moreover, NF-Υ, orchestrating the differential gene expression in response to acute alcohol intake, affects brain cell proliferation [70]. In particular, 6 h after ethanol (6 g/kg) administration in B6 and D2 mice, upregulation of NF-Υ in the brain was determined by microarray analysis [70]. In line with these findings, 6 h after ethanol administration in MITO-Luc mice, we observed a widespread BLI signal also in the brain region, undetectable at baseline and in saline-treated controls (Figure 6).



**Figure 6.** NF- $\kappa$ B-dependent luciferase activity in the MITO-Luc mouse model in response to acute ethanol administration. In vivo longitudinal bioluminescence imaging analysis of a representative animal 6 h after either saline or ethanol administration. The pseudo-color scale bar represents the relative bioluminescent signal intensities from the lowest (blue) to the highest (red); radiance is expressed in photons per second per square centimeter per steradian (photons/s/cm<sup>2</sup>/steradian).

#### 4. Discussion

Alcohol is a leading risk factor for disease burden and premature mortality, as stated by the World Health Organization in the Global Status Report on Alcohol and Health [71]. Each year, 3.3 million deaths worldwide are attributable to alcohol consumption, with an expected increasing trend over the next decade [72]. A recent study reported an increase in binge drinking behavior among the general population [73]. In particular, binge drinking is commonly diffused among younger adults. Even if they may later develop alcohol-use problems, most young age binge drinkers are not alcoholics or alcohol dependent. They consider heavy episodic alcohol intake as a “rite of passage” and generally fail to recognize the associated risks of perturbation of the physiological and neurodevelopmental changes occurring in adolescence. Emerging evidence from studies involving young human subjects indicates that acute drinking is responsible for the following side effects: morphometric anomalies in different brain areas, as assessed by functional magnetic resonance imaging [74,75]; neuroimmune system impairment, leading to long-term psychological and behavioral dysfunctions [76]; deficits in memory and attention functions [77]; negative effects on proliferation and differentiation potential of different types of progenitor/stem cells [22]; and gut microbiota dysbiosis [78]. Alcohol intake is also associated with several conditions, including tumors; immunological disorders; cardiovascular diseases; impaired injury healing; mental and behavioral disorders; gastrointestinal conditions; lung, skeletal and muscular diseases; reproductive disorders; and pre-natal harm [79,80]. Moreover, there are additional social and economic burdens connected with alcohol drinking and addiction.

Excessive consumption of alcoholic beverages may have a severe impact on the immune system, leading to impairments in host defense, susceptibility to viral infection, reduced healing, and increased risk of developing tumors in several anatomical sites [23,81,82]. Approximately 5.5% of all cancer deaths (770,000 per year worldwide) are attributable to alcohol, with a loss of about 19 years of potential life for each victim [79,82,83]. Although the contribution of the precise cellular and molecular processes of alcohol-associated carcinogenesis has not been fully clarified, a casual role of the genotoxic effect of the ethanol metabolite acetaldehyde and its by-products, the DNA damage induced by the increased level of reactive oxygen species, and the direct or indirect alterations of oncogenic or regulatory pathways have been proposed [23,84–87]. In particular, impaired hematopoiesis has been linked to the genotoxic action on hematopoietic stem cells (HSCs) of reactive aldehydes produced during ethanol metabolism [43,88]. In primitive BM-derived HSCs, preferential expression of the nuclear transcription factor  $\kappa$  (NF- $\kappa$ B) dictates self-renewal, proliferation, and survival [89–91]. Microarray analysis has shown that ethanol exposure determines the modulation of genes involved in cell proliferation, growth arrest, apoptosis, and DNA damage in peripheral blood both in animal models and humans [92]. Interestingly, NF- $\kappa$ B regulates the expression of several ethanol-responsive genes in mice subjected to acute alcohol intoxication [70]. Moreover, acting as a regulator of major histocompatibility complex (MHC) gene expression [93] and macrophage mat-



