2. Immunosuppressive effect of isopropanol: down-regulation of cytokine production results from the alteration of discrete transcriptional pathways in activated lymphocytes

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Journal of Immunology, 2008 Aug 15;181(4):2348-55.

Running title: Immunosuppressive properties of isopropanol.

Manuscript information: 63,873 characters, 5 figures, 48 references.

Keywords: immunosuppression, alcohol, T lymphocyte, NK cell, isopropanol.

Référence complète :

Résumé

L'isopropanol employé couramment pour des usages domestiques et représente la plus importante cause d'empoisonnement aigu aux alcools après l'éthanol. Bien que les effets de l'éthanol sur le système immunitaire aient été abondamment étudiés, il n'existe presqu'aucune donnée au sujet de l'isopropanol. Vu la structure similaire des deux molécules, nous avons émis l'hypothèse que l'isopropanol possédait aussi des propriétés immunomodulatrices. De fait, une exposition aiguë à l'isopropanol *in vitro* est néfaste pour l'activité des lymphocytes T et des cellules NK à des concentrations aussi faibles que 0,08-0,16% (13-26 mM). Le traitement à l'IPA ne perturbe pas la signalisation précoce en aval du récepteur, mais a un effet reproductible et dose-dépendant sur la translocation nucléaire des facteurs de transcription NFAT et AP-1.

De plus, dans un modèle d'intoxication aiguë à l'isopropanol, les animaux traités à l'alcool subissent une immunosuppression mesurée par la réduction de la présence d'IL-2 et d'IFN-γ dans le sérum en réponse à l'entérotoxine B staphylococcique. L'isopropanol a aussi réduit assez fortement la production de TNF-α de façon à faire survivre des souris à un choc toxique autrement létal induit par l'entérotoxine.

Ces résultats suggèrent que l'isopropanol est potentiellement immunosuppresseur pour les systèmes inné et adaptatif et ils ont une portée significative étant donné l'exposition fréquente de la population à ce produit chimique.

Abstract

Isopropanol (IPA) is widely used in household applications and constitutes a leading cause of acute alcohol intoxication second only to ethanol. Although the effects of ethanol on the immune system have been extensively studied, much less data is available on IPA. Given the structural similarity between the two molecules, we hypothesized that IPA could as well have immune modulatory properties. We report here that acute IPA exposure is detrimental to human T lymphocyte and NK cell activity *in vitro* in concentrations as low as 0.08-0.16% (13-26 mM). IPA treatment did not affect receptor-mediated early signaling but had a reproducible and dose-dependent effect on the nuclear translocation of the nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1). Furthermore, we show in a model of acute IPA intoxication that animals became immunosuppressed as judged by their reduced ability to release IL-2 and IFN-γ in the serum in response to the staphylococcal enterotoxin B. This effect was also associated to the down-regulation of TNF-α production and was sufficiently strong to rescue susceptible animals from enterotoxin-induced toxic shock. Our results suggest that IPA is potentially immunosuppressive to the adaptive and innate immune system and have broad significance given the exposure of the general population to this ubiquitous chemical.

Introduction

Short-chain alcohols have a multitude of biological effects, including cardiac and central nervous system depression. In addition, a considerable body of evidence indicates that ethanol is capable of modulating the immune function mediated by T cells, monocytes, macrophages and neutrophils (1-4). Ethanol also inhibits the leukocyte/endothelial cell interaction thereby limiting the inflammatory response (4). Although the *in vitro* and *in vivo* effects of ethanol have been well characterized, much less data is available on other alcohols. Isopropanol (IPA) [3] exposure is the second most common cause of acute alcohol intoxication in North America with about 20,000 cases reported each year to poison centers (5). IPA is readily available to most consumers as rubbing alcohol and as an ingredient of hand-sanitizing gels and other commonly used household solutions. In addition, IPA is

widely utilized in hospitals as an antiseptic for surgical scrub and for patient care. Occupational exposure may also occur in numerous industrial applications. Previous studies addressed the impact of IPA exposure on the central nervous system, general hematologic parameters, carcinogenesis, vascular permeability, urinary system, reproduction and development (6-9). However, no detailed analysis of the potential impact of IPA on the immune system is available.

Given the structural similarity between IPA and ethanol, we hypothesized that IPA could also have immune modulatory properties. We report here that IPA is detrimental to human T lymphocyte and NK cell activity *in vitro* in concentrations as low as 0.08-0.16% w/v (or 13-26 mM). These results were further substantiated in a mouse model of acute IPA intoxication in which animals were immunosupressed as judged by their reduced capacity to produce inflammatory cytokines. This immunosupression was sufficiently strong to protect susceptible animals from superantigen-induced lethal shock. 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

Mononuclear cells were prepared from 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 NK cells ($CD56^+$) and T cells ($CD8^+$/$CD4^+$) were obtained by using antibody-based EasySep® separation kits with magnetic nanoparticles according to the manufacturer's instructions (StemCell Technologies, Vancouver, Canada). Cells were kept in complete medium: RPMI 1640 (Invitrogen Canada, Burlington, Canada) supplemented with 10% heat-inactivated FBS (BioCell Inc., Drummondville, Canada). Isopropanol was purchased from BDH (Toronto, Canada)

In most experiments, T cells were activated for 5 h at 37°C with anti-CD3/CD28 antibody-coated magnetic beads (Invitrogen). When indicated, alternative T cell activation protocols

were used: a) pre-treatment 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 for 3 min at 37°C with 10 μg/ml goat anti-mouse IgG (Invitrogen); b) treatment with 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma, St Louis, MO) and 200 ng/ml ionomycin (Sigma) for 5 h. Human NK cells were activated for 24 h by treatment with 50 μg/ml polyinosinic:polycytidylic acid (poly (I:C)) (Sigma) in presence of 10 U/ml IL-2 and 0.01 ng/ml IL-12 (Feldan Bio Inc., St-Laurent, Canada).

CFSE Staining: Freshly purified T cells were labeled with 10 μM CFSE (Invitrogen) 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% (w/v) IPA. 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 IL-2. FACS analysis of cell divisions and surface marker expression was performed on a XL flow cytometer (Beckman Coulter Inc., Miami, FL).

Western Blot and Luciferase Assay

Western blot: Purified T cells were activated for 3 minutes at 37°C by anti-CD3/anti-IgG antibodies as described above with or without 0.6% (w/v) IPA. The cells were 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). The membranes were first probed with ZAP-70-specific antibodies: rabbit anti-human ZAP-70 (99F2, 1/1000, Cell Signaling Technology, Danvers, MA) and mouse anti-human ZAP-70 (pY319)/Syk (pY352) (17a, 1/5000, BD Biosciences, Mississauga, Canada); then, they were washed, and incubated with 1/15000 dilutions of the antibodies IRDye 800CW goat anti-rabbit IgG and IRDye 680 goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE). Detection and quantification was performed with the Odyssey Infrared Imaging System (Li-Cor Biosystems).

