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Methanol Induces a Discrete Transcriptional Dysregulation that Leads to Cytokine Overproduction in Activated Lymphocytes

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Methanol is an important cause of acute alcohol intoxication; it is ubiquitously present at home and in the workplace. Although the existing literature provides a reasonable insight into the immunological impact of ethanol and to a much lesser extent of isopropanol, much less data are available on methanol. We hypothesized on structural grounds that methanol would share the immunosuppressive properties of the two other short-chain alcohols. We report here that methanol increases the proliferative capacity of human T lymphocytes and synergizes with the activating stimuli to augment cytokine production. The cytokine upregulation was observed *in vitro* at methanol concentrations as low as 0.08% (25mM) as measured by interleukin-2, interferon-γ, and tumor necrosis factor-α release in T cells. Methanol did not affect the antigen receptor–mediated early signaling but promoted a selective and differential activation of the nuclear factor of activated T cells family of transcription factors. These results were further substantiated in a mouse model of acute methanol intoxication in which there was an augmented release of proinflammatory cytokines in the serum in response to the staphylococcal enterotoxin B. Our results suggest that methanol has a discrete immunological footprint of broad significance given the exposure of the general population to this multipurpose solvent.

Key Words: **immunomodulation; leukocyte; T lymphocyte; cytokine; methanol; immunotoxicology.**

The use of short-chain alcohols is deeply ingrained into a variety of aspects of our everyday lives. These substances have a multitude of biological effects, and a considerable body of evidence indicates that ethanol is capable of modulating the immune function mediated by T cells, monocytes, macrophages, and neutrophils (Goral and Kovacs, 2005; Saeed *et al.*, 2004; Szabo *et al.*, 2007; Taieb *et al.*, 2002). In addition, we have recently reported that isopropanol is detrimental to human T lymphocyte and natural killer cell activity, and acute intoxication may lead to acute immunosuppression (Désy *et al.*, 2008). Although the existing literature on short-chain alcohols provides a reasonable insight into the immunological impact of ethanol and to a much lesser extent of isopropanol, much less data are available on other alcohols. Exposure of the general population to dietary and environmental sources of methanol, the simplest alcohol, usually does not amount to dangerous levels (Shelby *et al.*, 2004). Yet, methanol exposure is an important cause of acute alcohol intoxication with more than 2000 cases reported each year to poison centers in North America (Bronstein *et al.*, 2008). Methanol is readily available to consumers as a component of several household solutions, such as varnishes, paints, windshield washer fluids, antifreeze, and adhesives (Lanigan, 2001). The ingestion of as little as 6 ml of methanol can cause toxicity; on the other hand, there are individuals who survived the intake of more than 500 ml of this alcohol (Brahmi *et al.*, 2007; Hantson *et al.*, 2000; Martens *et al.*, 1982). Regardless of the type of treatment provided, hemodialysis, alcohol dehydrogenase inhibition, or substrate competition, some 15–36% of the intoxicated patients die (Brent *et al.*, 2001; Hunderi *et al.*, 2004; Liu *et al.*, 1998). In surviving patients, the blood alcohol concentration (BAC) recorded at hospital admission ranges broadly from 20 to 1290 mg/dl (0.02–1.29% or 6.24–402 mmol/l) (Brahmi *et al.*, 2007; Brent *et al.*, 2001; Cowen, 1964; Gonda *et al.*, 1978; Hantson *et al.*, 2000, 2005; Hovda *et al.*, 2005; Hunderi *et al.*, 2004; Kostic and Dart, 2003; Liu *et al.*, 1998; Lushine *et al.*, 2003; Martens *et al.*, 1982; Verhelst *et al.*, 2004; Wu *et al.*, 1995). The highest methanol concentrations are often associated with permanent visual impairment. However, there are documented cases of treated patients who recover without visual sequelae despite BAC levels higher than 600 mg/dl (0.6% or 187 mmol/l) (Brent *et al.*, 2001; Lushine *et al.*, 2003; Martens *et al.*, 1982; Wu *et al.*, 1995).

Previous studies addressed the effect of methanol on the central nervous system, the sensory system, the gastrointestinal tract, renal function, metabolism, genetic stability, carcinogenesis, reproduction, and development (Barceloux *et al.*, 2002; Hantson and Mahieu, 2000; Shelby *et al.*, 2004). In addition, existing reports suggest a potential immunosuppressive activity

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of methanol in rats (Parthasarathy *et al*., 2007; Zabrodskii *et al*., 2008). However, the latter studies involved repeated exposure of the rodents to large doses of the alcohol over several days or weeks, a situation that is not likely to be met in the clinical setting of acute intoxication. To our knowledge, no detailed analysis of the impact of methanol on the human immune system and of the underlying mechanisms of such potential effect is yet available.

Given the structural similarity between methanol, ethanol, and isopropanol, we hypothesized that methanol would share the immune modulatory properties of the two other alcohols and that these biological effects would be mediated through similar molecular mechanisms. We report here that methanol works in a different way by inducing the upregulation of the proliferative capacity and effector function of human T lymphocytes *in vitro* in concentrations as low as 0.08% wt/vol (or 25mM). We also show that a specific transcriptional dysregulation underlies the immunological impact of methanol. These results are further substantiated in a mouse model of acute methanol intoxication in which the production of proinflammatory cytokines is dysregulated. All concentrations tested *in vitro* and *in vivo* were within the clinically relevant range observed in the first hours of acute methanol intoxication. The full understanding of the pathophysiology of methanol poisoning, including the putative immune dysregulation, may be instrumental in rescuing patients that do not respond to therapy. Our results have broad significance taking into account the potential exposure of the general population to this ubiquitous chemical.

MATERIALS AND METHODS

Cell Isolation, Culture, Activation, and Proliferation Analysis

This study was approved by the Institutional Clinical Research Ethics Committee (L'Hôtel-Dieu de Québec/Centre hospitalier universitaire de Québec [L'HDQ-CHUQ]). Mononuclear cells were prepared from the peripheral blood from healthy volunteers by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Piscataway, NJ). Written informed consent was obtained from all donors. More than 95% pure populations of human T cells ($CD8^+$/$CD4^+$) were obtained with EasySep separation kits (StemCell Technologies, Vancouver, Canada). Cells were kept in complete medium: RPMI 1640 (Invitrogen Canada, Burlington, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS; BioCell Inc., Drummondville, Canada). Methanol (99.9% pure) was purchased from Fisher Scientific (Pittsburgh, PA).

In most experiments, T cells were activated with anti-CD3/CD28 antibody–coated magnetic beads (Invitrogen Canada) at 37°C for 1 or 5 h as specified in the text. When indicated, three alternative T-cell activation protocols were used: (1) pretreatment for 20 min on ice with 1 μg/ml mouse anti-human CD3 monoclonal antibody (CD3-2; Mabtech, Nacka Strand, Sweden), followed by washing and incubation at 37°C for 3 min with 10 μg/ml goat anti-mouse IgG (Invitrogen Canada); (2) pretreatment for 20 min on ice with 1 μg/ml anti-human CD3 (CD3-2) and 5 μg/ml anti-human CD28 (CD28.2; BioLegend, San Diego, CA) mouse monoclonal antibodies, followed by washing and incubation at 37°C for 15 min with 10 μg/ml anti-IgG; and (3) treatment with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 200 ng/ml ionomycin (Sigma, St Louis, MO) for 5 h.

Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining: Freshly purified T cells were labeled with 10μM CFSE (Invitrogen Canada) in PBS/1% FBS for 10 min at 37°C and further incubated in RPMI/10% FBS for 5 min on ice; then, cells were washed and activated with anti-CD3/CD28 antibody–coated beads for 5 h with or without 0.6% (wt/vol) methanol. The activating beads were magnetically removed; the cells were washed and incubated for 72 h in 96-well plates (10^6 cells/ml) in complete medium without exogenous interleukin (IL)-2. FACS analysis of cell divisions and surface marker expression was performed on a XL flow cytometer (Beckman Coulter Inc., Miami, FL).

Cytokine Analysis

Measurements of human IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α in cell culture supernatants and murine IL-2 and IFN-γ in serum samples were performed with specific cytokine ELISA kits according to the manufacturer's instructions (Mabtech). Murine TNF-α levels were measured with the mouse TNF-α ELISA MAX Standard Kit (BioLegend) following the protocol provided by the manufacturer.

ELISA-Based Transcription Factor Activation Assay

Nuclear proteins were extracted using the Active Motif Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions; the total protein concentration of the lysates was determined by the Bradford assay (Bio-Rad, Hercules, CA). The nuclear translocation of the nuclear factor of activated T cells (NFAT), activator protein-1 (AP-1), and nuclear factor-κB (NF-κB) was analyzed as follows: NFATc1 activation was measured with the TransAM NFATc1 Kit; NFATc2 activation was measured with a modified NFATc1 TransAM Kit (the anti-NFATc2 4G6-G5 antibody was used to replace the original anti-NFATc1 primary antibody in the kit); c-Fos and c-Jun activation was measured with the TransAM AP-1 kit; and p50 and p65 activation was measured with the TransAM NF-κB Kit. ELISA-based TransAM kits were used according to the manufacturer's instructions (Active Motif). Briefly, nuclear extracts were incubated with plate-bound transcription factor–specific oligonucleotides; the plates were washed and further incubated with transcription factor–specific antibodies. Addition of a horseradish-conjugated secondary antibody and the 3,3′,5,5′-tetramethylbenzidine substrate produced a colorimetric reaction measurable in a spectrophotometer.

Inhibitor compounds (Sigma) were used as follows: cyclosporin A, 1 μg/ml; BAY 11-7082, 5μM; SP600125, 25μM; SB202190, 10μM; and PD98059, 50μM.

Electrophoretic Mobility Shift Assay

Purified T cells were stimulated with anti-CD3/CD28 antibody–coated beads for 1 h in presence of the indicated amounts of methanol. Nuclear extracts were prepared with the Active Motif Extraction Kit and used for DNA-binding analysis with a double-stranded oligonucleotide carrying the NFAT recognition sequence from the distal ARRE-2 in the human IL-2 promoter (5′-GGAGGAAAAACTGTTTCATAGAAGGCGT-3′). The electrophoretic mobility shift assay (EMSA) probe was end labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase treatment. Binding reactions were performed with 5 μg of nuclear protein in 10mM Tris-HCl (pH 7.5), 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM dithiothreitol, 50mM NaCl, 6% glycerol, 1 μg bovine serum albumin, and 0.2 μg poly(dI)-poly(dC) at room temperature for 20 min. Protein-DNA complexes were separated in a 6% nondenaturing polyacrylamide gel. Supershift assays were performed by preincubating the samples on ice with antibodies against NFATc1 (7A6) or c2 (G1-D10) (Santa Cruz Biotechnology, Santa Cruz, CA) before the addition of the labeled probe.

Western Blot and Luciferase Assay

Western blot. Purified T lymphocytes or Jurkat cells were activated as mentioned above for 3 min (ZAP-70) or 15 min (NFAT) in presence or absence of 0.6% (wt/vol) methanol. Samples for ZAP-70 analysis were lysed in SDS buffer (Désy *et al*., 2008), and those for NFAT analysis were prepared with the Nuclear Extract Kit (Active Motif). Proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted as described (Désy *et al*., 2008).

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Primary antibodies for ZAP-70 were the rabbit anti-human total ZAP-70 (99F2, 1/1000; Cell Signaling Technology, Danvers, MA) and the mouse anti-human phospho-ZAP-70 (17a, 1/5000; BD Biosciences, Mississauga, Canada); in the case of NFAT analysis, the primary antibodies were the mouse anti-human NFATc2 (4G6-G5, 1/200; Santa Cruz Biotechnology) and the control rabbit anti-HDAC-1 (H-51, 1/200; Santa Cruz Biotechnology). Secondary antibodies were the IRDye 800CW goat anti-rabbit IgG and/or IRDye 680 goat anti-mouse IgG (1/20,000; Li-Cor Biosciences, Lincoln, NE). Detection and quantification were performed with the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Luciferase assay. The generation of Jurkat cells carrying the firefly luciferase gene driven by the NFAT synthetic promoter is described elsewhere (Désy *et al.*, 2008). Jurkat-luciferase cells were stimulated with PMA/ionomycin with or without methanol treatment as indicated in the text. Lysates for luciferase assays were prepared with the passive lysis buffer (E1941; Promega, Madison, WI) and analyzed in a Lumat 9501 luminometer (Berthold, Nashua, NH). Relative luciferase units were calculated in relation to the unstimulated negative control after normalization to total protein content measured by the Bradford assay (Bio-Rad).

In Vivo *Studies*

Seven- to 13-week-old female BALB/c mice were bought from The Jackson Laboratory (Bar Harbor, ME). All tests respected the ethical guidelines set by the Institutional Animal Protection Committee (CPA-CHUQ). Food and water were provided *ad libitum*. Animals received sc 5 μg of staphylococcal enterotoxin B (SEB; Toxin Technology Inc., Sarasota, FL) for cytokine induction and were sacrificed by CO_2 asphyxiation 4 h after administration for IL-2, IFN-γ, and TNF-α serum analysis. Methanol was injected ip (2 g/kg). The BAC was determined by gas chromatography with a 3900 GC unit (Varian, Palo Alto, CA).

Statistical Analysis. One-way ANOVA followed by Dunnett's multiple comparison posttest was performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA) on data presented in all figures. p Values < 0.05 were considered significant.

RESULTS

The Immune Modulatory Effect of Methanol Is Distinct from that of Other Short-Chain Alcohols

We have previously reported that isopropanol downregulates the production of IL-2 *in vitro* and *in vivo* (Désy *et al.*, 2008), and our expectation was that the closely related methanol would exhibit a similar dampening effect on lymphocytes. In the present study, we have investigated first whether methanol exposure *in vitro* would have any impact on the ability of human peripheral T lymphocytes to produce IL-2 once activated by antibody cross-linking of the T-cell receptor (TCR). Although this cytokine peaked at 24- to 48-h poststimulation, it was readily measurable within 5 h (mean = 1.15 ng/ml ± 0.07 SEM, $n = 6$). Unlike our earlier results with isopropanol, activated T cells produced more IL-2 when treated with methanol as measured in the culture supernatants by ELISA (Fig. 1A, black bars). The increase in cytokine production was observed at methanol doses as low as 0.08%. The observed effect was decoupled from any nonspecific cytotoxicity as the cell viability of methanol-treated samples in the concentration range that upregulated IL-2 by 27–70% was similar to that of untreated control cells (Fig. 1A, gray bars). Methanol in absence of stimulation did not have any effect.

