4. The dysregulation of the monocyte/macrophage effector function induced by isopropanol is mediated by the defective activation of distinct members of the AP-1 family of transcription factors

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Short title: Immunomodulation by isopropanol.

Résumé

L'isopropanol est la deuxième cause la plus commune d'intoxication aiguë aux alcools à courte chaîne. Les effets immunomodulateurs des alcools à chaîne courte autre que l'éthanol s'exercent par une interférence avec l'activation du *nuclear factor of activated T cells* (NFAT) avec ou sans la participation additionnelle de l'*activator protein-1* (AP-1). Dans ce chapitre, nous examinons l'effet immunomodulateur de l'isopropanol dans des conditions non-dépendantes de NFAT : soit, la sécrétion de cytokines inflammatoires par des monocytes stimulés au lipopolysaccharide (LPS). Notre hypothèse était que, dans ce contexte, l'isopropanol n'aurait qu'un effet ténu ou encore aucun effet.

À notre étonnement, le défaut d'activation d'AP-1 s'est avéré suffisant pour causer une dérégulation sévère et dose-dépendante de l'activité des monocytes *in vitro,* et ce à des concentrations aussi basses que 0,16% (26 mM). Trois effets ont été observés : l'interleukine (IL)-1β et l'IL-8 demeurent inchangés; l'IL-6 est accru; tandis que le TNF-α et CCL2 sont diminués. Les fonctions effectrices des macrophages dérivés des monocytes sont aussi compromises.

Nos résultats montrent que, dans des monocytes activés au LPS, la signalisation précoce en aval du *Toll-like receptor 4* (TLR4) est préservée puisque l'isopropanol ne change pas l'activité kinase de l'*IL-1 receptor associated kinase 1* (IRAK1). Les sentiers de signalisation de NF-κB et des MAPK p38 et JNK sont demeurés insensibles à l'alcool. À l'opposé, l'activation de l'*extracellular signal-regulated protein kinase* (ERK) et ultimement de c-Fos et JunB est réduite.

La dérégulation des cytokines induite par l'alcool a été confirmée dans un modèle murin d'intoxication à l'isopropanol dont la production de TNF-α en réponse au LPS a pratiquement été abolie. L'ampleur de cet effet de l'isopropanol est telle que des souris ont pu être sauvées d'un choc toxique induit par le LPS.

Ces données s'ajoutent au peu de connaissances disponibles sur l'immunotoxicologie de l'isopropanol, un produit chimique ubiquitaire auquel la population est grandement exposée.

Abstract

Isopropanol is the second most common cause of short-chain alcohol acute intoxication. Non-ethanolic short-chain alcohols mediate their immunomodulatory effect by interfering with NFAT activation with or without additional AP-1 involvement. In the present study, we examined the immunomodulation induced by isopropanol in conditions that are not reliant on NFAT: the inflammatory cytokine response of lipopolysaccharide-stimulated monocytes. Our hypothesis was that isopropanol acute exposure would have an attenuated effect or no consequence in this setting. To our surprise, the impairment of AP-1 activation was sufficient to mediate a severe and dose-dependent phenotype in human monocytes *in vitro* at alcohol concentrations as low as 0.16% (or 26 mM). There were three outcomes: IL-1β/IL-8 were unaltered; IL-6 was up-regulated; and TNF-α/CCL2 were down-regulated. The effector function of human monocyte-derived macrophages was also compromised. Our results showed that TLR4 early signaling was preserved, as isopropanol did not change the IRAK1 kinase activity in LPS-stimulated cells. The NF-κB signaling cascade and the p38/JNK modules of the MAPK pathway were alcohol-insensitive. Conversely, the activation of ERK and, ultimately, of c-Fos and JunB were impaired. The alcohol-induced cytokine dysregulation was confirmed in a mouse model of isopropanol intoxication in which the production of TNF-α in response to LPS challenge was virtually abolished. The magnitude of this alcohol effect was sufficiently high to rescue animals from LPS-induced toxic shock. Our data contribute to the dismal body of information on the immunotoxicology of isopropanol, one of the most ubiquitous chemicals to which the general population is significantly exposed.

Keywords: immunomodulation, immunosuppression, isopropanol, monocyte, macrophage, cytokine, immunotoxicology

Introduction

Deliberate or accidental exposure to isopropanol is the second most common cause of acute alcohol intoxication in North America with over 20,000 cases recorded each year

(Bronstein *et al.*, 2009). Alcoholic patients, children, and suicidal individuals are the ones most likely to be poisoned (Zaman *et al.*, 2002). Isopropanol is used in multiple industrial applications but is also easily available to most consumers as rubbing alcohol and as an ingredient of several household products such as hand-sanitizing gels. Although there is a considerable body of information on the general biological effects of isopropanol in animal models of acute, subchronic and chronic exposure (Burleigh-Flayer *et al.*, 1994, 1997; Kapp *et al.*, 1996; Kasuga *et al.*, 1992), surprisingly little is known about its immunotoxicology. Conversely, it is well documented that ethanol modulates the immune system directly or indirectly by interfering with the function of a variety of cells such as T lymphocytes, monocytes, macrophages, dendridic cells, neutrophils, and endothelial cells (Goral and Kovacs, 2005; Oak *et al.*, 2006; Saeed *et al.*, 2004; Szabo *et al.*, 2007; Taieb *et al.*, 2002; Zhao *et al.*, 2003). In our previous work, we have demonstrated that the molecular events that underlie the immunomodulation induced by short-chain alcohols have different flavors that are specific to each alcohol despite their considerable structural similarity (Désy *et al.*, 2008, 2010). Although many of the biological effects of ethanol on the immune system have been attributed to a dysregulation of the nuclear factor-κB (NF-κB) signaling pathway, other short-chain alcohols seem to keep this signaling cascade unaltered (Désy *et al.*, 2008, 2010; Oak *et al.*, 2006; Saeed *et al.*, 2004; Szabo *et al.*, 2007). Instead, they mediate their immunomodulation by interfering with the activation of the nuclear factor of activated T cells (NFAT) family of transcription factors with or without additional involvement of the activator protein-1 (AP-1). Thus, while methanol up-regulates NFATc2 nuclear translocation in lymphocytes (Désy *et al.*, 2010), isopropanol down-regulates the activation of NFATc1 and AP-1 in T lymphocytes and NK cells (Désy *et al.*, 2008). In the present study, we sought to examine the immunomodulation induced by isopropanol in a stimulation model that is less reliant on the NFAT family of transcription factors. We chose to study the monocyte inflammatory cytokine response to lipopolysaccharide (LPS) because it involves a well-defined signal transduction pathway that leads to NF-κB and AP-1 activation and does not require NFAT (Kawai and Akira, 2010). Our hypothesis was that isopropanol acute exposure would have an attenuated effect or no consequence in this setting. Our results revealed that the impairment of AP-1

activation was sufficient to cause a severe and dose-dependent phenotype in human monocytes *in vitro* at alcohol concentrations as low as 0.16% (or 26 mM). Similarly to what was reported for T lymphocytes and NK cells (Désy *et al.*, 2008), isopropanol did not change the NF-κB signaling cascade in activated monocytes; nevertheless, it produced an immune dysregulation that was mediated by ERK and, ultimately, by the c-Fos and JunB members of the AP-1 family of transcription factors. The immunosuppressive potential of this alcohol was validated *in vivo* and had sufficiently high magnitude to rescue mice from LPS-induced toxic shock.

Materials and Methods

Cell isolation, culture, and stimulation. 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. Monocytes were isolated from mononuclear cells by plastic adherence (Fuss *et al.*, 2009; Szabo and Mandrekar, 2008) and maintained in RPMI 1640 (Invitrogen Canada, Burlington, Canada) supplemented with 10% heat-inactivated FBS (BioCell Inc., Drummondville, Canada). Monocyte-derived macrophages were generated by cultivating monocytes in RPMI 1640 supplemented with 18% heat-inactivated autologous serum for 8 days as described (Szabo and Mandrekar, 2008). Human primary cells were used in most *in vitro* experiments to strengthen the quality of the data. Established cell lines were used only in a few instances to limit the volume of blood drawn from the donors.