Luciferase assay: Jurkat-luc cells were stimulated with PMA/ionomycin with or without IPA treatment as indicated in the text. Lysates for luciferase assays were prepared with the passive lysis buffer (E1941, Promega, Madison, WI), mixed with reaction solution (25 mM

glycylglycine, pH7.8, 10 mM $MgSO_4$, 5 mM ATP), and analysed in a Berthold Lumat 9501 luminometer (Berthold, Nashua, NH) after the addition of D-luciferin (Fisher Scientific, Pittsburgh, PA) to a final concentration of 0.1 mM. 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, Hercules, CA).

Generation of Jurkat-luc cells: Stable Jurkat-luc cells were generated by lentiviral transduction. Vector generation and transduction conditions: The synthetic promoter used in our studies contains three copies of the human distal IL-2 NFAT binding site placed upstream of the -77 to +45 region of the human IL-2 promoter. The firefly luciferase gene driven by this synthetic promoter was cloned in the Nhe I and Xho I sites of a modified version of pRRL-5pme (10) to generate pLV-iluc. This parental version of pRRL-5pme also carries the zeocin resistance gene driven by the PGK promoter. Lentiviral supernates were generated in 293T cells by transient transfection (11). Three plasmids: pMDLg/RRE, pRSV-rev and pMD.VSV-G were cotransfected with pLV-iluc. The supernates containing lentivirus were harvested 48 and 72 hours after transfection, filtered through a 0.45 μm filter, and frozen at -80^{o}C until use. Jurkat cells were transduced overnight in 24-well plates at 2.5 x 105 cells/ml with 0.5 ml viral supernate plus 0.5 ml fresh medium and 8 μg/ml polybrene (Sigma). Stable Jurkat-luc cells were generated after two rounds of 4-day zeocin selection (Invitrogen).

Cytokine analysis

Measurements of human or murine IL-2 and IFN-γ in cell culture supernates and murine IL-2 and IFN-γ in serum samples were performed with specific cytokine ELISA kits according to the manufacturer's instructions (Mabtech). Briefly, 96-well plates were coated with the relevant capture antibody (hIL-2: IL-2 I, mIL-2: 1A12, hIFN-γ: 1-D1K, mIFN-γ: AN18) and incubated with serially diluted standards or unknown samples; then, they were washed and incubated with a biotinylated detection antibody (hIL-2: IL-2 II-biotin, mIL-2: 5H4-biotin, hIFN-γ: 7-B6-1-biotin, mIFN-γ: R4-6A2-biotin) followed by streptavidin-horseradish peroxidase. The plates were read at 450 nm (or 620 nm) after treatment with 3,3',5,5'- Tetramethylbenzidine (TMB) substrate solution.

Murine TNF-α levels were measured with the mouse TNF-α Enzyme Immunometric Assay Kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instructions.

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

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). NFAT activation was measured with the TransAM NFATc1 kit; c-Fos and c-Jun activation was measured with the TransAm AP-1 kit; 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 TMB substrate produced a colorimetric reaction measurable in a spectrophotometer.

Cytotoxicity assays

The cytotoxic activity was analysed in standard 4 h ^{51}Cr-release assays as reported elsewhere (11). The targets were labeled with $Na^{51}CrO_4$ for 2 h at 37^{o}C. Tests were performed in presence or absence of 0.6% (w/v) IPA in triplicate. Freshly purified NK cells and peripheral T lymphocytes were used as effectors. Target cells were the NK-sensitive K562 cell line and a control autologous lymphoblastoid cell line in the NK cells assays; Targets for T cell assays were the OKT3 hybridoma and a control hybridoma specific to MAGE-A9 (kindly provided by Dr. Alain Bergeron, Laval University).

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). Animals received subcutaneously 5 µg staphylococcal enterotoxin B (SEB) (Toxin Technology Inc., Sarasota, FL) for cytokine induction and were sacrificed by CO_2 asphyxiation 2 h or 4 h after administration for IL-2/TNF-α or IFN-γ serum analysis, respectively.

For the analysis of murine T cell subsets, mice received 20 μg SEB intravenously with (or without) an intraperitoneal injection of 2 g/kg isopropanol. The animals were sacrificed 160 min later for spleen isolation; $CD4^+$ and $CD8^+$ T cell populations were purified by using antibody-based cell separation kits with magnetic nanoparticles (StemCell Technologies) according to the manufacturer's instructions. Purified cells (97-98% pure) were cultured in vitro without isopropanol for 18 h and the supernates were checked for the presence of IFN-γ by ELISA.

Toxic shock was induced with a subcutaneous injection of 10 μg SEB after presensitization with 20 mg D-galactosamine (Sigma) as reported elsewhere (12). IPA was injected intraperitoneally (2 g/kg). Mice were checked hourly for 72 h. Animals that survived the 72 h experiment were followed for 5 days. The blood alcohol concentration (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, except when indicated otherwise. The Student's t test was used in figures 2.2B, 2.4C and 2.4D. Survival curves were determined by the Kaplan-Meier method (fig. 5E). p values < 0.05 were considered significant.

Results

Isopropanol interferes with the production of IL-2 and the proliferative capacity of antigen-receptor-activated peripheral T lymphocytes

The IL-2 gene is transcribed following antigen-specific activation of the T cell receptor (TCR). In this study, we have investigated first whether IPA exposure *in vitro* would have any impact on the ability of human peripheral lymphocytes to produce IL-2 once activated by antibody cross-linking of the TCR. These cells produced less IL-2 when treated with IPA as measured in the culture supernates by ELISA (fig. 2.1A, black bars). The reduction in cytokine production was observed at IPA doses as low as 0.16%. The observed alcohol effect was not the consequence of nonspecific cytotoxicity as the cell viability of IPA-

treated samples in the concentration range that produced 36 to 86% IL-2 inhibition was similar to that of untreated control cells (fig. 2.1A, gray bars).

Given the importance of the IL-2 autocrine loop for the expansion of antigen-specific cells *in vivo*, we asked if the reduced production of IL-2 translates into a lower proliferative capacity of the activated lymphocytes. Purified T cells were labeled with CFSE, activated, and analysed 72 h after by flow cytometry. Although the 5-h TCR cross-linking led to 1-4 divisions in about 40% of the cells in absence of exogenous IL-2 (mean: 39.69 ± 2.06 SEM, n: 4), cell proliferation was much less pronounced in presence of IPA treatment (mean: 18.82 ± 4.53 SEM, n: 4). The means of IPA-treated and untreated cells differ with a p value of 0.0058 (Student's t test). One representative experiment is shown in figure 2.1B.