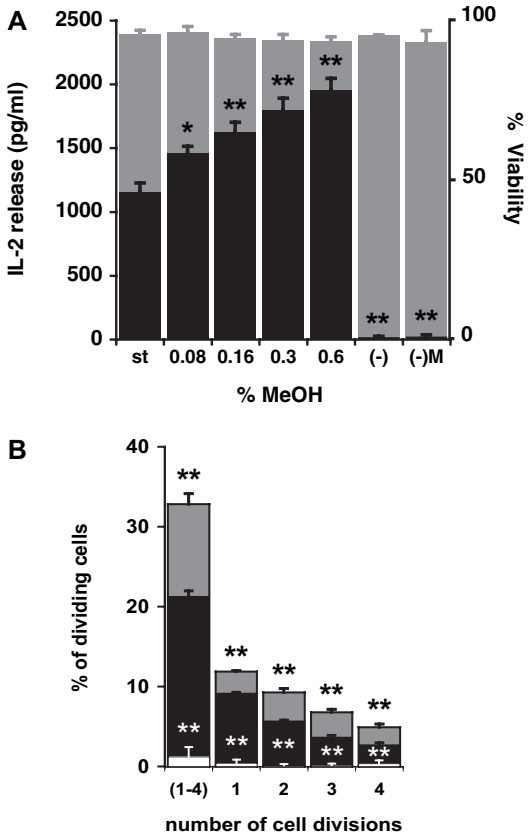

FIG. 1. Biological effect of methanol treatment on human T lymphocytes *in vitro*. (A) Methanol enhances IL-2 production in peripheral blood T lymphocytes: Purified T cells were stimulated with anti-CD3/CD28 antibody–coated beads for 5 h in presence of 0.08, 0.16, 0.3, and 0.6% (wt/vol) methanol. The IL-2 concentration in the supernatants was measured by ELISA and is depicted as means ± SEM in the black bar histogram. The cell viability is shown as means ± SEM in the gray bar histogram (**$p < 0.01$ relative to the *st* positive control group, $n = 6$). Panel symbols: *st*, stimulated cells in absence of methanol; (-), unstimulated cells; (-)M, unstimulated cells in presence of 0.6% (wt/vol) methanol; MeOH, methanol. (B) Proliferation of peripheral blood T cells in presence of methanol: Freshly purified T lymphocytes were labeled with CFSE and stimulated with anti-CD3/CD28 antibody–coated beads for 5 h with or without methanol. After removal of the activating beads and the alcohol, the cells were further incubated for 72 h in absence of exogenous IL-2. The number of cell divisions was counted by flow cytometric analysis and the mean percentage of dividing cells ± SEM was plotted as a histogram. White bars: unstimulated cells; black bars: stimulated cells; gray bars: cells stimulated in presence of 0.6% (wt/vol) methanol. For each cell division group, the means of the negative control (white) and of the alcohol-treated stimulated sample (gray) were compared with the mean of the positive control (black); **$p < 0.01$, $n = 3$.

Given the importance of the IL-2 autocrine loop for the expansion of antigen-specific cells *in vivo*, we have asked if the increased release of IL-2 translated into a higher proliferative capacity of the activated lymphocytes. Purified peripheral T cells were labeled with CFSE and activated for 5 h; then, they were washed and further incubated for 72 h before

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analysis by flow cytometry. Figure 1B shows that although the 5-h TCR cross-linking led to one to four divisions in about 20% of the cells in absence of exogenous IL-2 (mean = 21.35% ± 0.71 SEM, $n = 3$), cell proliferation was more pronounced if methanol was present during the 5-h stimulation (mean = 32.82% ± 1.31 SEM, $n = 3$). Significant differences between treated and untreated samples were also found when cells were analyzed according to the number of divisions completed by the end of the experiment (Fig. 1B).

Methanol Does Not Compromise Early Signaling Following TCR Activation of Lymphocytes

Methanol could act on the cell membrane in a way reminiscent of the effect of ethanol on ion channels and neurotransmitter receptors (Arevalo *et al.*, 2008; Aryal *et al.*, 2009). It could conceivably interact with the TCR directly, thereby amplifying antigen-dependent signal transduction in lymphocytes. To address this possibility, we have examined the phosphorylation status of ZAP-70, a key downstream molecule that is activated soon after TCR engagement. Figure 2A shows that signaling through the TCR itself was not affected by methanol because the ZAP-70 activation proceeded as efficiently as in untreated cells following anti-CD3 antibody cross-linking.

IL-2 Upregulation Does Not Involve the Transcriptional Pathways Affected by Other Short-Chain Alcohols

Because methanol did not present an obvious impact on early TCR signaling, the possibility of a positive regulation of IL-2 transcription by the alcohol was examined. A precedent in favor of this scenario was set by earlier studies which revealed that ethanol, and more recently isopropanol, mediate their impact on the immune system through the reduction of the nuclear translocation of transcription factors capable of binding the promoter regions of key cytokine genes (Désy *et al.*, 2008; Mandrekar *et al.*, 2007; Saeed *et al.*, 2004; Szabo *et al.*, 2007). In order to dissect the relative impact of methanol on transcription factors relevant to IL-2 production, we have measured the nuclear translocation of NF-κB (p50/p65) and AP-1 (c-Jun/c-Fos) in TCR-stimulated purified human T cells exposed to different concentrations of methanol. Panels B and C in Fig. 2 show that lymphocyte activation by anti-CD3/CD28 antibodies led to 1.7- and 2.7-fold nuclear increase in the amount of NF-κB p50 and p65, respectively. However, the activation of NF-κB remained unaffected by methanol treatment at all tested concentrations. The compound BAY 11-7082, an inhibitor of IκBα phosphorylation, reduced the nuclear content of p50/p65 in the same experiments to levels lower than those of the unstimulated control. AP-1 followed the same pattern observed for NF-κB with increase in the amount of c-Jun (10-fold) and c-Fos (20-fold) in the nucleus following activation by anti-CD3/CD28 antibodies (Figs. 2D and 2E). None of the examined concentrations of methanol had an effect on AP-1 c-Jun/c-Fos nuclear content. Instead, lymphocyte stimulation in the presence of the c-Jun N-terminal kinase inhibitor SP600125 or the combination of inhibitor drugs PD98059 (MEK1)/SB202190 (p38) produced about 50% of the maximal c-Jun/c-Fos nuclear content (*st*).