The human monocytic line Mono Mac 6 (Ziegler-Heitbrock *et al.*, 1988) and the murine macrophage cell line P388D1 (Koren *et al.*, 1975) were cultivated in RPMI 1640 containing 10% FBS. Murine monocytes were purified from bone marrow or from spleens (Swirski *et al.*, 2009) by using an antibody-based negative selection kit with magnetic nanoparticles (StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions.

Monocytes and macrophages were activated for cytokine production for 24 h at 37°C with 1 μg/ml ultra pure lipopolysaccharide from *Escherichia coli* O111:B4 (List Biological Laboratories, Campbell, CA). When indicated, alternative cell activation protocols were used: protocol a, 15 min stimulation with 1 μg/ml LPS at 37^{o}C (Western blot and *in vitro* kinase assay); protocol b, 1 h stimulation with 1 μg/ml LPS at 37^{o}C (Transcription factor activation assays); protocol c, 1 h stimulation with labeled *E. coli* at 37^{o}C (Flow cytometry-based phagocytosis analysis); and protocol d, 2 h stimulation with labeled *E. coli* at 37^{o}C (Microscopy-based phagocytosis analysis).

The data on primary cells in the various assays utilized in this work was obtained from multiple donors. The means ± SEM from independent experiments are indicated in each figure. In addition, two technical replicates per sample were used within each independent ELISA experiment. Isopropanol was purchased from BDH (Toronto, Canada) and was 99.5% pure.

Cytokine analysis. Measurements of human TNF-α, IL-1β, IL-6, and IL-8 or murine IL-6 in cell culture supernatants and murine TNF-α, IL-6, and CCL2 in serum samples were performed with specific cytokine ELISA kits according to the manufacturer's instructions (BioLegend, San Diego, CA). Human CCL2 levels were measured with the human CCL2 (MCP-1) ELISA Ready-SET-Go! kit (Ebioscience, San Diego, CA) as recommended by the manufacturer. Supernatants from human cells were used undiluted for IL-1β analysis or diluted 1:20, 1:10, 1:100, and 1:4 for TNF-α, IL-6, IL-8, and CCL2 measurements, respectively. Supernatants from murine cells were diluted 1:4 for IL-6 analysis. Murine sera were diluted 1:25, 1:50, and 1:5 for TNF-α, IL-6, and CCL2 measurements, respectively. Briefly, 96-well plates were coated with the relevant capture antibody and incubated with serially diluted standards or unknown samples; then, they were washed and incubated with the biotinylated detection antibody followed by streptavidin-horseradish peroxidase. The plates were read at 450 nm after sequential treatment with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution and phosphoric acid.

It is worth mentioning that short chain alcohols may interfere with the sensitivity of certain TNF-α ELISA kits at high concentrations (von Maltzan and Pruett, 2011). In our experiments, TNF-α ELISA assays were conducted with supernatants diluted 20 times and harvested after a 24 h-long incubation period with LPS at 37°C. Isopropanol has an evaporation rate of 2.83 as compared to 1.4 for ethanol and 0.3 for water (n-BuAc = 1.0); thus, only negligible isopropanol amounts were still present at the time of processing of diluted supernatants. As remarked for the ELISA assays for TNF-α measurement in cell supernatants, the serum samples were also diluted (25 times) before analysis. Only trace amounts of isopropanol were left in the diluted serum, < 0.01% or < 1.5 mM, without accounting for metabolic clearance, which is likely to reduce these levels even further.
Inhibitor compounds (Sigma, St Louis, MO) were used as follows: BAY 11-7082: 5 μM; SB202190: 10 μM; SP600125: 25 μM; and PD98059: 50 μM.

Western blot. Purified cells were activated for 15 min at 37°C with LPS as described above with or without 0.6% (w/v) isopropanol. The cells were washed in alcohol-free buffer and lysed in sodium dodecyl sulfate (SDS) sample buffer (2% w/v SDS, 0.25 M β-mercaptoethanol, 10% v/v glycerol, 0.05 M Tris-HCl, pH 6.8, 0.004% w/v bromophenol blue); lysates were separated in 12% polyacrylamide gels and blotted onto nitrocellulose filters (Hybond-C, GE Healthcare, Piscataway, NJ). All antibodies were purchased from Cell Signaling Technology (Danvers, MA) unless indicated otherwise. The membranes were first probed with the following antibodies: IKKα/β detection, anti-phospho-IKKα/β rabbit monoclonal antibody (16A6, 1:1000) and anti-β-tubulin mouse monoclonal antibody for protein loading control (TUB 2.1, 1:4000, Sigma); p38 detection, anti-phospho-p38 MAPK mouse monoclonal antibody (28B10, 1:2000) and anti-total p38 rabbit polyclonal antibody (In-house, 1:10000); JNK detection, anti-phospho-SAPK/JNK rabbit polyclonal antibody (Thr^{183}/Tyr^{185}, 1:1000) and anti-β-tubulin mouse monoclonal antibody for protein loading control (TUB 2.1, 1:4000); and ERK detection, anti-phospho-p44/42 MAPK mouse monoclonal antibody (E10, 1:2000) and anti-total p42 MAPK rabbit polyclonal antibody (In-house, 1:5000, Huot *et al.*, 1995). The membranes were subsequently washed and

incubated with 1/15000 dilutions of the antibodies IRDye 800CW goat anti-rabbit IgG and/or IRDye 680 goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE). Detection and quantification were performed with the Odyssey Infrared Imaging System (Li-Cor Biosystems).

Interleukin-1 receptor-associated kinase 1 (IRAK1) immunoprecipitation and in vitro kinase assay. Mono Mac 6 cells were stimulated with LPS for 15 min at 37^{o}C in presence or absence of 0.6% (w/v) isopropanol. Cells were washed in alcohol-free PBS and submitted to lysis and IRAK1 immunoprecipitation with reagents from the Roche protein G immunoprecipitation kit (Roche Diagnostics, Laval, Canada) according to the manufacturer's instructions. The anti-IRAK1 rabbit polyclonal antibody (Millipore, Billerica, MA) was used to generate immunocomplexes. Sample input was equalized for protein content after quantification with the DC Protein Assay (Bio-Rad, Hercules, CA). Washed immunocomplexes were resuspended in kinase buffer (25 mM Tris HCl, pH 7.2, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 2 mM DTT) and added to the myelin basic protein substrate in presence of ^{32}P-γ-ATP and cold ATP for 30 min at 30^{o}C. The kinase reaction was stopped by adding SDS sample buffer and an aliquot was separated in 12% SDS-PAGE. Gels were dried and subsequently analysed and quantified in a phosphoimager.

Phagocytosis assays. P388D1 cells were incubated at 37^{o}C with *E. coli* bioparticles labeled with either pHrodo or Alexa Fluor 488 (Invitrogen Canada) in presence or absence of isopropanol as indicated. Phagocytosis was analysed with a TE300 microscope (Nikon, Melville, NY) and quantified with the MetaVUE software (Molecular Devices, Sunnyvale, CA). Confocal imaging was performed with a FluoView FV1000 microscope and the FluoView application software (Olympus Canada, Markham, Canada). Alternatively, samples were analysed in a XL flow cytometer (Beckman Coulter Inc., Miami, FL). Quenching of surface-bound Alexa Fluor 488 bacteria was achieved by incubating the cells with 0.2% (w/v) trypan blue in PBS.

ELISA-based transcription factor activation assay. Cells were washed in alcohol-free buffer before the generation of nuclear lysates. 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). c-Fos, Fra1, FosB, c-Jun, JunD and JunB activation was measured with the TransAm AP-1 kit; 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 TMB substrate produced a colorimetric reaction measurable in a spectrophotometer.

In vivo studies. 7-13-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 5 μg LPS subcutaneously for cytokine induction and were sacrificed by CO_2 asphyxiation 90 min or 180 min after administration for TNF-α or IL-6/CCL2 serum analysis, respectively. Toxic shock was induced with a subcutaneous injection of 0.2 μg LPS after presensitization with 20 mg D-galactosamine (Sigma) as reported elsewhere (Tsytsykova and Goldfeld, 2000). Animals were checked hourly in the beginning of the protocol and were followed for 5 days. Isopropanol was injected intraperitoneally (2 g/kg) in the experiments for cytokine induction analysis and for toxic shock protection.

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, except when indicated otherwise. The Student's t test was used in figures 4.3A, 4.3B and 4.4A. Survival curves were determined by the Kaplan-Meier method (fig. 4.5E). p values < 0.05 were considered significant.