uration [94], NF- $\kappa$ B is involved in the immune response. Consistently, in the MITO-Luc mouse model, in which the luciferase gene expression is driven by the activity of a NF- $\kappa$ B-dependent cyclin B2 promoter, we observed a rapid increase in BM luciferase activity after acute ethanol intake. Interestingly, NF- $\kappa$ B-driven luciferase activity in hematopoietic organs was then reduced to baseline levels in approximately 4 weeks. This result is in agreement with a previous report describing that alcohol-dependent BM damage affecting hematopoiesis is reversible after abstinence for a month [41]. We previously reported that the activation of microglial cells induced by supraphysiological levels of unconjugated bilirubin can induce luciferase emission in the brain of MITO-Luc mice [28]. In line with these findings, we observed an increment in brain BLI emission 6 h after acute ethanol administration, consistent with possible proliferation of microglial cells, which has been described as one of the pathophysiological consequences of alcohol intake [95–97].

Along with ethanol as its major metabolic by-product, acetaldehyde is also considered as a carcinogen by the International Agency for Research on Cancer. As a matter of fact, ethanol, directly or through its metabolic intermediates, determines an impairment of neuronal [98,99], hepatic [100], and hematopoietic progenitor cell proliferation [22,101]. Specifically, the susceptibility of HSCs to acetaldehyde has been demonstrated [43] and toxicity on BM precursor cells was observed in heavy drinkers [102]. Both in mice [45] and humans [103], in the absence of the protective role of the Fanconi anemia DNA repair pathway and the activity of an isoform of aldehyde dehydrogenases (ALDH2), ethanol's genotoxic effect results in severe depletion of the HSCs pool [104]. Harmful effects of in utero alcohol exposure on hematopoiesis in BM and spleen have been described in experimental models [105,106]. Furthermore, epidemiological studies demonstrated the association between maternal alcohol consumption during pregnancy and development of childhood leukemia, suggesting a possible toxic role of ethanol and/or its metabolites on fetal bone marrow hematopoiesis [107]. Moreover, acetaldehyde produced during ethanol oxidation mediates G2/M cell cycle arrest [108]. Interestingly, about 1–2% of nucleated BM cells, including hematopoietic, mesenchymal, and endothelial precursors, express high levels of ALDH [109], implicated in the regulation of cell proliferation and resistance to exogenous stress [110]. In particular, ALDH modulation affects HSCs' self-renewal via inhibition of retinoic acid signaling [111]. We used our model to evaluate the response to pharmacological modulation of ALDH as a potential therapeutic target to combat aldehyde toxicity [112]. Understanding the mechanisms of in vivo aldehyde production and de-toxication is particularly relevant considering that more than 560 million people in the world, being characterized by the presence of the ALDH2\*2 genetic variants [113,114], are more susceptible to aldehyde toxicity. Indeed, we determined that ALDH activation restored, at least in part, physiological cell proliferation upon alcohol intake, while inhibition of ALDH activity had a detrimental effect.

Overall, our experimental data indicate that acute alcohol administration affects cell proliferation in the bone marrow and spleen. The study was performed on a transgenic animal model, which offers unique opportunities to dissect in vivo the pathophysiological effects of alcohol on cell proliferation. Caution should be exercised in translating information obtained from alcohol studies performed on rodents into clinical treatments in humans since no animal model completely reproduces all the complex aspects of human alcohol consumption and intoxication [26]. Nonetheless, the results are broadly consistent with clinical data observed in alcoholic patients indicating that alcohol and its metabolite acetaldehyde exert a cytotoxic effect on hematopoietic cells [21,55] and further support the notion that ALDH might represent a druggable target for protection of alcohol and aldehyde toxicity.

## 5. Conclusions

Alcohol intake determines a transient modulation in the bioluminescence emission in the MITO-Luc mouse, where the light intensity is associated with cellular proliferative status. Activation of ALDH activity and administration of an antioxidant compound partially protected the animals from a reduction of cell proliferation in BM and spleen; conversely, inhibition of ALDH activity had a detrimental effect. Collectively, these data are in agreement with a previously established impairment on the antitumor immune response associated with the cytotoxic effect on HSCs exerted by aldehydes produced by both ethanol and methanol metabolism. The proposed experimental model might be instrumental for further in vivo elucidation of the mechanisms of aldehydes' endogenous production and de-toxification.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/cancers13194999/s1>, Figure S1: Uncropped WB.

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