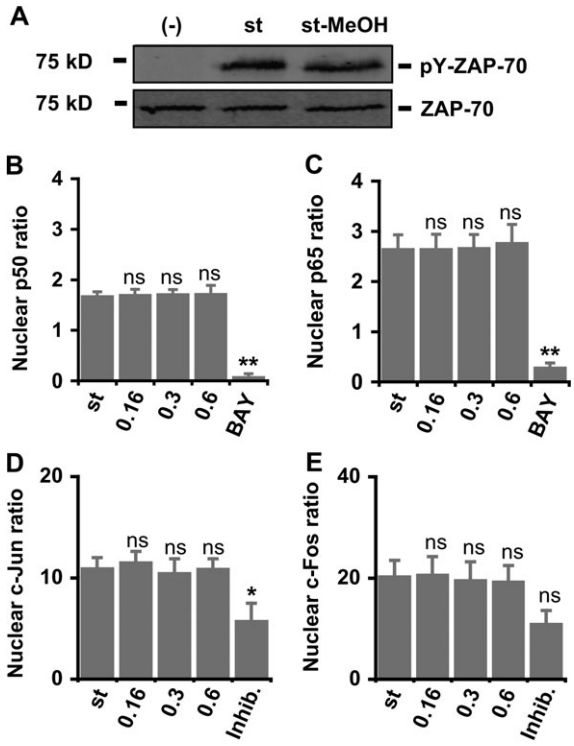

FIG. 2. Methanol impact on early TCR signaling and on transcription factor activation. (A) Methanol treatment does not affect ZAP-70 phosphorylation following TCR activation: Human peripheral blood T cells were stimulated for 3 min with anti-CD3/anti-IgG in presence (st-MeOH) or absence (*st*) of 0.6% (wt/vol) methanol and processed for SDS/polyacrylamide gel electrophoresis. One representative blot of three is shown (mean densitometric units ± SEM: 29.8 ± 6.9 [*st*]; 28.5 ± 7.9 [st-MeOH], $p > 0.05$). (B and C) NF-κB nuclear translocation does not change with isopropanol treatment: Human peripheral blood T cells were stimulated with anti-CD3/CD28 antibody–coated beads for 5 h in presence of 0.16, 0.3, and 0.6% (wt/vol) methanol. Nuclear extracts were incubated with immobilized p50- or p65-binding oligonucleotides in 96-well plates; the amount of bound transcription factor was assessed with a p50- or a p65-specific antibody by ELISA. The nuclear transcription factor ratio was calculated by dividing the sample value by the value of the unstimulated control in absence of methanol. Data is presented as means ± SEM (ns means not significant or $p > 0.05$, $^{**}p < 0.01$ relative to the *st* control group, $n = 3$ for B and $n = 3$ for C). (D and E) Methanol does not affect AP-1 nuclear translocation: Human peripheral blood T cells were stimulated as described in panels (B and C) in presence of the indicated amounts of methanol. Nuclear extracts were incubated with immobilized c-Jun– or c-Fos–binding oligonucleotides in 96-well plates; the amount of bound transcription factor was assessed with a c-Jun– or a c-Fos–specific antibody by ELISA. The nuclear transcription factor ratio was calculated by dividing the sample value by the value of the unstimulated control in absence of methanol. Data are presented as means ± SEM (ns, not significant $p > 0.05$, $^{*}p < 0.05$ relative to the *st* control group, $n = 3$ for D and $n = 3$ for E). Stimulation in presence of inhibitor compounds is indicated as follows: BAY: BAY 11-7082 (panels B and C); inhib.: SP600125 (panel D); inhib.: PD98059/SB202190 (panel E); other symbols are as in Figure 1A.

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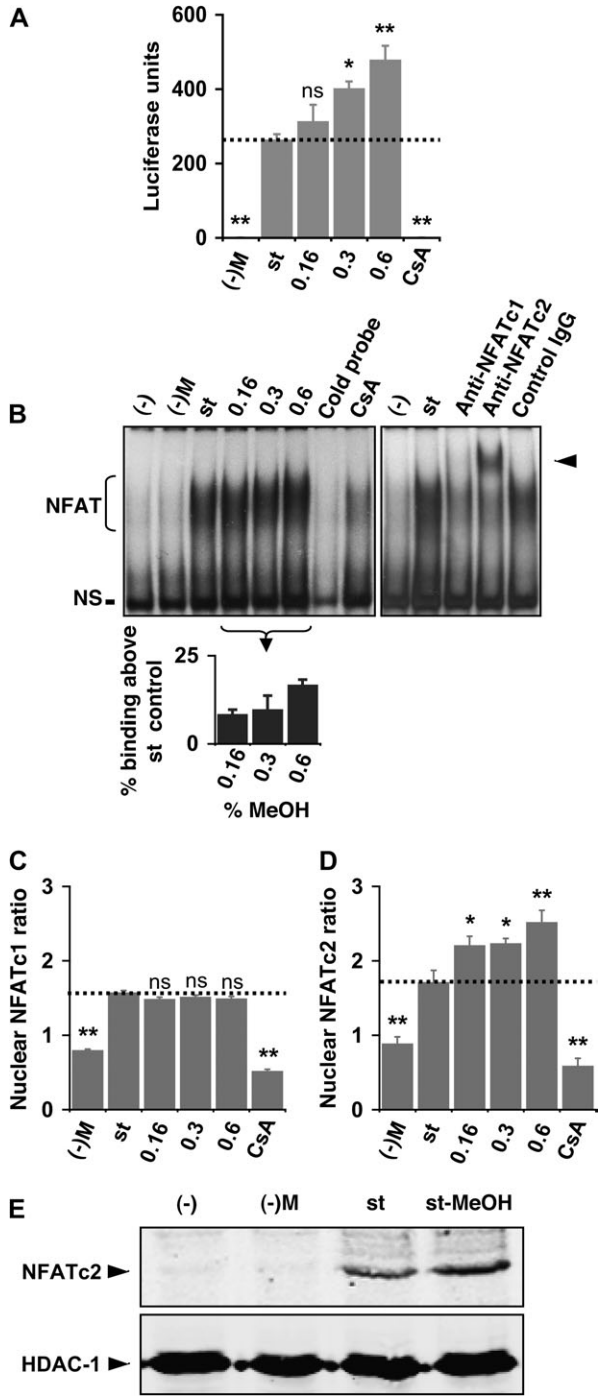