Results

Isopropanol changes the cytokine/chemokine release profile of activated human monocytes

Monocytes are capable of releasing several cytokines in response to Gram-negative bacteria (da Silva Correia *et al.*, 2001; Miyake, 2006). The acquisition of effector function is a highly choreographed process that may begin with the recognition of pathogen-derived lipoglycans, as illustrated by the engagement of lipopolysaccharides by the MD2/Toll-like receptor 4 (TLR4) complex, and culminates with the transcriptional activation of several genes encoding immunologically relevant proteins (Miyake, 2006). In the present study, we have investigated whether isopropanol exposure *in vitro* would have any impact on the ability of human monocytes to produce cytokines once activated by lipopolysaccharides. Three distinct outcomes were observed as illustrated in figure 4.1. The releases of the proinflammatory cytokine IL-1β and of the chemokine IL-8 were unaltered even at the highest isopropanol concentrations tested. The production of IL-6 was up-regulated by 53% at the lowest alcohol dilution and more than doubled at the highest isopropanol concentration as measured in the culture supernatants by ELISA. In contrast, monocytes treated with isopropanol produced lower amounts of the proinflammatory cytokine TNF-α and of the chemokine CCL2. This down-regulation was observed at isopropanol doses as low as 0.16%. The observed effect was not the consequence of nonspecific cytotoxicity as the cell viability of alcohol-treated samples in the concentration range that produced inhibition levels of 30-41% for TNF-α and of 25-64% for CCL2 in figure 4.1 was similar to that of untreated control cells (data not shown and Désy *et al.*, 2008). Moreover, in agreement with our previous data on the effect of isopropanol and the closely related methanol on lymphocytes (Désy *et al.*, 1998, 2010), alcohol treatment alone had no impact on the release of any tested cytokine by monocytes. This was evident even for IL-6, whose negligible production in unstimulated cells was not augmented by isopropanol (Mean release ± SEM: 105 ± 5.8 pg/ml in unstimulated cells and 97 ± 11.9 pg/ml in alcohol-treated unstimulated cells, n: 3).

The immunomodulatory effect of isopropanol extends to differentiated macrophages

Monocytes are attracted to injured sites where they differentiate into macrophages (Swirski *et al.*, 2009), which play a pivotal role in the regulation of the inflammatory response. The finding that cytokine release by human monocytes is affected by isopropanol led us to predict a similar impact on the function of derived macrophages. Panel A in figure 4.2 shows that exposure to this alcohol *in vitro* creates an identical profile of cytokine dysregulation in monocytes and macrophages with down-regulation of TNF-α/CCL2 and up-regulation of IL-6 production. Besides their secretory function, tissue macrophages are also active phagocytes involved in pathogen clearance and removal of necrotic material (Park *et al.*, 2011). Thus, it was conceivable that the alcohol-induced dysfunction could extend to their ability to ingest large particles. We have tested this assumption by examining the internalization of fluorescent bacteria in a murine macrophage cell line in presence and absence of isopropanol. Figure 4.2 shows representative micrographs (panel B) and a quantitative assessment of the cellular fluorescence intensity (panel C); phagocytosis was reduced by 34% at 0.3% isopropanol exposure and by more than 50% at the highest concentration tested. These results were further substantiated by flow cytometric analysis, which revealed similar levels of phagocytosis impairment, 37% and 67%, in cells exposed to 0.3% and to the highest alcohol concentration, respectively (Figure 4.2, panel D).

Early signaling events triggered by Toll-like receptor 4 activation are not compromised in human monocytes acutely exposed to isopropanol

Isopropanol could exert its modulatory effect via direct interaction with molecules that initiate or facilitate signaling in response to lipoglycans. LBP lipid transferase initially promotes the incorporation of LPS aggregates into the plasma membrane and their subsequent transfer to CD14 (Miyake, 2006). The latter ultimately presents LPS to the MD2/TLR4 complex, inducing receptor clustering, recruitment of key adaptor molecules to the TLR4 cytoplasmic domain, and downstream signaling (da Silva Correia *et al.*, 2001; Miyake, 2006). In order to address the possibility that isopropanol would interfere with the

membrane-based early events that characterize lipoglycan recognition, we have examined the kinase activity of IRAK1. This enzyme is recruited by the MyD88 adaptor protein early on following LPS stimulation and is central to the activation of NF-κB and AP-1 transcription factors and the resulting production of proinflammatory cytokines (Kawai and Akira, 2010). Panel A in figure 4.3 shows that IRAK1 is not affected by alcohol exposure as measured by its ability to phosphorylate a model substrate.

The biological effect of isopropanol is not mediated by an alteration of the NF-κB signaling pathway in LPS-activated human monocytes

Once we have established that isopropanol does not change early TLR4 signaling, we checked whether NF-κB activation was compromised by examining the phosphorylation status of the IκB kinase (IKK). This enzyme consists of regulatory (γ) and catalytic (α and β) subunits, whose activity is found downstream of IRAK1/TRAF6/TAK1 in the TLR4 signal transduction pathway (Hayden and Ghosh, 2008; Israël, 2010); upon LPS stimulation, the IKK complex undergoes transautophosphorylation or is directly phosphorylated by TAK1 on serine residues in the T loop domain of the catalytic subunits (Hayden and Ghosh, 2008). Western blot analysis with an antibody that recognizes phosphorylated IKKs ($Ser^{176/180}$ in IKKα and $Ser^{177/181}$ in IKKβ) revealed that IKK activation is similar in alcohol-treated and untreated samples following LPS exposure (Figure 4.3, panel B). These findings were supported by the measurement of the nuclear translocation of the NF-κB p65 subunit in TLR4-stimulated human monocytes treated or not with isopropanol. Figure 4.3C shows that monocytes activated by LPS remained unaffected by alcohol treatment. The compound BAY 11-7082, an inhibitor of IκBα phosphorylation, reduced the nuclear content of p65 in the same experiments to levels lower than those of the unstimulated control.

Isopropanol selectively modulates the activation of the extracellular signal-regulated protein kinase (ERK) module of the mitogen-activated protein kinase (MAPK)

superfamily and reduces the nuclear translocation of discrete components of the AP-1 transcription factor in LPS-treated human monocytes

As our results have revealed that the NF-κB pathway is insensitive to isopropanol exposure, we have decided next to examine the three modules of the MAPK signaling cascade, p38, JNK, and ERK, in LPS-stimulated monocytes. Panel A in figure 4.4 shows that there is no clear alcohol effect on the phosphorylation of p38 and JNK. Conversely, the phosphorylation of the activation loop residues Thr^{185} and Tyr^{187} in ERK2 was 37% less efficient in presence of isopropanol. One of the best-studied effects of ERK is the initiation of transcription of the immediate early gene c-*fos* through the activation of Elk-1 (Yoon and Seger, 2006). The c-Fos transcription factor is detectable as early as 20 min after stimulation and is readily phosphorylated at Ser^{374} by ERK and on additional sites by ERK-activated kinases or by an extended ERK activity (Lallemand *et al.*, 1997; Murphy *et al.*, 2002; Yoon and Seger, 2006). In order to dissect the impact of isopropanol on ERK substrates, we have measured the nuclear translocation of phosphorylated c-Fos in TLR4-stimulated monocytes exposed to different concentrations of the alcohol. Figure 4.4B, left panel, shows that monocyte activation by LPS led to a 3-fold increase in the amount of c-Fos in the nucleus. The same stimulation in presence of 0.16% isopropanol led to a 2.4-fold increase in nuclear c-Fos (or 68% of the maximal c-Fos nuclear content above the unstimulated cell baseline). The alcohol effect was dose-dependent and the increase of this transcription factor in the nucleus was down to 1.7 fold (or 33% of the maximal content) at the highest isopropanol concentration. The same stimulatory conditions in presence of a combination of inhibitor drugs of the MAPK signaling cascade, PD98059 (MEK1)/SB202190 (p38)/SP600125 (JNK), produced levels of nuclear c-Fos lower than those of the unstimulated cells.