Early signaling following TCR activation is preserved in isopropanol-treated lymphocytes

IPA could conceivably interact with the T cell receptor directly thereby severing antigen-dependent signal transduction in lymphocytes. To address this possibility we have examined the phosphorylation status of the key downstream adaptor molecule ZAP-70. Figure 2.2A shows that signaling through the T cell receptor itself was not affected by IPA, as the ZAP-70 activation proceeded as efficiently as in untreated cells following anti-CD3 antibody cross-linking. Figure 2.2B presents the densitometric analysis of three Western blots, one of which is depicted in panel A. Next, we have examined the cytosolic Calcium levels induced by T cell activation in presence of IPA. Intracellular Calcium oscilations regulate a variety of biological processes, including antigen-dependent, TCR-mediated T cell activation (13). IPA did not affect the cytosolic Calcium increase observed after TCR triggering or treatment with the Calcium ionophore ionomycin (data not shown).

Isopropanol blocks IL-2 production via transcriptional inhibition

The lack of an obvious impact of the IPA treatment on early TCR signaling led us to examine the possibility of a negative effect on IL-2 transcription. For this purpose, we have generated a stable Jurkat subline carrying the firefly luciferase gene driven by a synthetic IL-2 minimal promoter shown previously to be responsive to PMA/ionomycin (Jurkat-*luc*) (14-15). IPA was capable of inhibiting in a dose-dependent manner the luciferase activity triggered by PMA/ionomycin in these cells (fig. 2.3A). IPA concentrations as low as 0.3%

had a significant dampening effect on IL-2 transcription as indicated by a 24% reduction in luciferase activity. Similar results were obtained by anti-CD3 antibody cross-linking in Jurkat-luc cells (data not shown).

Nuclear translocation of transcription factors is affected by isopropanol in activated T cells

The promoter used in the experiments shown in figure 2.3A contains three copies of the binding site for the nuclear factor of activated T cells (NFAT) placed upstream of the IL-2 core promoter and is highly responsive to the Ca^{++}/NFAT signaling pathway (14-15). However, the regulation of the IL-2 gene following TCR triggering is more complex and involves the participation of transcription factors activated by two additional major signal transduction pathways (16). In order to dissect further the relative impact of IPA on these molecules, we have measured the nuclear translocation of NFAT (NFATc1), nuclear factor-κB (NF-κB: p50/p65), and activator protein-1 (AP-1: c-Jun/c-Fos) in TCR-stimulated purified human T cells exposed to different concentrations of IPA. Figure 2.3B shows that lymphocyte activation by anti-CD3/CD28 antibodies led to a 2-fold increase in the amount of NFAT in the nucleus. The same stimulation in presence of 0.6% IPA led only to a 1-fold increase in nuclear NFAT (or 54% of the maximal NFAT nuclear content above the unstimulated cell baseline). This effect was dose-dependent with the highest inhibition observed for the highest IPA concentrations. The calcineurin inhibitor cyclosporine A was used as a control in the same stimulatory conditions with little variation in nuclear NFAT content (17% less than the nuclear content baseline of unstimulated cells). Activation of AP-1 in presence of IPA followed the same pattern observed for NFAT with 55% of the maximal c-Jun nuclear content above the unstimulated cell baseline achieved at the highest IPA concentration (fig. 2.3E). Activation by anti-CD3/CD28 antibodies in presence of the c-Jun N-terminal kinase inhibitor SP600125 produced 42% of the maximal c-Jun nuclear content above the unstimulated cell baseline. The same T cell stimulatory conditions produced 45.4% of the maximal c-Fos nuclear content in presence of the highest IPA concentration and 88.3% in presence of the MEK1 inhibitor PD98059 (fig. 2.3F). In contrast to the results obtained with NFAT and AP-1, activation of NF-κB remained unaffected by IPA treatment at all tested concentrations (fig. 2.3C/D). 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.

Production of the inflammatory cytokine interferon-γ by human peripheral T lymphocytes and NK cells is inhibited by isopropanol

The NFAT and AP-1 families of transcription factors play a major role in the expression of several cytokines, including IFN-γ (17-22). The finding that the inhibition of IL-2 production in IPA-treated T cells is associated with reduced nuclear translocation of c-Jun, c-Fos and NFAT led us to speculate that IFN-γ expression would also be affected in these cells. First, we have examined the IFN-γ release in peripheral T lymphocytes following activation with anti-CD3/CD28 antibodies in presence of different IPA concentrations (fig. 2.4A). Treatment with 0.16% IPA led to a 35% reduction in IFN-γ release. The higher IPA concentrations tested, 0.3%, 0.6% and 1.2% produced inhibitory effects of 55%, 84% and 98%, respectively. Cyclosporin A in the same experimental conditions led to a virtually complete IFN-γ inhibition (data not shown).

The above results have encouraged us to extend our analysis to another immune cell capable of producing large amounts of IFN-γ. Purified human NK cells have been stimulated in vitro with poly (I:C) in presence of IL-2 and IL-12 and exposed to different quantities of IPA. Figure 4B shows that concentrations as low as 0.08% were active. Treatment with 0.08%, 0.16% and 0.3% IPA reduced the IFN-γ release by stimulated NK cells by 31%, 40% and 87%, respectively. The two highest IPA concentrations produced an almost complete inhibitory effect with only background levels of IFN-γ being released. NK cells were > 95% viable at all IPA concentrations tested (data not shown).

Isopropanol reduces the cytotoxic activity of T lymphocytes and NK cells in vitro

The identification of the negative impact of isopropanol on the production of IFN-γ by T and NK cells has prompted us to examine if this effect was extended to other effector functions. Panel C in figure 4 shows that the cytotoxic activity of purified human peripheral T lymphocytes against OKT3 hybridoma cells was inhibited by about 20% in presence of IPA. OKT3 cells display the activating anti-CD3 antibody and work as a T cell target (23).

Similarly, the cytotoxicity of purified human NK cells against K562 target cells was inhibited by 30-40% in presence of IPA (Figure 2.4D).

Isopropanol inhibits the production of IL-2 and IFN-γ in vivo

After having demonstrated the negative impact of IPA treatment on IL-2 and IFN-γ production by lymphocytes *in vitro*, we have examined the relevance of these findings in a mouse model of acute IPA intoxication. Mice were administered IPA intraperitoneally, 2 g/kg, to generate a mean blood alcohol concentration of 200 mg/dl after 30 min (198 ± 3.704 SEM, n: 10). Induction of IL-2 and IFN-γ production *in vivo* was achieved by subcutaneous injection of the superantigen staphylococcal enterotoxin B. T lymphocytes with the relevant TCR Vβ chains undergo transient activation, cell proliferation, and begin massive cytokine production (12). As anticipated, injected SEB induced IL-2 levels of > 12 ng/ml after 2 h (fig. 2.5A) and > 1.6 ng/ml IFN-γ after 4 h (fig. 2.5B). IPA dampened the cytokine production substantially: 49.2% or 6.3 ng/ml IL-2 detected at 2 h, and 86.6% or 0.2 ng/ml IFN-γ detected at 4h. The differences in cytokine serum levels between animals treated and untreated with IPA were statistically significant as indicated in figures 2.5A and 2.5B.