FIG. 3. Differential modulation of NFAT family members by methanol exposure *in vitro*. (A) Methanol increases the activation of a synthetic promoter containing NFAT binding sites: Jurkat-luciferase cells were stimulated with PMA/ionomycin in presence of 0.16, 0.3, and 0.6% (wt/vol) methanol for 5 h. Samples were lysed and assayed for luciferase activity. Results are presented as mean relative luciferase units per microgram of protein ± SEM (ns, not significant $p > 0.05$, $*p < 0.05$, $**p < 0.01$ relative to the *st* control group, $n = 4$). (B) Effect of methanol on the DNA-binding capacity of NFAT: Nuclear extracts were prepared from purified human peripheral blood T cells that have been stimulated with anti-CD3/CD28 antibody–coated beads for 1 h in presence of the indicated methanol concentrations and were analyzed by EMSA. One representative autoradiograph is depicted. The histogram under the autoradiograph presents the percentage NFAT binding above the level of the stimulated positive control (*st*). The densitometric analysis was prepared from three independent experiments. The detached right-hand side of the autoradiograph shows the supershift analysis with the anti-NFATc1 (7A6) and anti-NFATc2 (G1-D10) antibodies. The NFAT complex is indicated on the left, and the supershifted band is indicated by the arrow on the right. NS: nonspecific band. (C) Methanol does not change NFATc1 nuclear content: Purified human peripheral blood T lymphocytes were stimulated with anti-CD3/CD28 antibody–coated beads for 1 h in presence of 0.16, 0.3, and 0.6% (wt/vol) methanol. Nuclear extracts were incubated with immobilized NFAT-binding oligonucleotides in 96-well plates; the amount of retained transcription factor was assessed with an NFATc1-specific antibody by ELISA. The nuclear NFATc1 ratio was calculated by dividing the sample value by the value of the unstimulated control in absence of methanol. Data are presented as means ± SEM (ns: $p > 0.05$, $**p < 0.01$ relative to the *st* control group, $n = 3$). (D) Methanol upregulates NFATc2 nuclear content: Purified human peripheral blood T cells were stimulated as described in panel (C) in presence of the indicated amounts of methanol. Nuclear extracts were incubated with immobilized NFAT-binding oligonucleotides in 96-well plates; the amount of retained transcription factor was assessed with an NFATc2-specific antibody by ELISA. The nuclear NFATc2 ratio was calculated by dividing the sample value by the value of the unstimulated control in absence of methanol. Data are presented as means ± SEM ($*p < 0.05$, $**p < 0.01$ relative to the *st* control group, $n = 3$). (E) Measurement of NFATc2 in the nucleus by Western blot. Jurkat cells were stimulated for 15 min with anti-CD3/CD28/anti-IgG in presence (st-MeOH) or absence (*st*) of 0.6% (wt/vol) methanol and the nuclear fractions were processed by SDS/polyacrylamide gel electrophoresis. Histone deacetylase (HDAC-1) was used as a loading control. One representative blot of four is shown (Mean densitometric units ± SEM: 26.2 ± 2.6 [*st*]; 35.9 ± 3.1 [st-MeOH], $p < 0.05$, $n = 4$). Figure symbols: CsA indicates stimulation in presence of cyclosporin A; other symbols are as in Fig. 1A. The dashed line in (A) indicates the promoter activation level of the stimulated positive control (*st*). The dashed lines in (C) and (D) indicate the ratio of the relevant NFAT in the stimulated positive control (*st*).

Methanol Increases IL-2 Production in Activated T Cells via Differential Modulation of NFAT Family Members

Given the apparent lack of effect of methanol on the pathways leading to the activation of NF-κB and AP-1, we have turned our attention to the NFAT family of transcription factors. NFAT synergizes with NF-κB and AP-1 to promote the maximal activation of the IL-2 gene (Attema *et al.*, 2002). First, we have tested the impact of different concentrations of methanol on a stable Jurkat subline carrying a firefly luciferase gene that is highly responsive to the Ca^{++}/NFAT signaling pathway. This reporter gene is driven by a synthetic minimal IL-2 promoter containing three copies of the human distal IL-2 NFAT binding site (Désy *et al.*, 2008; Durand *et al.*, 1988; Shaw *et al.*, 1988). Methanol was capable of augmenting the luciferase activity triggered by PMA/ionomycin in these cells in a dose-dependent manner (Fig. 3A). Methanol concentrations as low as 0.3% had a significant enhancement effect on IL-2 transcription as indicated by a 52% increase in luciferase activity in relation to the untreated positive control (*st*). The upregulation reached 81% at the highest dose. Similar results were obtained by anti-CD3 antibody cross-linking in Jurkat-luciferase cells (data not shown).

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The interference of methanol with NFAT activation that was suggested by the results of the reporter assays was corroborated by data obtained from EMSAs. Nuclear extracts were prepared from human peripheral blood T lymphocytes that had been activated or not in presence of methanol. Figure 3B shows one representative experiment in which there was more binding of the probe to NFAT in the nuclear extracts from methanol-treated samples in a dose-dependent fashion. The percentage increase in binding in relation to the untreated positive control (*st*) was measured by densitometry in three independent experiments and plotted in the associated histogram. Figure 3B also presents a supershift analysis with antibodies against NFATc1 and NFATc2. Figure 3B shows that in our experimental conditions, 1 h-stimulation with anti-CD3/CD28 antibodies, both NFAT proteins are available in the nucleus to form complexes on the target promoter regions.

NFATc1 and c2 are highly homologous in their DNA-binding domains and are capable of recognizing the same nucleotide sequences. The similarity between these transcription factors is also reflected in the distribution of calcineurin-docking sites and phosphorylated serine–rich motifs (Macian, 2005; Srinivasan and Frauwirth, 2007). Thus, we predicted that the upregulatory transcriptional effect of methanol would extend to both NFAT family members, given their overall structural similarity. The results of the transcription factor activation assays presented in Figures 3C and 3D disprove that hypothesis. Although T-cell activation increased the nuclear content of NFATc1 1.6-fold, there was no additional change when methanol was added even at the highest concentration (Fig. 3C).

T-cell activation also led to a 1.7-fold increase in NFATc2 nuclear content but activation in presence of methanol raised the NFATc2 nuclear content 2.2-fold (29% more than activation alone) at the lowest concentration and 2.5-fold (47% more than activation alone) at the highest concentration (Fig. 3D). The calcineurin inhibitor cyclosporin A was used as a control in the same stimulatory conditions and reduced NFATc1 and c2 to about 50% of the nuclear content of resting T cells. The methanol-induced effect on NFATc2 was corroborated by Western blot analysis of the nuclear fraction of activated T cells which, showed a 27% increase in the dephosphorylated form of the transcription factor in alcohol-treated samples (Fig. 3E).

Release of Proinflammatory Cytokines by Human Peripheral T Lymphocytes Is Upregulated by Methanol

The DNA consensus motif 5′-T/AGGAAA-3′ recognized by NFAT is found in the 5′ region of several genes encoding proteins of immunological relevance (Macian, 2005; Porter and Clipstone, 2002). Thus, our finding that methanol increases IL-2 production through the dysregulation of NFATc2 could herald a wider impact of this alcohol on the immune system. The potentially extended immune modulatory effect of methanol was examined by checking two other cytokines that are central