We have examined next the fate of other members of the AP-1 family of transcription factors in the same experimental conditions as above. Our results have revealed that isopropanol exposure does not have any impact on the nuclear translocation of c-Jun, JunD, Fra1 and FosB (data not shown). We have found, however, a measurable alcohol effect on JunB activation. Similar to c-Fos, the JunB protein appears rapidly after cell activation and is encoded by an immediate early gene whose transcription is induced by two important

types of ERK substrates, the kinases RSK2 and MSK1/2 (Cargnello and Roux, 2011; Lallemand *et al.*, 1997). The right panel in figure 4.4B shows that LPS activation produced a 2.2-fold augmentation in nuclear JunB. Monocytes stimulated in presence of 0.16% isopropanol experienced a 1.8-fold increase in nuclear JunB (or 68% of the maximal JunB nuclear content above the unstimulated cell baseline). The alcohol effect was also dose-dependent and the LPS-induced increase in nuclear JunB was 1.4 fold (or 36% of the maximal content) at the highest alcohol concentration. The JunB nuclear content in TLR4-activated monocytes was similar to that of control unstimulated cells when the PD98059/SB202190/SP600125 kinase inhibitors were used.

Isopropanol inhibits the production of TNF-α and CCL2 in vivo and confers protection from LPS-induced toxic shock

The biological effect of isopropanol on the immune response to LPS was subsequently tested in a mouse model of acute alcohol intoxication. Mice were administered isopropanol intraperitoneally, 2 g/kg, to generate a mean blood alcohol concentration of 200 mg/dl after 30 min. The production of TNF-α, IL-6, and CCL2 was induced *in vivo* by subcutaneous injection of LPS. As anticipated, LPS triggered TNF-α levels of > 1700 pg/ml after 90 min (Fig. 4.5A) and > 13000 pg/ml CCL2 after 180 min (Fig. 4.5D). Isopropanol exposure led to a statistically significant down-regulation of the serum release of these cytokines: 86% or 240 pg/ml TNF-α detected at 90 min, and 76% or 3195 pg/ml CCL2 detected at 180 min. The differences in cytokine serum levels between animals treated and untreated with the alcohol were statistically significant as indicated in figures 4.5A and 4.5D. Similarly, we expected that isopropanol would increase IL-6 production in this mouse model in a way that would resemble its impact on LPS-stimulated human monocytes *in vitro*. To our surprise, LPS challenge *in vivo* was not associated with higher serum levels of IL-6 in the context of isopropanol intoxication (Fig. 4.5B). This finding made us consider the possibility that murine and human primary monocytes would respond differently to LPS as regards the alcohol modulation of IL-6 release. Indeed, upon LPS-stimulation, purified murine monocytes were not responsive to isopropanol *in vitro* as illustrated in figure 4.5C.

Our results revealed that isopropanol is a powerful negative regulator of the inflammatory cytokine TNF-α both *in vitro* and *in vivo*. TNF-α is copiously produced and plays a central role in the pathophysiology of LPS-induced lethal shock (Galanos and Freudenberg, 1993; Tsytsykova and Goldfeld, 2000). Therefore, it was our assumption that isopropanol treatment could protect mice from toxic shock. In order to address this issue, we have sensitized BALB/c mice with 20 mg D-galactosamine by intraperitoneal injection. All sensitized animals developed toxic shock within 16 h following subcutaneous LPS challenge (10/10) (Fig. 4.5E). A very different picture was produced by alcohol administration, a single injection of 2 g/kg had a dramatic effect since it completely prevented LPS-induced toxic shock, and all animals survived. These mice were followed for 5 days after LPS challenge and were indistinguishable from sensitized control groups receiving PBS or isopropanol. No death occurred in groups injected with isopropanol (10/10) or PBS (10/10) in absence of LPS.

Discussion

Short-chain alcohols mediate a variety of biological effects through nonspecific mechanisms at high concentrations, usually in the 500 mM range (Dwyer and Bradley, 2000). Nevertheless, they may interact with specific targets and induce loss of function of ion channels, neurotransmitter receptors, enzymes, and adhesion molecules at more physiologically relevant levels (Jung *et al.*, 2005; Ren *et al.*, 2003; Shahidullah *et al.*, 2003). The inhibitory effect of ethanol on LPS-induced TLR4 triggering in macrophages has been suggested to result from its partition into cell membranes (Dai *et al.*, 2005; Szabo *et al.*, 2007); in this setting, the alcohol would reduce the capacity of micro-domains to recruit and/or retain relevant molecules and compromise the cascade that relays the signal generated by receptor ligation on the cell membrane to the nucleus. In a similar fashion, it was conceivable that isopropanol could change LPS binding to CD14 and the stability and/or conformation of the MD2/TLR4 complex thereby dampening or virtually aborting downstream signaling. This scenario would be compatible with our findings that mostly revealed loss of function of LPS-stimulated monocytes after isopropanol treatment, IL-6

up-regulation being the notable exception. Our results, however, showed that TLR4 early signaling is preserved during isopropanol acute exposure (Figure 4.3A). We found that isopropanol acts downstream of the cell membrane as it does not change the kinase activity of IRAK1 in LPS stimulated cells. This is in sharp contrast with ethanol, which affects IRAK1 activity in line with the model described above.

Further down in the activation cascade, the kinase TAK1 is capable of triggering the two major pathways leading to proinflammatory cytokine production during LPS stimulation, MAPKs and NF-κB signaling (Cargnello and Roux, 2011; Israël, 2010; Kawai and Akira, 2010). Our data suggest that isopropanol initiates its effect downstream of TAK1, as the function of this enzyme remains unchanged since the activation of the p38, JNK, and NF-κB signaling cascades proceeds normally (Figs. 4.3B, 4.3C and 4.4A). We believe that isopropanol may interact directly with ERK2 because ERK1 phosphorylation is not affected, indicating that the upstream MEK kinases are functional (Fig. 4.4A). Although MEK1/2 are not completely interchangeable, MEK1 can phosphorylate both ERK1/2 *in vitro* (Xu *et al.*, 2001) and *in vivo* in MEK2-deficient mice (Bélanger *et al.*, 2003). Thus, it is unlikely that MEK2 loss of function could account for the lower ERK2 phosphorylation observed in isopropanol-treated samples because the MEK2 kinase activity could be operationally replaced by that of MEK1.

Unphosphorylated ERK2 is virtually idle but undergoes a dramatic conformational change upon cell activation resulting from the posttranslational modification of the Thr^{183} and Tyr^{185} residues (Yoon and Seger, 2006). This change is accompanied by the acquisition of a catalytic activity five orders of magnitude higher than basal levels and the ability to translocate into the nucleus (Chuderland and Seger, 2005). There are multiple ERK substrates and, among these, c-Fos has been studied extensively (Yoon and Seger, 2006). The nuclear content of c-Fos is determined by the level of direct ERK-mediated phosphorylation, which stabilizes the c-Fos protein, and by the very fast transcriptional activation of the c-*fos* gene via Elk-1, which is also an ERK-dependent process (Cargnello and Roux, 2011; Murphy *et al.*, 2002; Yoon and Seger, 2006). In agreement with the results presented in figure 4.4, lower ERK activity is expected to be associated with a diminished c-Fos phosphorylation and nuclear presence (Murphy *et al.*, 2002). c-Fos dimerizes with

Jun proteins to form the AP-1 transcription factor (Lallemand *et al.*, 1997). It is noteworthy that the promoter region of the gene that encodes the chemokine CCL2 displays two AP-1 binding sites (Kok *et al.*, 2009; Martin *et al.*, 1997; Shyy *et al.*, 1995; Sutcliffe *et al.*, 2009). Thus, the lower activation and nuclear translocation of c-Fos that are associated to isopropanol exposure could account for the down-regulation of CCL2 production by stimulated monocytes as illustrated in figure 1. Nevertheless, although there are AP-1 sites also in the proximal TNF-α promoter, monocytes stimulated with whole bacteria or LPS assemble rather unique TNF-α enhanceosomes that include AP-1 heterodimers lacking c-Fos (Barthel *et al.*, 2003; Tsai *et al.*, 2000). It is conceivable that the other transcription factor that we found to be altered in our experiments, JunB, mediates the TNF-α down-regulation induced by acute alcohol exposure. The transcription of the immediate early gene *junB* is coordinated by the ERK-activated substrate kinases RSK2 and MSK1/2, which activate CREB by phosphorylation at Ser^{133} thereby allowing the recruitment of CBP and p300 to the *junB* promoter (Cargnello and Roux, 2011). JunB appears as a protein 20-40 min after the initiation of MAPK signaling and is expected to be down-regulated when the catalytic activity of ERK is diminished (Lallemand *et al.*, 1997). Figure 4.4B, right panel, shows that the nuclear content of transcriptionally active JunB is indeed compromised by isopropanol. In support to this scenario, a recent report described the participation of JunB in the induction of TNF-α in LPS-stimulated myeloid cells and showed JunB binding to the TNF-α promoter by chromatin immunoprecipitation (ChIP) (Gomard *et al.*, 2010). At this point in time, we cannot exclude that additional factors encoded by immediate early genes downstream of ERK may contribute to the alcohol effect. The Elk-1-dependent Egr-1 transcription factor, for instance, was shown to integrate the TNF-α enhanceosome and to participate in the transcriptional activation of this gene in LPS-stimulated monocytic cells (Barthel *et al.*, 2003; Shi *et al.*, 2002; Tsai *et al.*, 2000). This in line with the observation that the MEK1 (ERK) inhibitor PD98059 blocks Elk-1, Egr-1, and TNF-α expression in LPS-stimulated cells (Guha *et al.*, 2001; Shi *et al.*, 2002).