Isopropanol treatment impacts both $CD4^+$ and $CD8^+$ T lymphocytes in vivo

Both $CD4^+$ and $CD8^+$ T lymphocytes may respond to SEB to produce cytokines. We have examined next how these T cell subsets were affected by isopropanol in our in vivo experimental model. BALB/c mice were injected with SEB intravenously to activate $CD4^+$ and $CD8^+$ T cells carrying the responsive TCRs; isopropanol was provided intraperitoneally. It has been shown that a minimal T cell/APC conjugate time of 2 h is required for commitment to cytokine production and cell proliferation (24). Basing on this premise, we have followed the animals for 160 minutes, after which they were sacrificed; their splenocytes were harvested, and separated into $CD4^+$ and $CD8^+$ T cell populations. Purified T cell subsets were incubated for 18 h at 37^{o}C and culture supernates were analysed by ELISA for IFN-γ production. Figure 2.5C shows that isopropanol exposure in

vivo reduced the IFN-γ release by $CD4^+$ T cells in vitro by 74%. Similarly, the IFN-γ production in vitro by $CD8^+$ T cells decreased about 70% when animals from which they derived were exposed to IPA for 160 min (fig. 2.5D).

The immunosuppressive effect of IPA confers protection to animals from SEB-induced toxic shock

The ability of IPA to prevent or substantially reduce the production of cytokines in response to SEB *in vivo* led us to wonder whether this immunosuppressive effect could delay the development of toxic shock in susceptible animals. In order to investigate this possibility, we have sensitized BALB/c mice with 20 mg D-galactosamine by intraperitoneal injection. All sensitized animals succumbed to toxic shock within 14 h after receiving SEB subcutaneously (12/12) (fig. 2.5E). In contrast, a single injection of 2 g/kg IPA had a dramatic effect since it delayed or completely aborted the development of SEB-induced toxic shock in all animals. Seven out of twelve animals survived the 72-h experiment; they were followed for up to 5 days after SEB injection and were indistinguishable from sensitized control groups receiving PBS or IPA as regards activity, grooming, eating and drinking behavior. No animal injected with IPA (12/12) or PBS (12/12) in absence of SEB died.

In addition to IL-2 and IFN-γ, the inflammatory cytokine TNF-α is copiously produced and plays a central role in the pathophysiology of superantigen-induced lethal shock (12). TNF-α gene transcription was shown by other investigators to be regulated by NFAT (25). Our results revealed IPA as a negative regulator of NFAT nuclear translocation *in vitro* and as an immunosuppressive agent capable of protecting mice from toxic shock. Therefore, it was a logical assumption that IPA treatment would block TNF-α production *in vivo*. Indeed, figure 2.5F shows that mice treated with SEB produced significant amounts of TNF-α in the first 2 h after injection as opposed to animals that received IPA + SEB.

Discussion

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 (> 2% w/v or > 500 mM) (26). 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 (27-29). 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 (26-29). As suggested for other short-chain alcohols (26, 30), IPA could displace water molecules from such pockets and establish contact with the proteins via hydrogen bonds that would be stabilized by van der Waals forces in the hydrophobic region. These interactions would ultimately produce a local distortion and alteration in protein function.

Another interpretation for the effects of IPA would result from the possible interference with the capacity of membrane micro-domains to recruit and/or retain molecules involved in signaling, thus compromising the formation of the immunological synapse. Given the central role played by lipid rafts in amplifying receptor-mediated signals in immune cells (3), it is conceivable that IPA could affect surface molecules, such as the TCR, directly by inducing unfavorable conformational changes or, indirectly, by disrupting lipid-protein interactions. A similar model has recently been evoked to explain the ethanol inhibition of LPS-mediated Toll-like receptor 4 (TLR4) signaling in macrophages (3).

Upon engagement of the relevant ligand, the TCR triggers a phosphorylation cascade that is followed by a biphasic increase in intracellular Ca^{++}. The initial wave derives from the intracellular stores and is rapidly trailed by the extra-cellular influx regulated by Ca^{++} release-activated Ca^{++} (CRAC) channels (13). We initially examined if IPA would exert its immunosuppressive effect by interference with the CRAC-regulated Ca^{++} influx and the subsequent calcineurin-dependent activation of NFAT. There was some support for this possibility given the reported association of other alcohols with ion channels, often altering their function (26-27, 29). We failed to show any IPA-induced change in the pattern of intracellular Calcium increase that follows TCR triggering or ionomycin treatment (data not shown). The fact that TCR-mediated early signaling as measured by ZAP-70 phosphorylation and Calcium release is preserved indicates that the effect of IPA is downstream of the cell membrane. We cannot discard, however, that higher IPA

concentrations could also affect lipid rafts in a way reminiscent of the model suggested for ethanol on the TLR4 receptor (3) but this remains to be experimentally tested.

The inhibition reported here was observed *in vitro* at IPA concentrations as low as 0.08% (13 mM) as measured by IFN-γ release in NK cells and 0.16% (26 mM) as measured by IL-2 and IFN-γ release in T cells. These concentrations are equal to or lower than those of ethanol used in previous studies that reported a statistically significant impact on immune cells (1-4). Many of the biological effects of ethanol on the immune system have been associated to a reduced nuclear translocation of NF-κB, a transcription factor capable of binding the promoter regions of multiple cytokines (3-4). In contrast, we found that IPA does not affect NF-κB but has a reproducible and dose-dependent effect on the nuclear translocation of AP-1 and NFAT. This finding supports the view that IPA exerts its impact on immune cells through the interaction with selective pathways rather than a membrane-based nonspecific down-modulation of the immune cell activation. NFAT and AP-1 have been shown to modulate synthesis of the three cytokines examined in this paper (IL-2, IFN-γ and TNF-α) (14-15, 17-22, 25). As expected, NFAT nuclear translocation and cytokine release was blocked by cyclosporine A in activated lymphocytes. Nevertheless, IPA differed from cyclosporine A in that it did not target the calcineurin phosphatase activity (data not shown). Thus it must interact directly with NFAT or with downstream molecules involved in its nuclear translocation, such as importin β1, or molecules involved in its phosphorylation in the nucleus, such as glycogen synthase kinase 3. Similarly, IPA may also interact directly with c-Jun and/or c-Fos compromising the formation and/or function of the AP-1 dimer as the phosphorylation pattern of upstream molecules such as p38 remains unchanged (data not shown).