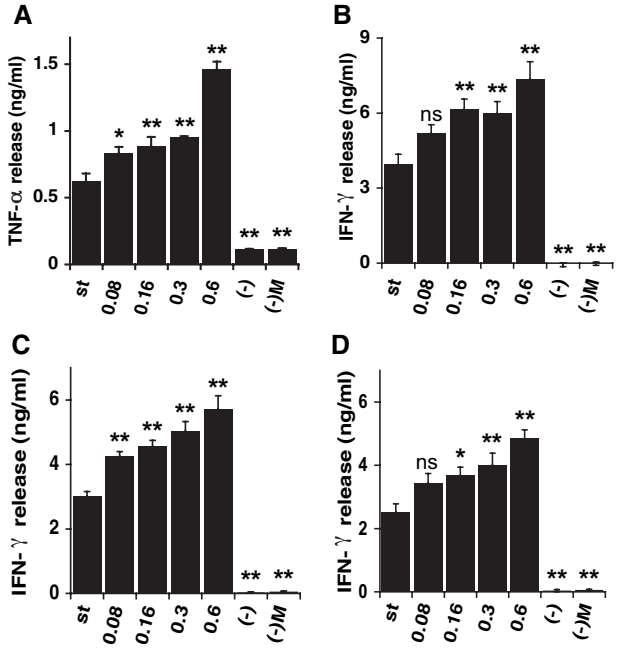

FIG. 4. Effect of methanol on proinflammatory cytokine release by human peripheral blood T lymphocytes *in vitro*. (A) Methanol increases TNF-α production in peripheral blood T lymphocytes: T cells were stimulated with anti-CD3/CD28 antibody–coated beads for 5 h in presence of 0.08, 0.16, 0.3, and 0.6% (wt/vol) methanol. The TNF-α concentration in the supernatants was measured by ELISA. Results are presented as means ± SEM (*$p < 0.05$, **$p < 0.01$ relative to the *st* control group, $n = 3$). (B) Methanol increases IFN-γ production in peripheral blood T lymphocytes: T cells were stimulated and treated as in panel (A). The amount of IFN-γ released in the supernatants was measured by ELISA. Results are presented as means ± SEM (ns, not significant $p > 0.05$, **$p < 0.01$ relative to the *st* control group, $n = 4$). (C) Methanol increases IFN-γ production in $CD8^+$ T lymphocytes: Purified $CD8^+$ T cells were stimulated and treated as in panel (A). The IFN-γ concentration in the supernatants was measured by ELISA. Results are presented as means ± SEM (**$p < 0.01$ relative to the *st* control group, $n = 7$). (D) Methanol increases IFN-γ production in $CD4^+$ T lymphocytes: Purified $CD4^+$ T cells were stimulated and treated as in panel (A). ELISA results are presented as means ± SEM (ns: $p > 0.05$, *$p < 0.05$, **$p < 0.01$ relative to the *st* control group, $n = 4$). Figure symbols are as in Figure 1A.

to the immune response and that are known to be inducible by the Ca^{++}/NFAT signaling pathway: TNF-α and IFN-γ (Macian, 2005; Porter and Clipstone, 2002; Teixeira *et al.*, 2005; Tsytsykova and Goldfeld, 2000). Peripheral T lymphocytes were activated with anti-CD3/CD28 antibodies in presence of different methanol dilutions. Similarly to IL-2, TNF-α and IFN-γ peaked at later times but were already measurable 5 h after stimulation (mean = 0.65 ng/ml ± 0.04 SEM, $n = 3$ for TNF-α and 3.93 ng/ml ± 0.41 SEM, $n = 4$ for IFN-γ). The cytokine release increased in a dose-dependent fashion in methanol-exposed cells, with an average augmentation ranging from 33 to 133% for TNF-α (Fig. 4A) and 32 to 86% for IFN-γ (Fig. 4B).

Next, we examined whether the immune modulatory impact of methanol was equally extended to different T-cell subsets by looking at their IFN-γ production. Peripheral T lymphocytes

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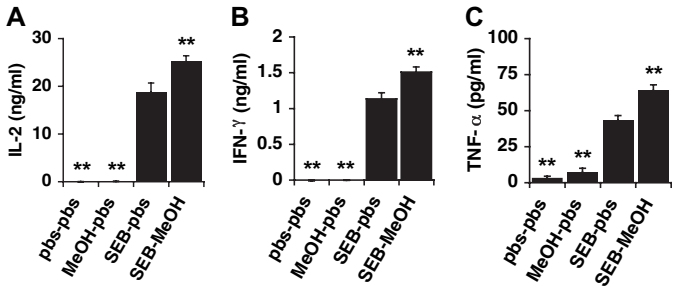

FIG. 5. Immunological impact of methanol *in vivo*. (A) IL-2 production is upregulated in mice acutely exposed to methanol: BALB/c mice received 5 μg SEB sc plus the ip injection of either 2 g/kg methanol (SEB-MeOH group) or saline (SEB/pbs group). Additional control groups received either saline sc plus methanol ip (pbs-MeOH) or saline only (pbs/pbs). Animals were sacrificed 4 h after injections, and serum IL-2 levels were quantified by ELISA. Results are presented as means ± SEM (**$p < 0.01$ relative to the SEB/pbs group, $n = 6$ per group). (B) IFN-γ production increases in mice acutely exposed to methanol: BALB/c mice were injected and sacrificed as above. Serum IFN-γ was measured by ELISA. Means ± SEM are shown (**$p < 0.01$ relative to the SEB/pbs group, $n = 5$ per group). (C) Acute exposure to methanol augments TNF-α production: BALB/c mice were injected and sacrificed as above. The mean TNF-α serum concentration ± SEM was determined by ELISA (**$p < 0.01$ relative to the SEB/pbs group, $n = 7$ per group).

were separated in $CD8^+$ (Fig. 4C) and $CD4^+$ (Fig. 4D) subpopulations and activated with anti-CD3/CD28 antibodies in presence or absence of methanol. Figure 4C shows that methanol concentrations as low as 0.08% possessed an immune modulatory effect. Treatment with 0.08, 0.16, and 0.3% methanol upregulated the IFN-γ release by stimulated $CD8^+$ cells relative to the untreated positive control (*st*) by 41, 51, and 67%, respectively. The highest methanol concentration almost doubled the production of the cytokine in stimulated cells. A similar trend was observed with $CD4^+$ lymphocytes: Methanol concentrations of 0.16, 0.3, and 0.6% raised the IFN-γ release above the level achieved by activation alone by 45, 59, and 92%, respectively (Fig. 4D). Lymphocytes were > 97% viable at all tested methanol concentrations (data not shown and Fig. 1A).

Methanol Upregulates the Production of IL-2 and Proinflammatory Cytokines In Vivo

After having demonstrated the upregulatory impact of methanol treatment on IL-2, TNF-α, and IFN-γ production by lymphocytes *in vitro*, we examined the relevance of these findings in a mouse model of acute methanol intoxication. Mice were administered methanol ip, 2 g/kg, to generate a mean BAC of 238 mg/dl after 30 min (238.4 ± 13.7 SEM, $n = 3$). Cytokine production was triggered *in vivo* by sc injection of the superantigen SEB. In these conditions, T lymphocytes with the relevant TCR Vβ chains undergo transient activation, cell proliferation, and begin massive cytokine production (Tsytsykova and Goldfeld, 2000). As anticipated, SEB alone induced IL-2 (18.8 ng/ml; Fig. 5A), IFN-γ (1.1 ng/ml; Fig. 5B), and TNF-α (43.3 pg/ml; Fig. 5C). Methanol treatment enhanced the SEB-stimulated cytokine production substantially: 33% for IL-2/IFN-γ and 47% for TNF-α. Serum levels of 25.1 ng/ml IL-2, 1.5 ng/ml IFN-γ, and 64.1 pg/ml TNF-α were detected 4 h after SEB/methanol administration. The differences in cytokine serum levels between animals treated and untreated with methanol were statistically significant as indicated in Figure 5.

DISCUSSION

Short-chain alcohols have the ability to partition into cell membranes and to denature proteins by promoting the formation of α-helices and/or by disrupting tertiary structures; these effects are largely nonspecific and are typically observed at high concentrations (> 500mM) (Dwyer and Bradley, 2000). At more physiologically relevant concentrations, alcohols have been shown to induce loss of function of specific proteins such as ion channels, neurotransmitter receptors, enzymes, and adhesion molecules (Aryal *et al.*, 2009; Jung *et al.*, 2005; Ren *et al.*, 2003; Shahidullah *et al.*, 2003). Structural and biophysical data suggest that binding to the target proteins occurs at discrete sites that are constituted by hydrophobic pockets lined by nonpolar amino acids (Aryal *et al.*, 2009; Dwyer and Bradley, 2000; Jung *et al.*, 2005; Ren *et al.*, 2003; Shahidullah *et al.*, 2003). These interactions would ultimately produce a local distortion and alteration in protein function.