The finding that isopropanol acute exposure was associated to JunB down-regulation can also help us to understand the paradoxical up-regulation of IL-6 presented in figure 4.1. It

has recently been shown by ChIP and luciferase reporter assays that JunB-containing AP-1 complexes act as powerful repressors of transcriptional activity in the context of the IL-6 promoter (Pflegerl *et al.*, 2009). Most importantly, JunB-deficient cells produce larger amounts of IL-6 than their wild-type counterparts (Meixner *et al.*, 2008; Pflegerl *et al.*, 2009). Therefore, the selective and simultaneous reduction of c-Fos and JunB in the nucleus could account for the alcohol-induced down-regulation of TNF-α/CCL2 and up-regulation of IL-6 in LPS-stimulated cells *in vitro*.

It is not surprising that isopropanol induces a similar pattern of cytokine modulation in monocytes and macrophages (Figs. 4.1 and 4.2A) as these cells represent partially overlapping differentiation stages of the same lineage (Valledor *et al.*, 1998). This modulation is likely to be mediated by the dysregulation of the c-Fos and JunB transcription factors as discussed above. Moreover, we have found that macrophages were less efficient in internalizing bacteria in presence of isopropanol (Fig. 4.2B/C/D), a finding that is reminiscent of the impact of ethanol on phagocytosis (Boé *et al.*, 2010; Goral *et al.*, 2008; Karavitis *et al.*, 2008). ERK has been reported to participate in the Fcγ receptor- and complement-mediated phagocytosis in neutrophils and macrophages (García-García *et al.*, 2002; García-García and Rosales, 2002; Jehle *et al.*, 2006; Mansfield *et al.*, 2000). MAPK signaling also contributes to the uptake of non-opsonized particles by macrophages, although the p38 pathway is believed to play the predominant role (Blander and Medzhitov, 2004). The reduced phagocytosis observed in our experiments with isopropanol may result from impaired ERK signaling. With over 150 direct phosphorylation targets (Yoon and Seger, 2006), ERK kinases are truly pleiotropic and have several possible paths to impact phagocytosis. Thus, the alcohol effect could be indirectly mediated by one of the first identified targets of ERK, cytosolic phospholipase A_2, which has been suggested to be involved in phagocytosis (García-García *et al.*, 2002; García-García and Rosales, 2002; Lin *et al.*, 1993); alternatively, such an effect could be a consequence of reduced phosphorylation of cytoskeletal elements by ERK (Mansfield *et al.*, 2000; Yoon and Seger, 2006). This matter will be addressed in future investigations.

The immunomodulation by isopropanol of the monocyte/macrophage effector function was demonstrated *in vitro* at concentrations as low as 0.16% (26 mM), which are comparable to

the concentrations of ethanol associated to a biological effect on immune cells in other studies (Goral and Kovacs, 2005; Oak *et al.*, 2006; Saeed *et al.*, 2004; Szabo *et al.*, 2007; Taieb *et al.*, 2002; Zhao *et al.*, 2003). The potential of isopropanol to modulate the TLR4-mediated inflammatory cytokine response to LPS was confirmed *in vivo* in a mouse model of acute alcohol intoxication. To put in perspective the relevance of the alcohol doses used in our *in vivo* experiments, we should consider the blood alcohol concentrations in acutely poisoned patients. Although there are thousands of cases of isopropanol intoxication recorded each year (Bronstein *et al.*, 2010), clinical reports with detailed information on time and volume of ingestion are relatively scarce in the medical literature. Patients may survive blood isopropanol concentrations as high as 560 mg/dl (0.56% or 93 mM), while others may succumb to much lower concentrations (Lacouture *et al.*, 1983). This disparity comes from the fact that clinical measurements have often been made hours after ingestion and underestimate the serum alcohol levels present in the early phase of the intoxication (Daniel *et al.*, 1981; Gaudet and Fraser, 1989; King *et al.*, 1970; Mueller-Kronast *et al.*, 2003; Rich *et al.*, 1990; Rosansky, 1982). Concentrations above 400 mg/dl are considered life-threatening and generally require dialysis (Emadi and Coberly, 2007; Lacouture *et al.*, 1983). We have injected mice with 2 g/kg isopropanol to generate a blood alcohol concentration of 200 mg/dl (0.2% or 33 mM) after 30 min; this level is lower than the reported average sublethal isopropanol blood concentration in severely intoxicated humans (310 mg/dl after 7 h) (Ekwall and Clemedson, 1997) and is comparable to the concentration range that is active *in vi*tro (starting at 0.16% or 26 mM). As suggested by the results of our *in vitro* experiments, intoxicated mice had a severe impairment in their response to LPS as measured by a substantial drop in serum TNF-α and CCL2 (7.4- and 4- fold, respectively). There was, however, no significant effect of isopropanol on IL-6 production in animals challenged with LPS. The latter result suggested that mouse and human cells are differently susceptible to alcohol modulation of IL-6 production. This assumption was confirmed by exposing purified murine monocytes *in vitro* to LPS in presence or absence of isopropanol. We found that murine cells responded to LPS but were indeed insensitive to alcohol modulation of the IL-6 release. The regulation of the IL-6 promoter is rather intricate and involves the concerted action of several transcription factors (Dendorfer *et al.*, 1994;

Vanden Berghe *et al.*, 1999). Although the proximal region of the mouse and human IL-6 promoters share consensus sequences for AP-1, NF-κB, NFAT, C/EBP, and CREB, there is divergence further upstream from the transcription start site (Allen *et al.*, 2010; Samuel *et al.*, 2008). It is conceivable that the differential alcohol modulation of the IL-6 response to LPS reflects a distinct set of interactions of JunB-containing AP-1 dimers with other factors that are recruited in accordance with the contextual profile of each promoter; this issue, however, goes beyond the scope of this paper. Overall, the immunological impact of isopropanol *in vivo* is suppressive and corroborates our previous data obtained with lymphocytes. The magnitude of the TNF-α down-regulation led us to speculate that isopropanol could rescue mice from LPS-induced toxic shock syndrome. We found that all animals injected with LPS after presensitization with D-galactosamine developed a fulminant toxic shock with a median survival of 10.5 h. In stark contrast, all mice treated with isopropanol survived without any signs of the syndrome.

Our results have clinical implications given the possibility that patients acutely intoxicated with isopropanol may also be acutely immunosuppressed. This scenario should be considered in cases of severe poisoning, especially if underlying infection and/or trauma is present. Moreover, the general consumer has easy access to a wide range of products that contain high concentrations of isopropanol such as hand sanitizers and rubbing alcohol. Isopropanol is readily absorbed by the gastrointestinal tract but its absorption through intact adult skin is very poor (Brown *et al.*, 2007; Kirschner *et al.*, 2009; Kraut and Kurtz 2008). With that in mind, limited transdermal absorption may occur with an estimated skin permeation coefficient (k_p) of 4-15 x 10^{-4} cm/h (Boatman *et al.*, 1998; Clewell *et al.*, 2001; Cronin *et al.*, 1999; Frasch, 2002; Turner *et al.*, 2004). Although topical application was documented in a few cases of isopropanol poisoning (Arditi and Killner, 1987; Dyer *et al.*, 2002; Leeper *et al.*, 2000), most conventional topical uses are likely to produce only negligible systemic alcohol levels (Brown *et al.*, 2007; Kirschner *et al.*, 2009). In addition, the direct immunomodulatory impact of isopropanol on healthy skin, if any, is likely to be transitory and confined to resident/infiltrating immune cells. From another standpoint, however, one should perhaps be cautious when applying isopropanol-containing gels or solutions to diseased skin. It should be noticed that a typical 70% alcohol solution is over

400 times more concentrated than the minimal biologically active amount *in vitro* (0.16% or 26 mM). In this regard, a recent report has identified the down-regulation of JunB and the associated up-regulation of IL-6 production in keratinocytes in systemic lupus erythematosus (Pflegerl *et al.*, 2009). As the JunB/IL-6 dysregulation could potentially be exacerbated by isopropanol, one should be prudent in using topical isopropanol in these patients until further investigation is conducted to clarify the risks, if any.