In order to address the immunosuppressive effects of IPA in an *in vivo* setting, we have used a model of acute intoxication. Deliberate or accidental ingestion of IPA ranks second as a cause of alcohol poisoning according to the 2005 annual report of the American Association of Poison Control Centers (5). Acute intoxication usually occurs in alcoholic patients, children, and suicidal individuals (31). The blood IPA concentration can reach levels as high as 560 mg/dl (0.56% or 93 mM); many of the reported measurements have been made hours after ingestion and may underestimate the serum amounts present in the

early phase of the intoxication (32-37). Nevertheless, a concentration above 400 mg/dl is usually considered life-threatening and demands a more aggressive intervention such as dialysis (38-39). In our model, we have injected mice intraperitoneally with 2 g/kg IPA to generate a blood alcohol concentration of 200 mg/dl (0.2% or 33 mM) after 30 min; this level is under the reported average sublethal blood concentration in intoxicated humans (310 mg/dl) (40) and is well within the concentration range that we have shown to be biologically active *in vi*tro (starting at 0.08-0.16% or 13-26 mM). Our results indicate that during this state of acute intoxication the animals are immunosuppressed as judged by their reduced ability to release IL-2 or IFN-γ in the serum in response to SEB. The magnitude of this immunosupression was further assessed by monitoring the survival of animals injected with SEB after presensitization with D-galactosamine. We were initially uncertain about the outcome of this particular experiment as we thought that it might be too stringent. In fact, all presensitized animals developed a fulminating toxic shock syndrome with a median survival of 9 h after SEB injection. Nevertheless, in contrast to the untreated animals, the syndrome did not occur or had its development delayed in all mice treated with IPA and the majority survived. This is in line with the massive suppressive effect of IPA on the production of IL-2, IFN-γ and TNF-α. It is believed that the production of copious amounts of IFN-γ, and especially of TNF-α, plays a major role in the development of the syndrome (12, 25).

An obvious consequence of our findings is the assumption that any individual acutely intoxicated by IPA may also be acutely immunosuppressed. This assumption could be easily tested in a clinical setting and may provide the basis for the establishment of precautionary measures in the emergency room to deal with this predicament. The issue would be particularly relevant in circumstances in which an underlying infection or trauma complicate the clinical picture.

The implications of our results could be also extrapolated to areas other than acute systemic intoxication. IPA is used in many industrial applications and occupational exposure by inhalation or other routes may occur (8). One of the major weaknesses of the literature on IPA is the virtual absence of solid information about chronic toxicity in humans. Our experiments were not designed to address this issue. Nevertheless the results reported here

for acute exposure indicate that immunological parameters may serve as a sensitive endpoint for IPA toxicity that could be included in future studies on the long-term effects of this chemical.

Intact adult skin is not an efficient route of IPA absorption; the skin permeation coefficient (k_p) is estimated to be in the order of 4-15 x 10^{-4} cm/h (41-43). Yet, dermal absorption does occur (41, 44-45) and a few cases of systemic IPA intoxication after topical exposure have been reported in the literature (46-48). IPA is present, often in a high concentration (60-95%), in hand sanitizer gels/solutions and many household products readily available over the counter. Taking into consideration that our results show a significant biological effect *in vitro* with IPA concentrations as low as 0.08-0.16% (13-26 mM), it is reasonable to question if the application to the skin of a product that is 500-1000 times more concentrated would have similar consequences even in the context of poor dermal absorption. As regards intact normal adult skin, it is likely that the immunosuppressive effect, if any, would be transitory and only relevant to the immune cells present in the treated skin itself. Nevertheless, future studies to address this issue are warranted given the widespread and poorly regulated use of this chemical.

The *in vitro* data presented here suggests that acute IPA exposure reduces the ability of lymphocytes to produce proinflammatory cytokines, and thus may compromise the innate and adaptive immune system; in addition, acute intoxication led to acute immunosupression *in vivo*, an effect that was sufficiently strong to rescue susceptible animals from enterotoxin-induced toxic shock. These results are directly relevant in the context of acute IPA intoxication and constitute a rational for the inclusion of immunological endpoints into the design of future studies to address chronic and topical IPA exposure.

Acknowledgements

The authors wish to thank: G. Crabtree for the IL-2 minimal promoter; J. Gosselin and M. Dufour for Calcium experiments; L. Nadeau for gas chromatography; D. Richard for access to the infrared imaging system; P. Salmon and D. Trono for lentivectors; D. L'Héreault and H. Dombrowski for blood collection; J. Charron, F. Lyon, J. Filley and M. Vauzelle for helpful discussions; Anne Julien and Richard Boutet for valuable technical assistance.

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Footnotes

[1] This work was supported by grants from the National Sciences and Engineering Research Council of Canada and the Canada Foundation for Innovation.

[2] Address correspondence and reprint requests to Dr. Pedro O. de Campos-Lima, Laval University Cancer Research Center, McMahon St 9, Quebec City, QC, G1R 2J6, Canada.

[3] Abbreviations used in this paper: IPA, isopropanol, isopropyl alcohol, 2-propanol; SEB, staphylococcal enterotoxin B.

Figures and Legends

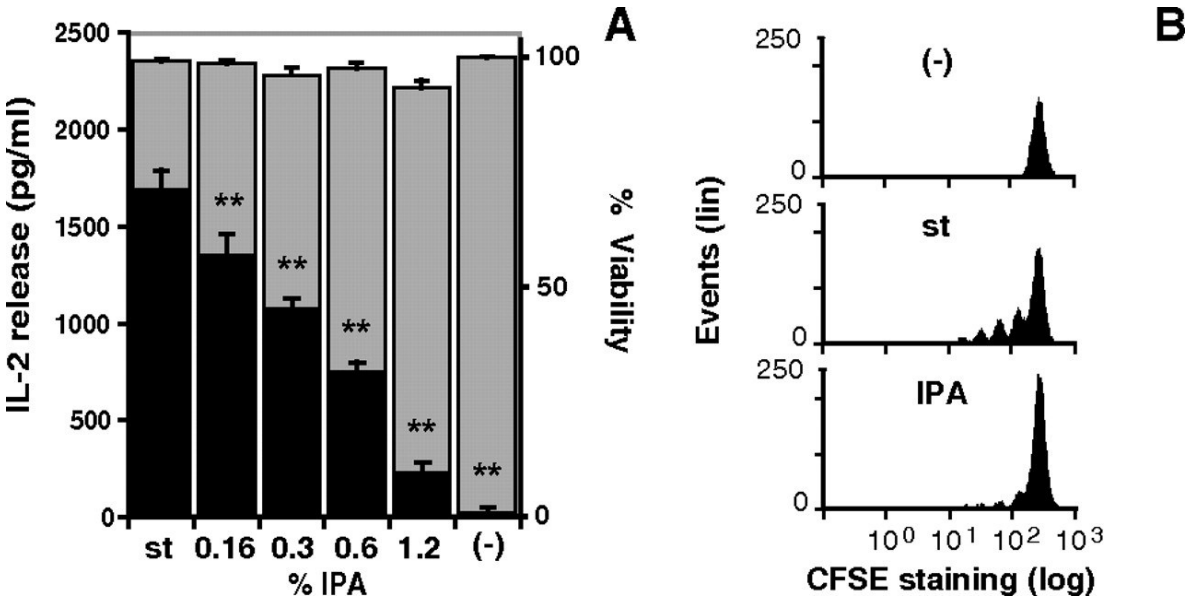

Figure 2.1 - Biological effect of Isopropanol treatment *in vitro*.