Many of the biological effects of ethanol on the immune system have been attributed to reduced nuclear translocation of NF-κB (Saeed *et al.*, 2004; Szabo *et al.*, 2007). In addition to ethanol, isopropanol is the only other alcohol that has been studied with regard to the transcriptional regulation of its immune modulatory properties (Désy *et al.*, 2008). Isopropanol differs from ethanol in that it does not compromise NF-κB but reduces the nuclear content of the transcription factors AP-1 and NFAT. We have assumed on the grounds of structural similarity to the above alcohols that methanol might possess an acute immunosuppressive effect possibly involving an alteration of similar activation pathways. Instead, our results revealed that methanol synergizes with the activating stimuli and augments the T-cell cytokine production. Methanol *per se* did not induce any effect in our experiments, indicating that its target(s) must be preactivated (Figs. 1, 3, 4, and 5 and data not shown). The source of T-cell activation in healthy humans may derive from the exposure to environmental non-self antigens or from the reactivation of persistent infection by common agents such as Epstein-Barr virus that often lead to clonal T-cell expansions (de Campos-Lima *et al.*, 1997; Lalonde *et al.*, 2007). Antigen-independent activation of T cells also occurs in physiological conditions and is important to maintain the T-cell repertoire in the steady state (Goldrath *et al.*, 2002). It is noteworthy that the inebriation that follows the excessive consumption of any alcohol, including methanol, is frequently associated to trauma and, thus, may increase the risk of infection and pathogen-driven T-cell activation (Fitzgerald et al.,

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2007; Saxena *et al*., 1987). The cytokine release upregulation reported here was observed *in vitro* at methanol concentrations as low as 0.08% (25mM) as measured by IL-2, TNF-α, and IFN-γ release in T cells. These concentrations are equal to or lower than those of short-chain alcohols used in previous studies that reported a statistically significant impact on immune cells (Goral and Kovacs, 2005; Saeed *et al*., 2004; Szabo *et al*., 2007; Taieb *et al*., 2002). Another aspect that distinguishes the methanol effect is the lack of interference with the pathways leading to the activation of NF-κB and AP-1. Methanol does affect the NFAT nuclear content but very differently from isopropanol. Although the NFATc1 activation remained unaffected even at the highest doses tested, methanol induced an enhancement of the NFATc2 nuclear content following TCR engagement.

Conceivably, alcohols could modulate the immune cell activation by interfering with several steps along the cascade that relay the signal generated by the recognition of an antigenic ligand on the cell membrane to the nucleus. Alcohols could partition into the cell membrane altering the capacity of microdomains to recruit and/or retain molecules involved in signaling, thus compromising the formation of the immunological synapse. The inhibitory effect of ethanol on the lipopolysaccharide-induced Toll-like receptor 4 signaling in macrophages has been suggested to unfold along these lines (Szabo *et al*., 2007). Methanol could affect surface molecules, such as the TCR, directly by inducing conformational changes or, indirectly, by disrupting lipid-protein interactions. We tested this possibility by measuring ZAP-70 phosphorylation in methanol-treated samples. This tyrosine kinase has a critical role in signaling downstream of the TCR; it binds to the CD3-zeta and phosphorylates the key adaptor protein LAT (Smith-Garvin *et al*., 2009). Our results indicate that methanol acts downstream of the cell membrane as ZAP-70 activation proceeds normally following TCR triggering. It is still possible, however, that methanol could affect lipid rafts at higher concentrations not examined in this study.

The phosphorylation cascade downstream of ZAP-70 leads to an increase in intracellular Ca^{++} (Smith-Garvin *et al*., 2009); the phosphatase calcineurin senses the elevated Ca^{++} levels and, in presence of calmodulin, activates the cytoplasmic moieties of the NFAT transcription factors, thereby allowing their migration into the nucleus (Smith-Garvin *et al*., 2009). Ethanol and longer alcohols have been shown to bind calmodulin at discrete sites enhancing its affinity for Ca^{++} and changing its impact on calcineurin function (Ohashi *et al*., 2004). Methanol could conceivably change the phosphatase activity of calcineurin perhaps indirectly via calmodulin binding. We have tested this hypothesis by measuring the calmodulin/calcineurin activity in activated cells and found it to be preserved in presence of methanol (data not shown). In addition, we have observed that the calcineurin inhibitor cyclosporin A overrides the alcohol-induced NFATc2 upregulation and the consequent increase in cytokine release (data not shown).

Methanol could also influence the shuttling of NFATc2 into the nucleus by interfering with the function of importin β1. However, one would expect the closely related NFATc1 to be affected; a more general impact extended to other transcription factors that bind importin β1, such as c-Fos, would be anticipated as well (Malnou *et al*., 2007). The similarity of the poststimulation nuclear content of c-Fos and NFATc1 in methanol-treated and untreated samples does not support this contention. By the same token, methanol could interfere with the activity of maintenance kinases that keep the NFAT in the cytoplasm in resting T cells, such as casein kinase 1 (CK1). However, the differential modulation of NFATc2 is unlikely to result from the dysregulation of the activity of CK1 because this enzyme recognizes the serine-rich motif SRR1 in both NFATc1 and c2 (Macian, 2005; Okamura *et al*., 2004; Srinivasan and Frauwirth, 2007). Another possibility is that the alcohol inhibits the nuclear export of the NFAT proteins. Constitutive kinases, such as the glycogen synthase kinase 3 (GSK3) and CK1, as well as inducible kinases, such as p38 and the c-Jun N-terminal kinase (JNK), have been implicated in the rephosphorylation of the NFAT proteins that enables their export from the nucleus (Macian, 2005; Okamura *et al*., 2004; Srinivasan and Frauwirth, 2007). GSK3 and CK1 act on both NFATc1/c2 nuclear factors making these enzymes unlikely alcohol targets (Beals *et al*., 1997; Macian, 2005; Okamura *et al*., 2004; Srinivasan and Frauwirth, 2007). The same reasoning applies to the recently identified dual-specificity tyrosine phosphorylation–regulated kinase (DYRK) 1A, a priming kinase that allows additional phosphorylation by GSK3 and CK1 of both NFATc1/c2 nuclear factors (Arron *et al*., 2006; Gwack *et al*., 2006). As for the inducible kinases, JNK specifically targets the nuclear factor that is unchanged by methanol treatment, NFATc1 (Chow *et al*., 2000). On the other hand, p38 specifically phosphorylates NFATc2 at the first serine of the SRR1 motif (Gómez del Arco *et al*., 2000); methanol could conceivably induce an augmented retention of this nuclear factor by inhibiting p38. Nevertheless, p38 postactivation expression, phosphorylation pattern, and kinase activity were not different in alcohol-treated and untreated samples (data not shown). We favor the possibility that methanol interacts directly with NFATc2 perhaps inducing a conformational change that masks the nuclear-export signal or reduces the accessibility of phosphorylation sites (Fig. 6). This matter will be addressed in future studies.