The current work extends our previous findings in that it reveals yet another mechanism of isopropanol-induced immunosuppression, which is based on the disablement of downstream events in the TLR4 signaling cascade. Acute exposure to this alcohol dysregulates the effector function of monocyte/macrophages *in vitro* and compromises the cytokine response to LPS challenge *in vivo* to an extent that protects animals from otherwise lethal toxic shock. Our data contribute to the existing dismal body of information on the immunotoxicology of isopropanol, one of the most ubiquitous chemicals in the world.

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Figures and Legends

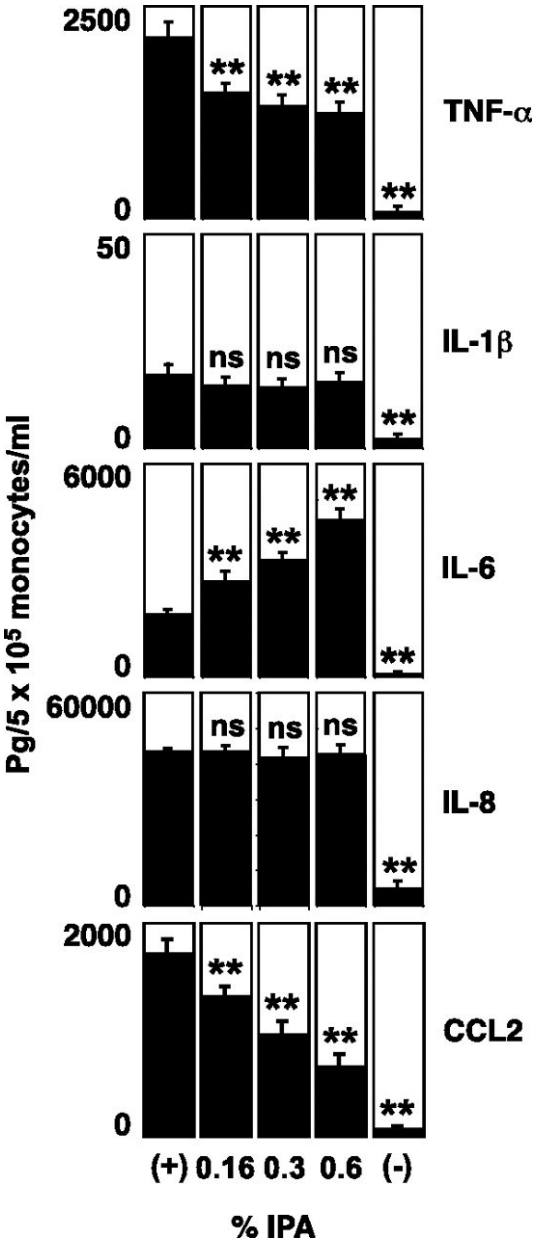

Figure 4.1 - Biological effect of isopropanol treatment *in vitro* on human monocytes.

Primary monocytes were stimulated with LPS for 24 h in presence of 0.16%, 0.3% and 0.6% (w/v) isopropanol. The concentrations of TNF-α, IL-1β, IL-6, IL-8 and CCL2 in the supernatants were measured by ELISA and are depicted as means ± SEM (*ns*: $p > 0.05$, **

$p < 0.01$ relative to the (+) control group; n: 4 for TNF-α, n: 3 for IL-6/IL-8, n: 5 for IL-1β/CCL2).

Figure symbols: (+) indicates cell activation in absence of isopropanol; (-) represents the unstimulated control in absence of isopropanol. *IPA* indicates isopropanol.

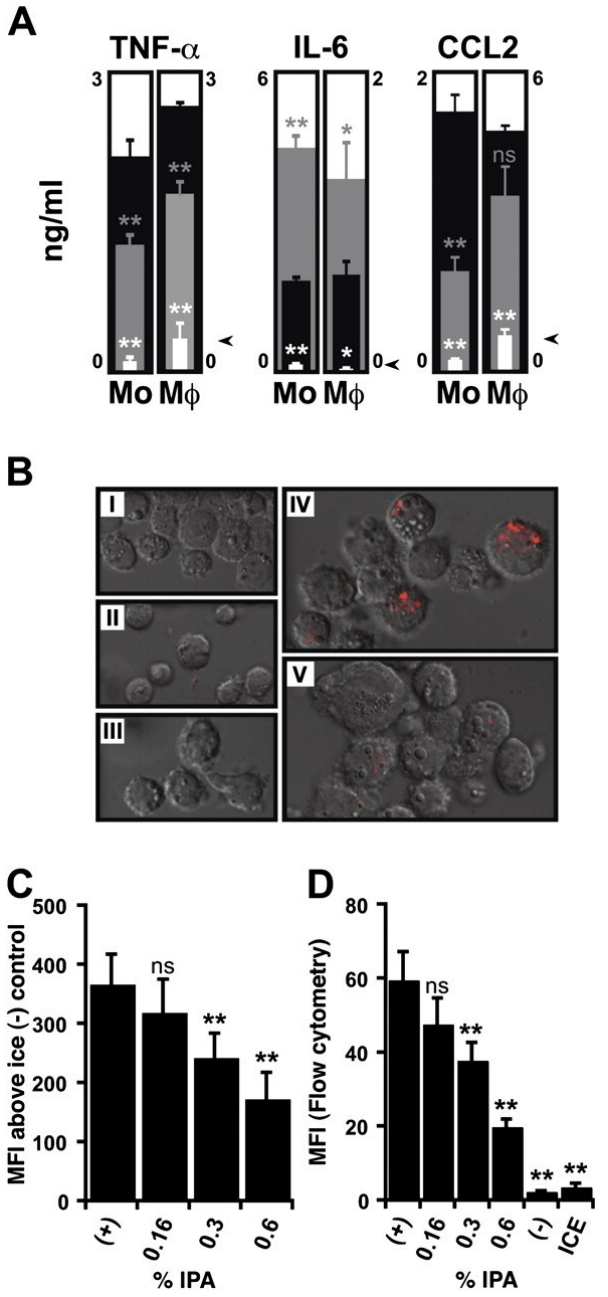

Figure 4.2 - Biological effect of isopropanol treatment *in vitro* on macrophages.

(A) Isopropanol treatment affects the cytokine production of human primary macrophages: Primary cultures of human macrophages (Mϕ) and monocytes (Mo) were stimulated with LPS in presence or absence of 0.6% (w/v) isopropanol and the supernatants were harvested after 24 h for measurement of TNF-α, IL-6, and CCL2 by ELISA. Concentrations are shown as means ± SEM. The positive controls (LPS-stimulation in absence of isopropanol)

are shown as black columns; the negative controls (unstimulated cells in absence of isopropanol) are shown as white columns; and the experimental samples (LPS-stimulation in presence of isopropanol) are shown as grey columns (*ns*: $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ relative to the positive control (black) group in each histogram; n: 4 for Mo/Mϕ TNF-α, n: 3 for Mo/Mϕ IL-6, n: 5 for Mo CCL2 and n: 3 for Mϕ CCL2). The arrows indicate the mean cytokine production in alcohol-treated unstimulated macrophages.

(B) Isopropanol interferes with macrophage phagocytosis: P388D1 cells were incubated with pHrodo *E. coli* for 2 h in presence or absence of 0.6% (w/v) isopropanol. These bioparticles fluoresce in acidic environment upon internalization. The panels show representative micrographs of cells without bioparticles (B-I), of cells incubated with bioparticles on ice in absence (B-II) or presence (B-III) of isopropanol, and of cells incubated with bioparticles at 37°C in absence (B-IV) or presence (B-V) of isopropanol.