(A) Isopropanol inhibits 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 0.16%, 0.3%, 0.6% and 1.2% (w/v) isopropanol. The IL-2 concentration in the supernates 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* control group, n: 4).

(B) Flow cytometric analysis of CFSE-labeled cells: one representative experiment of four is presented.

Figure symbols: *st* indicates cell activation in absence of isopropanol. (-) represents the unstimulated control in absence of isopropanol. *IPA* indicates presence of 0.6% (w/v) isopropanol.

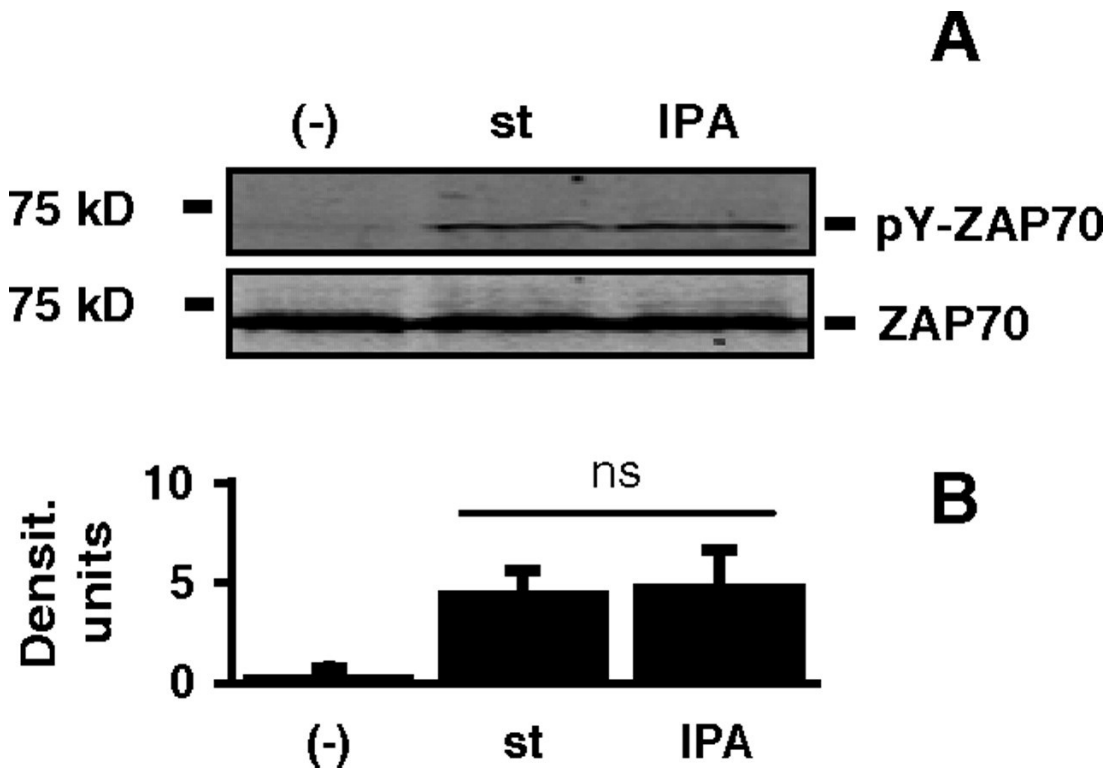

Figure 2.2 - Isopropanol and early TCR signaling

(A) Isopropanol treatment does not affect ZAP-70 phosphorylation following TCR activation: T cells were stimulated for 3 min with anti-CD3/anti-IgG in presence or absence of 0.6% (w/v) IPA and processed for SDS/PAGE. Western blots were probed with the monoclonal antibodies 17a (anti-human phospho-ZAP-70) and 99F2 (anti-human total ZAP-70). One representative blot of three is shown.

(B) Relative quantitation in relation to total ZAP-70 from 3 Western blots is shown as mean densitometric units ± SEM. Figure symbols are as in fig. 1. *ns:* $p > 0.05$.

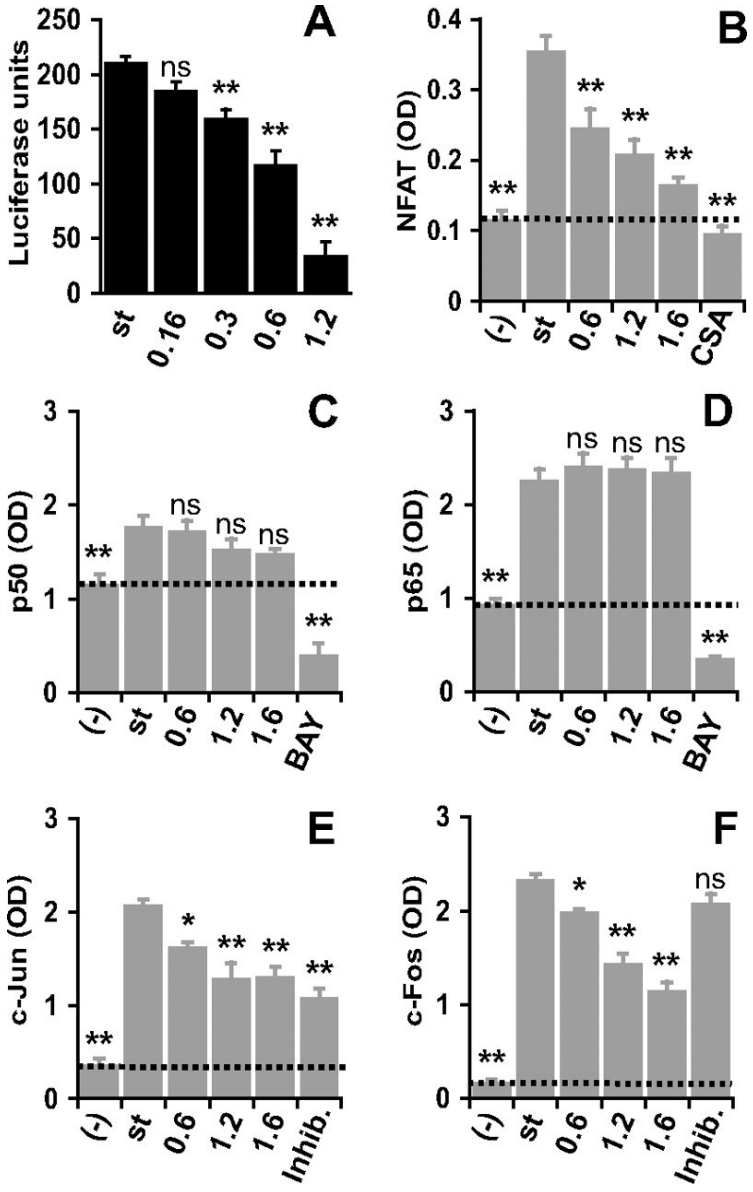

Figure 2.3 - Transcriptional effect of isopropanol.