Most of the acute toxicity of methanol has been attributed to its liver-produced formate metabolite (Barceloux *et al*., 2002). In this work, we have focused on the direct immune toxicity of the parent compound *in vitro*. In addition, we have used an *in vivo* model that largely excludes the metabolite components of the acute intoxication. Rodents break down formate very efficiently and even alcohol doses substantially higher than the one we have used in the *in vivo* experiments are not sufficient to induce formate accumulation (Lanigan, 2001; Shelby *et al*., 2004). Thus, the experimental dose and timeframe adopted in

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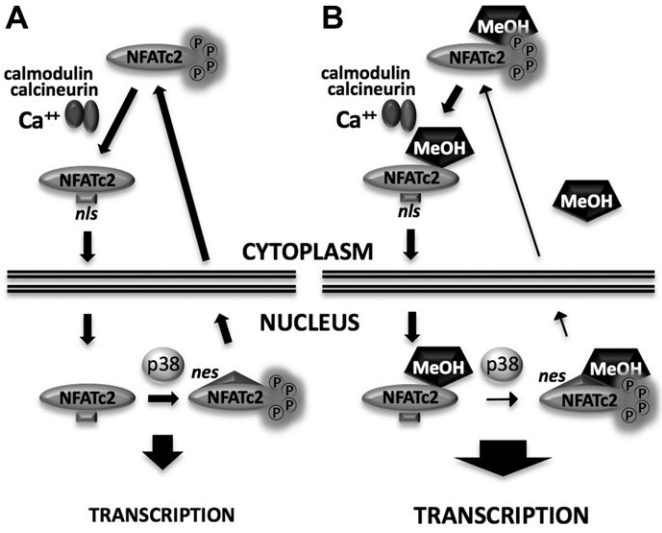

FIG. 6. Hypothetical model of the methanol transcriptional effect. The diagram illustrates a simplified model of NFATc2 activation in absence (A) or presence (B) of methanol. TCR triggering increases cytoplasmic Ca^{++} thereby activating calmodulin and unleashing the phosphatase activity of calcineurin. Dephosphorylation exposes the nuclear localization signal (nls) and allows the translocation of NFATc2 into the nucleus. Multiple kinases (GSK3, CK1, DYRK1A, and p38) rephosphorylate the NFAT proteins leading to their export from the nucleus; only p38 is indicated. Methanol may interact directly with NFATc2 producing a conformational change that masks the nuclear export signal (nes) or reduces the accessibility of phosphorylation sites.

our animal model facilitate the analysis of the immune impact attributable to methanol during the first hours of acute intoxication.

To put in perspective the relevance of the alcohol concentrations chosen for our studies, one should consider a few aspects of the methanol pharmacokinetics in the context of acute intoxication. Methanol is readily absorbed by the human gastrointestinal tract with peak blood levels achieved at 30–60 min after ingestion (Barceloux *et al*., 2002). As methanol *per se* does not have a very conspicuous clinical footprint and causes only a transient mild inebriation, the patients usually seek help much later after ingestion when the formate concentration builds up causing metabolic acidosis, visual disturbances, severe central nervous system depression, and gastrointestinal symptoms (Barceloux *et al*., 2002; Liu *et al*., 1998). The blood methanol concentration upon presentation at the hospital varies; the mean value was 179 mg/dl (0.179% or 56 mmol/l) in a large retrospective study conducted in the metropolitan Toronto area and 170 mg/dl (0.17% or 53 mmol/l) in the patients who entered the prospective trial of fomepizole for the treatment of methanol poisoning (Brent *et al*., 2001; Liu *et al*., 1998). These concentrations measured at the hospital are much lower than the peak values in the first hour postingestion because it may take up to 2 days for the patients to look for professional help. In saturating conditions of acute intoxication, methanol liver clearance follows zero-order kinetics with an estimated rate of 8.5 mg/dl/h (Jacobsen *et al*., 1988). Thus, a patient who presents at 48-h postingestion a methanol blood level just barely above the accepted treatment threshold of 20 mg/dl (0.02% or 6.24 mmol/l) would have had an earlier peak concentration of 420 mg/dl (0.42% or 131 mmol/l). This analysis is in line with a recent retrospective study of 173 poisoned patients for whom time-related blood alcohol data were available (Kostic and Dart, 2003); the individuals who presented themselves 24–48 h after ingestion had a median blood concentration of 110 mg/dl (0.11% or 34 mmol/l) as opposed to those few who sought medical attention within 6 h after ingestion and had a median methanol concentration of 300 mg/dl (0.3% or 94 mmol/l). Therefore, all methanol concentrations in our *in vitro* experiments, including the highest concentration used, are clinically relevant as they can either be measured upon hospital admission in surviving patients or can be inferred to have occurred during the first hours postingestion (Brahmi *et al*., 2007; Brent *et al*., 2001; Gonda *et al*., 1978; Hantson *et al*., 2000; Kostic and Dart, 2003; Lushine *et al*., 2003; Martens *et al*., 1982; Verhelst *et al*., 2004; Wu *et al*., 1995). In our *in vivo* model, we have injected mice ip with 2 g/kg methanol to generate a BAC of 238 mg/dl (0.238% or 74 mmol/l) after 30 min; in clinical terms, this time-related concentration compares with the peak methanol level in humans who present at 24-h postingestion a blood concentration of 42.5 mg/dl (0.04% or 13 mmol/l) (Jacobsen *et al*., 1988; Kostic and Dart, 2003). In addition, the dose we have chosen is well within the concentration range that we have shown to be biologically active *in vitro* (starting at 0.08% or 25mM). Our results indicate that during this state of acute intoxication, the animals had an enhanced immune reaction as judged by their ability to release IL-2, IFN-γ, and TNF-α in the serum in response to SEB.

One very peculiar aspect of methanol poisoning is the somewhat selective toxic injury inflicted on the basal ganglia, particularly the putamina, which may lead to parkinsonism (Barceloux *et al*., 2002; Reddy *et al*., 2007). There is evidence implicating the proinflammatory cytokine tumor necrosis factor in the pathogenesis of the most common cause of parkinsonism, Parkinson's disease (McCoy *et al*., 2008; Mogi *et al*., 1994). Our data show that NFATc2 is upregulated in methanol-treated cells; this transcription factor is capable of binding the promoter regions of multiple cytokine genes (Porter and Clipstone, 2002), including TNF-α. Indeed, we have found that methanol upregulates the TNF-α production both *in vitro* and *in vivo* (Figs. 4 and 5). These results feed the speculation that the methanol toxicity on the basal ganglia could be initiated by a conducive proinflammatory cytokine environment generated in the early phase of acute intoxication. By the same token, the upregulation of proinflammatory cytokines could play a role in the pathophysiology of methanol-induced acute pancreatitis, a condition that has been overlooked so far but that may be identified in half of the patients with severe acute intoxication (Hantson and Mahieu, 2000). These are tantalizing possibilities well worth investigating in future studies. If confirmed, they

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may provide the basis for the establishment of anti-inflammatory measures to counteract the proinflammatory properties of methanol in acutely poisoned patients. To our knowledge, this is the first in depth characterization of the immune toxicity of methanol in human lymphocytes. Our findings are directly relevant in the context of acute methanol intoxication and constitute a rationale for the inclusion of immunological end points into the design of future studies to address methanol exposure.

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