(C) Phagocytosis quantification by microscopy: P388D1 cells were incubated with pHrodo *E. coli* at 37°C for 2 h in presence of 0.16%, 0.3% and 0.6% (w/v) isopropanol. The fluorescence intensity values above background measured with the MetaVUE software are shown as means ± SEM (MFI; *ns*: $p > 0.05$, ** $p < 0.01$ relative to the (+) control group; n: 4). Background levels were established by incubating P388D1 cells with bioparticles in absence of isopropanol on ice for 2 h. (+) indicates incubation of P388D1 cells with bioparticles and without isopropanol at 37°C for 2 h. *IPA* indicates isopropanol.

(D) Phagocytosis quantification by flow cytometry: P388D1 cells were incubated with Alexa Fluor 488 *E. coli* bioparticles at 37°C for 1 h in presence of 0.16%, 0.3% and 0.6% (w/v) isopropanol. Quenching of surface-bound bacteria was performed as described in materials and methods. The mean fluorescence intensity ± SEM is presented (MFI; *ns*: $p > 0.05$, ** $p < 0.01$ relative to the (+) control group; n: 6). (-) indicates incubation of P388D1 cells in absence of bioparticles and without isopropanol at 37°C for 1 h; *ICE* indicates incubation of P388D1 cells with bioparticles and without isopropanol on ice for 1 h. *IPA* indicates isopropanol.

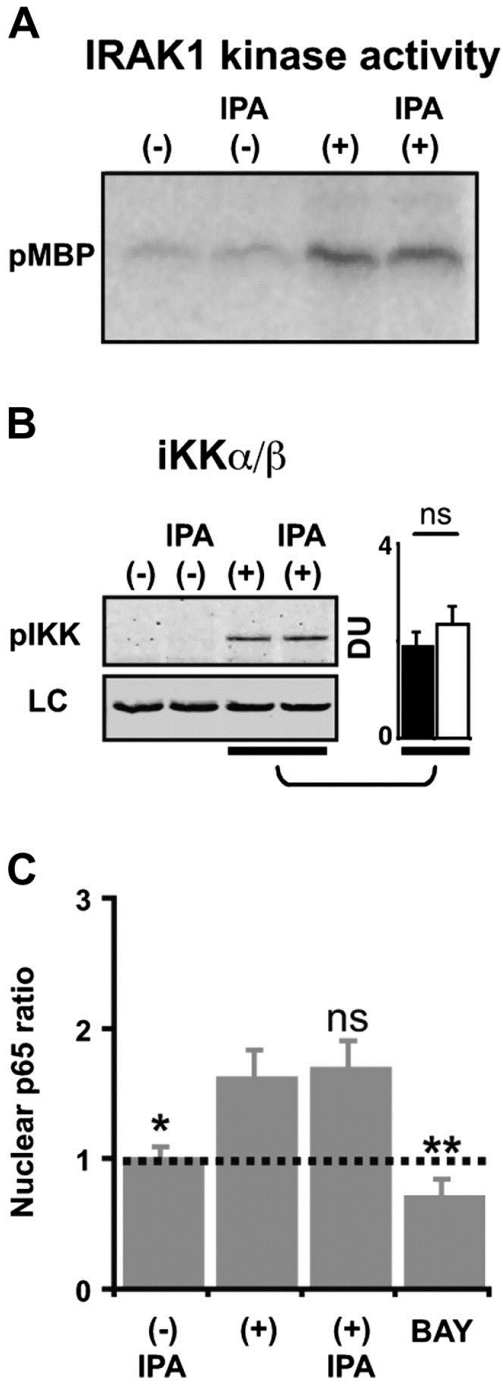

Figure 4.3 - Isopropanol acts downstream of the cell membrane and does not compromise the NF-κB signaling pathway.

(A) Isopropanol does not interfere with the activation of IRAK1: Mono Mac 6 cells were stimulated with LPS for 15 min in presence ((+)*IPA*) or absence (+) of 0.6% (w/v)

isopropanol; cell lysates were equalized for protein content and were used for IRAK1 immunoprecipitation as described in materials and methods. The enzymatic activity in the immunocomplexes was assessed by measuring ^{32}P--ATP incorporation into the myelin basic protein (*pMBP*) substrate after SDS-PAGE. One representative autoradiograph of four is depicted (Mean densitometric units ± SEM: 16.25 ± 2.0 (+); 15.31 ± 1.8 ((+)*IPA*), $p > 0.05$). (-) represents the unstimulated control in absence of isopropanol. (-)*IPA* represents the unstimulated control in presence of 0.6% (w/v) isopropanol.

(B) Isopropanol treatment does not affect IKKα/β phosphorylation following LPS stimulation: Human primary monocytes were stimulated for 15 min with LPS in presence ((+)*IPA*) or absence (+) of 0.6% (w/v) isopropanol and processed for SDS/PAGE. One representative Western blot is shown. The relative quantification of phosphorylated IKKα/β (*pIKK*) in relation to β-tubulin expression is depicted in mean densitometric units (*DU*) ± SEM on the right-hand side histogram (*ns:* $p > 0.05$, n: 3). (-) represents the unstimulated control in absence of isopropanol. (-)*IPA* represents the unstimulated control in presence of 0.6% (w/v) isopropanol. *LC* indicates loading control (β-tubulin).

(C) Nuclear translocation of the NF-κB p65 subunit is not affected by isopropanol: Primary human monocytes were stimulated with LPS for 1 h in presence or absence of isopropanol. Nuclear extracts were incubated with immobilized NF-κB-binding oligonucleotides in 96-well plates; the amount of bound transcription factor was assessed with 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 isopropanol. Data is presented as means ± SEM (*ns:* $p > 0.05$, ***** $p < 0.05$, ****** $p < 0.01$ relative to the (+) control group, n: 6).

The dashed line represents the baseline of p65 nuclear content in unstimulated cells. (-)*IPA* represents the unstimulated control in presence of 0.6% (w/v) isopropanol. (+) indicates LPS stimulation in absence of isopropanol. (+)*IPA* indicates LPS stimulation in presence of 0.6% (w/v) isopropanol. *BAY* indicates LPS stimulation in presence of the BAY 11-7082 inhibitor compound without isopropanol.