(A) Jurkat-*luc* cells were stimulated with PMA/ionomycin in presence of 0.16%, 0.3%, 0.6%, and 1.2% (w/v) isopropanol for 5 h. Samples were lysed and assayed for luciferase activity. Results are presented as mean relative luciferase units/µg of protein ± SEM (n: 3). (B) Isopropanol inhibits NFAT nuclear translocation: T cells were stimulated with anti-CD3/CD28 antibody-coated beads for 5 h in presence of 0.6%, 1.2% and 1.6% (w/v) isopropanol. Nuclear extracts were incubated with immobilized NFAT-binding oligonucleotides in 96-well plates; the amount of retained transcription factor was assessed

with a NFAT-specific antibody by ELISA. Data is presented as mean optical density (OD) units ± SEM (n: 6).

(C/D) NF-κB nuclear translocation is not affected by isopropanol: T cells were stimulated as described in panel B in presence of the indicated amounts of isopropanol. Nuclear extracts were incubated with 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. Data is presented as mean OD units ± SEM (n: 3 for C; n: 3 for D).

(E/F) Isopropanol inhibits AP-1 nuclear translocation: T cells were stimulated as described in panel B in presence of the indicated amounts of isopropanol. Nuclear extracts were incubated with 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. Data is presented as mean OD units ± SEM (n: 3 for E; n: 3 for F).

The dashed line in 3B, 3C/D and 3E/F represents the baseline of the relevant transcription factor nuclear content in unstimulated cells. *ns:* $p > 0.05$, * $p < 0.05$, ** $p < 0.01$ relative to the *st* control group.

Stimulation in presence of inhibitor compounds are indicated as follows: cyclosporine A: *CsA*; BAY 11-7082: *BAY;* SP600125: inhib. (panel E); PD98059: inhib. (panel F); other symbols are as in fig. 1.

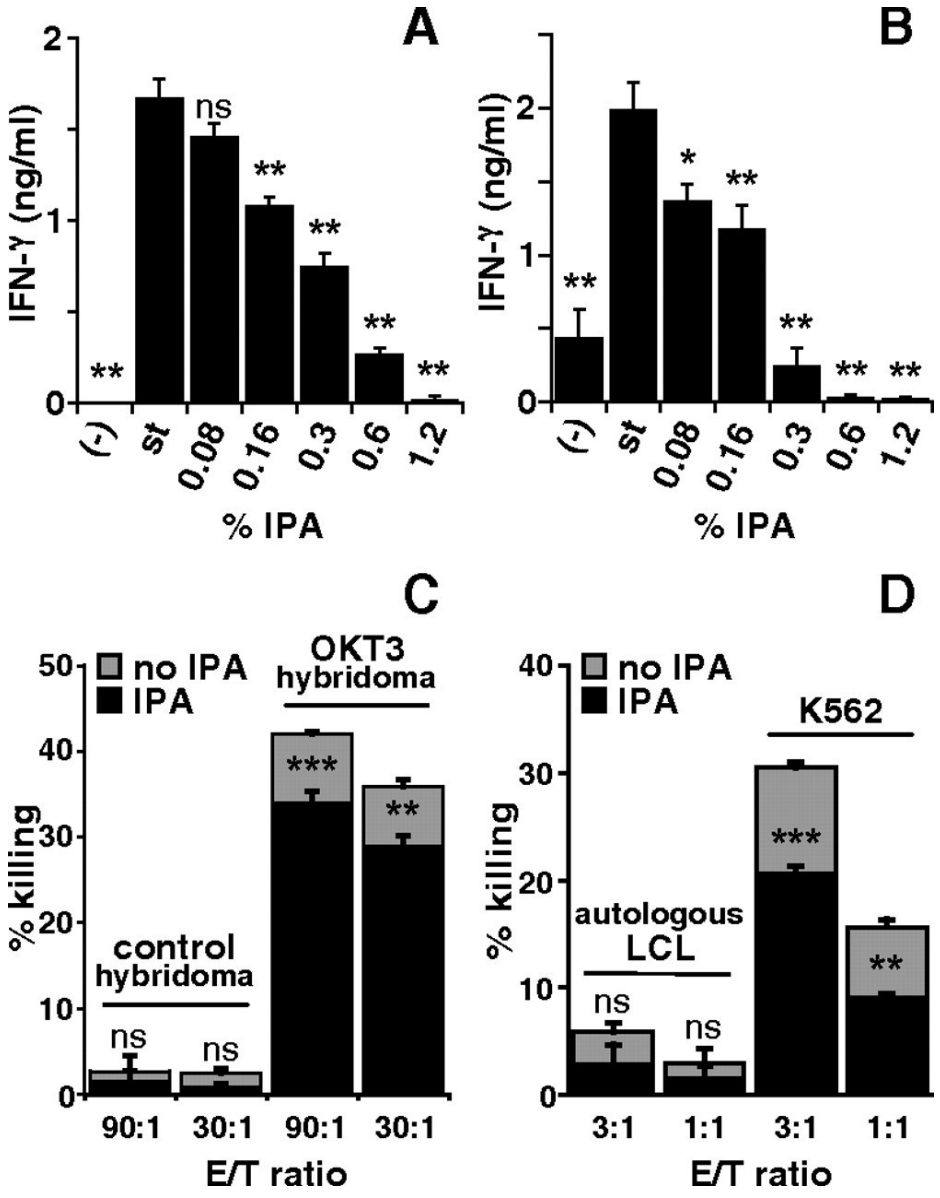

Figure 2.4 - Down-modulation of the effector function of human lymphocytes exposed to isopropanol *in vitro*.

(A) Isopropanol inhibits IFN-γ production in human 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%, 0.6% and 1.2% (w/v) isopropanol. The IFN-□ concentration in the supernates was measured by ELISA. Panel symbols: *st* indicates cell activation in absence of isopropanol. (-), represents the unstimulated control in absence of

isopropanol. Results are presented as means ± SEM (*ns*: $p > 0.05$, ** $p < 0.01$ relative to the *st* control group; n: 3).

(B) Isopropanol inhibits IFN-γ production in human NK cells: NK cells were stimulated by poly (I:C) in presence of IL-2/IL-12 for 24 h. Isopropanol was used in different concentrations, 0.08%, 0.16%, 0.3%, 0.6% and 1.2% (w/v), as shown. The IFN-γ concentration in the supernates was measured by ELISA. Panel symbols: *st* indicates cell activation in absence of isopropanol. (-) represents the unstimulated control in absence of isopropanol (complete medium plus IL-2/IL-12 without poly (I:C)). Results are presented as means ± SEM (* $p < 0.05$, ** $p < 0.01$ relative to the *st* control group; n: 3).

(C) Isopropanol decreases the cytotoxic activity of human T cells: The effector function of peripheral T cells against OKT3 hybridoma cells was analysed by Chromium-release assays in presence or absence of 0.6% (w/v) IPA as described in materials and methods. The effector/target ratios are indicated. Means ± SEM of untreated (grey columns) and IPA-treated (black columns) cells are shown in the bar histogram. The two means (treated versus untreated cells) were compared with the t test for each ratio: *ns*: $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$; n: 4.

(D) Isopropanol reduces the cytotoxic activity of human NK cells. The cytolytic function of peripheral NK cells against K562 cells was measured by Chromium-release assays in presence or absence of 0.6% (w/v) IPA as described in materials and methods. Means ± SEM of untreated (grey columns) and IPA-treated (black columns) cells are shown. The effector/target ratios are indicated. The two means (treated versus untreated cells) were compared with the t test for each ratio: *ns*: $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$; n: 4.

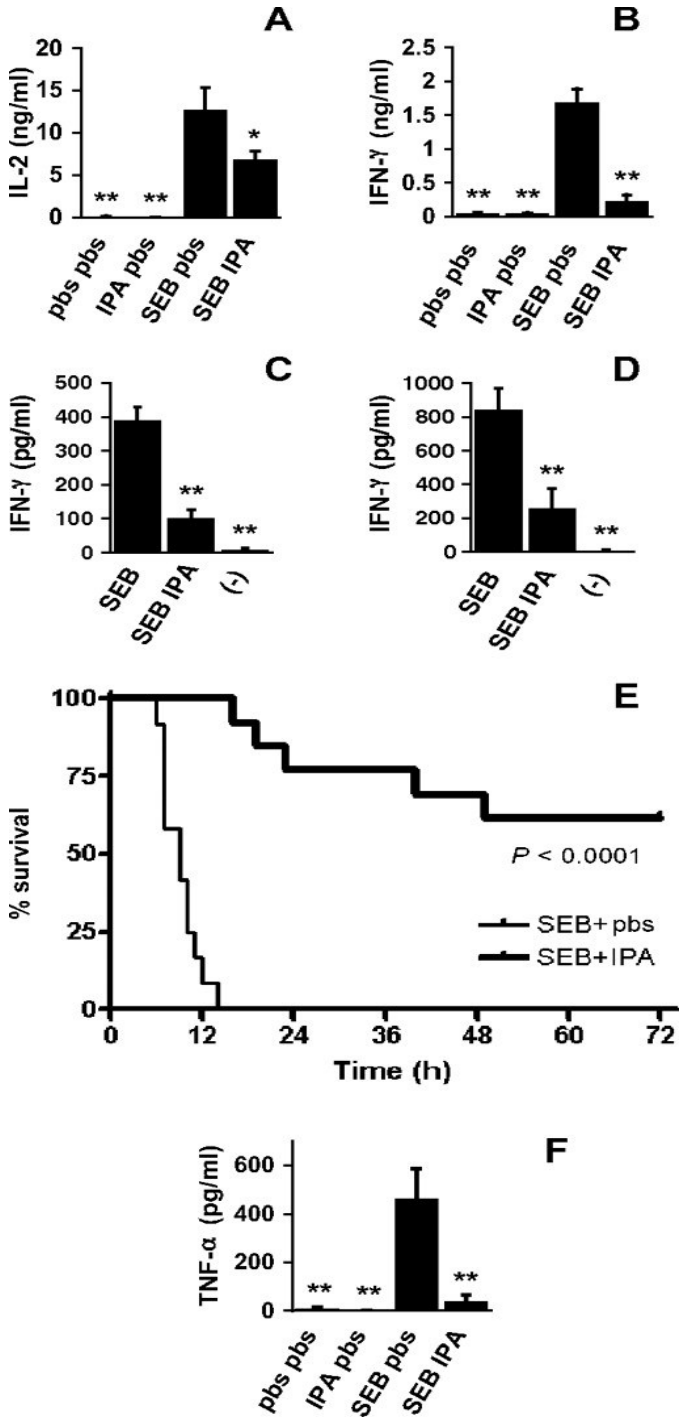

Figure 2.5 - Immunosuppressive effect of isopropanol *in vivo*.

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

saline subcutaneously plus isopropanol intraperitoneally (pbs/IPA) or saline only (pbs/pbs). Animals were sacrificed 2 h after injections, and serum IL-2 levels were quantified by ELISA. Results are presented as means ± SEM (* $p < 0.05$, ** $p < 0.01$ relative to the SEB/pbs group, n: 6/group).

(B) IFN-γ production is compromised in mice acutely exposed to isopropanol: BALB/c mice were injected as above and the animals were sacrificed after 4 h. Serum IFN-γ was measured by ELISA. Means ± SEM are shown (** $p < 0.01$ relative to the SEB/pbs group, n: 6/group).

(C) Isopropanol treatment in vivo inhibits the production of IFN-γ in vitro by $CD4^+$ T cells: Purified $CD4^+$ T cells were prepared from the spleens of animals that were treated (or not) with 2 g/kg isopropanol for 160 minutes before the sacrifice as described in materials and methods. The plotted results reflect the IFN-γ release after an 18-h culture period in vitro. SEB indicates that cells derived from animals that received only SEB (20 μg). SEB-IPA indicates that lymphocytes derived from mice that were injected with both SEB (iv) and isopropanol (ip). (-) indicates that cells came from animals that received neither SEB nor isopropanol. Results are presented as means ± SEM (** $p < 0.01$ relative to the SEB group, n: 5/group).

(D) Isopropanol exposure in vivo inhibits IFN-γ release in vitro by $CD8^+$ T lymphocytes: Purified $CD8^+$ T cells were prepared from the spleens of animals that were treated (or not) with isopropanol as described in panel 5C. The IFN-γ release was measured in supernates by ELISA after an 18-h culture period in vitro. The symbols SEB, SEB-IPA, and (-) are as in panel 5C. Results are presented as means ± SEM (** $p < 0.01$ relative to the SEB group, n: 4/group).

(E) Isopropanol protects mice from SEB-induced toxic shock: BALB/c mice were presensitized with 20 mg D-galactosamine; then, they were injected with 10 μg SEB subcutaneously plus 2 g/kg isopropanol intraperitoneally (SEB+IPA group). Alternatively, the presensitized animals were injected with 10 μg SEB subcutaneously plus saline intraperitoneally (SEB+pbs group). The Kaplan-Meier survival curve is presented ($p < 0.0001$, n: 12/group).

(F) TNF-α production is compromised in mice acutely exposed to isopropanol: Experimental groups were treated and labeled as in panel A. Animals were sacrificed 2 h after injections and serum TNF-α levels were quantified by ELISA. Results are presented as means ± SEM (** $p < 0.01$ relative to the SEB/pbs group, n: 6/group).