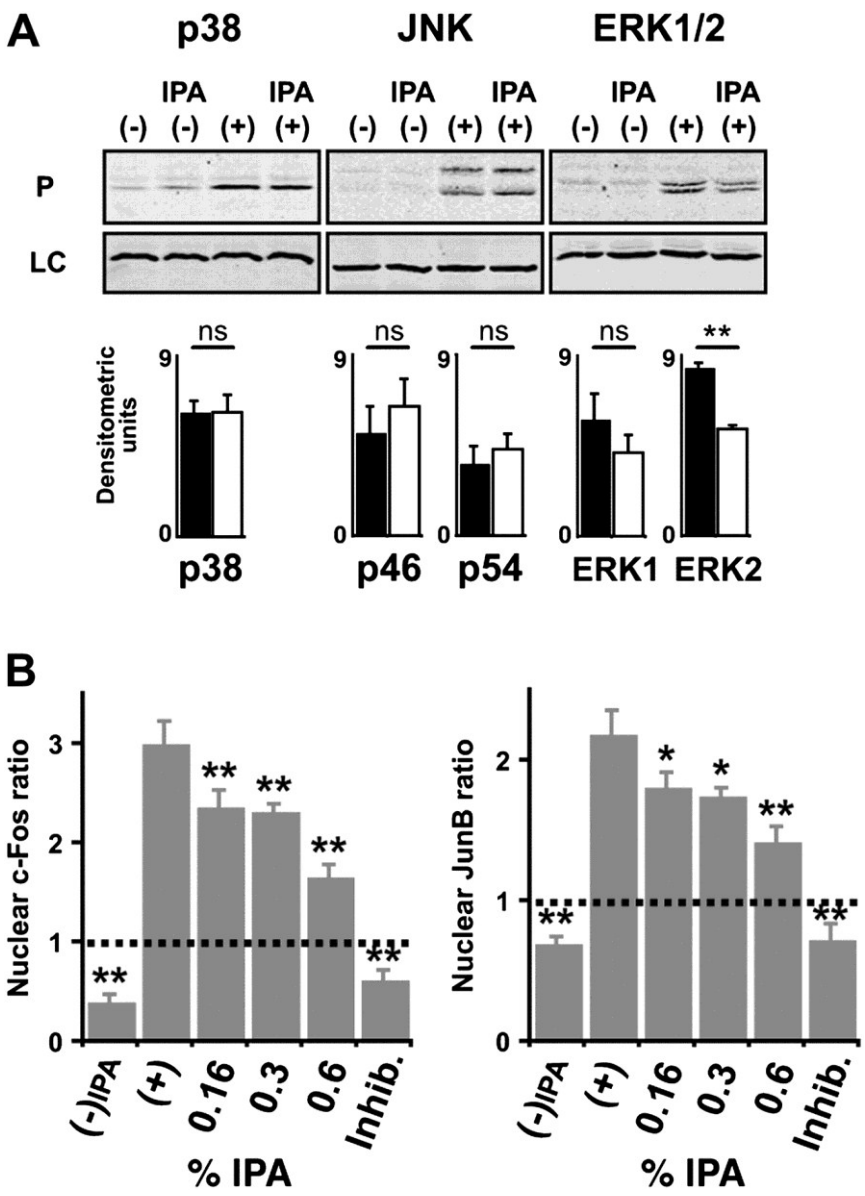

Figure 4.4 - Isopropanol induces a selective defect in the MAPK signaling cascade and alters the activation of discrete AP-1 family members.

(A) Isopropanol treatment inhibits the LPS-induced phosphorylation of ERK2 without compromising p38, JNK, and ERK1 activation: Human primary monocytes were stimulated for 15 min with LPS in presence ((+)*IPA*) or absence (+) of 0.6% (w/v) isopropanol and processed for SDS/PAGE. The upper blots show the expression of the phosphorylated forms (*P*) of p38, JNK p46/p54 and ERK1/2. The lower blots present the protein loading controls (*LC,* total p38; β-tubulin; and total ERK2). Relative quantifications

of the phosphorylated proteins in relation to the relevant loading control are depicted in mean densitometric units (*DU*) ± SEM under the representative Western blots (*ns:* $p > 0.05$, ** $p < 0.01$; n: 6 for p38, n: 5 for JNK, n: 3 for ERK). (-) represents the unstimulated control in absence of isopropanol. (-)*IPA* represents the unstimulated control in presence of 0.6% (w/v) isopropanol.

(B) Isopropanol inhibits the nuclear translocation of c-Fos and JunB: Primary human monocytes were stimulated with LPS for 1 h in presence of 0.16%, 0.3% and 0.6% (w/v) isopropanol. Nuclear extracts were incubated with immobilized AP-1-binding oligonucleotides in 96-well plates; the amount of bound transcription factor was assessed with a c-Fos- or JunB-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 isopropanol. Data is presented as means ± SEM (* $p < 0.05$, ** $p < 0.01$ relative to the (+) control group; n: 7 for c-Fos, n: 6 for JunB).

The dashed line represents the baseline of the relevant transcription factor nuclear content in unstimulated cells. (+) indicates LPS stimulation in absence of isopropanol. (-)*IPA* represents the unstimulated control in presence of 0.6% (w/v) isopropanol. *Inhib.* indicates LPS stimulation in presence of PD98059/SB202190/SP600125 kinase inhibitors without isopropanol. *IPA* indicates isopropanol.

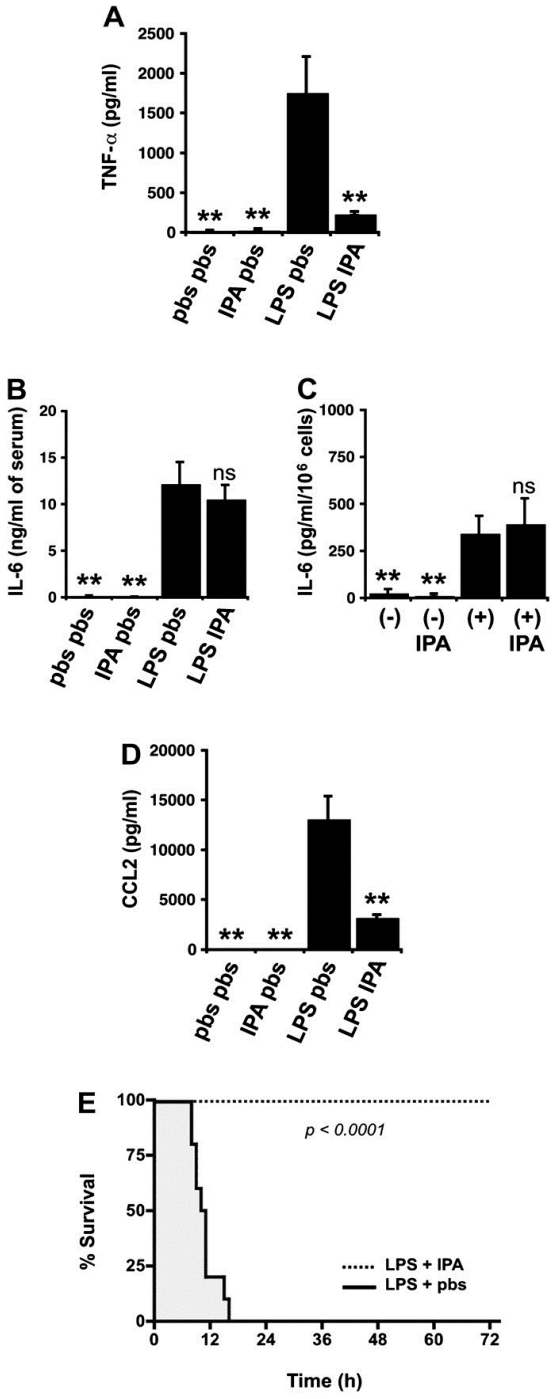

Figure 4.5 - Isopropanol-induced immunosuppression in vivo.

(A) TNF-α production is virtually stopped in mice acutely exposed to isopropanol: BALB/c mice received 5 μg LPS subcutaneously plus the intraperitoneal injection of either 2 g/kg isopropanol (LPS/IPA group) or saline (LPS/pbs group). Control groups received

either saline subcutaneously plus isopropanol intraperitoneally (IPA/pbs) or saline only (pbs/pbs). Animals were sacrificed 90 min after injections, and serum TNF-α levels were quantified by ELISA. Results are presented as means ± SEM (** $p < 0.01$ relative to the LPS/pbs group, n: 6/group).

(B) Acute exposure to isopropanol does not change the IL-6 production *in vivo* in response to LPS: BALB/c mice were injected as above and the animals were sacrificed after 180 min. Serum IL-6 was measured by ELISA. Means ± SEM are shown (*ns:* $p > 0.05$, ** $p < 0.01$ relative to the LPS/pbs group, n: 6/group).

(C) Isopropanol treatment in vitro does not interfere with the ability of mouse monocytes to produce IL-6 in response to LPS: Purified primary murine monocytes were stimulated with 1 μg/ml LPS for 24 h in presence ((+)*IPA*) or absence (+) of 0.6% (w/v) isopropanol. The levels of IL-6 in the supernatants were measured by ELISA and are shown as means ± SEM (*ns*: $p > 0.05$, ** $p < 0.01$ relative to the (+) control group; n: 3). (-) represents the unstimulated control in absence of isopropanol. (-)*IPA* represents the unstimulated control in presence of 0.6% (w/v) isopropanol. *IPA* indicates isopropanol.

(D) CCL2 production is compromised in mice acutely exposed to isopropanol: Experimental groups were treated and labeled as in panel A. Animals were sacrificed 180 min after injections, and serum CCL2 levels were quantified by ELISA. Results are presented as means ± SEM (** $p < 0.01$ relative to the LPS/pbs group, n: 6/group).

(E) Isopropanol confers full protection from LPS-induced toxic shock syndrome: BALB/c mice were presensitized with 20 mg D-galactosamine; then, they were injected with 0.2 μg LPS subcutaneously plus 2 g/kg isopropanol intraperitoneally (LPS+IPA group). Alternatively, the presensitized animals were injected with 0.2 μg LPS subcutaneously plus saline intraperitoneally (LPS+pbs group). The Kaplan-Meier survival curve is presented ($p < 0.0001$, n: 10/group).