The Size of the Unbranched Aliphatic Chain Determines the Immunomodulatory Potency of Short and Long Chain \textit{n}-Alkanols*

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Background: Aliphatic \textit{n}-alkanols are a family of ubiquitous substances that display general anesthetic properties in accordance to their degree of hydrophobicity. In addition, the immunomodulatory activity of one of its members, ethanol, has long been recognized. We reasoned that because unbranched aliphatic \textit{n}-alkanols are structurally very similar they might have an immunological impact that mirrors their anesthetic potency. We report the impact of the homologous \(C_1-C_{12}\) alcohol series on the ability of activated primary human lymphocytes to produce IFN-\(\gamma\). Methanol enhanced IFN-\(\gamma\) production whereas \(C_2-C_{10}\) alcohols reduced the release of this cytokine. The activity of the \textit{n}-alkanol series was observed within a wide concentration window ranging from millimolar levels for short chain alcohols to micromolar amounts for \(C_7-C_{10}\) alcohols. There was a clear correlation between immunomodulatory activity and hydrophobicity of the compounds, but a cutoff effect was evident at \(C_{11}\). \textit{n}-Alkanols were shown to act downstream of the cell membrane because T cell receptor early signaling was preserved. The activation of the nuclear factor of activated T cells (NFAT) was down-regulated progressively in accordance to the size of the \textit{n}-alkanol aliphatic chains with a clear downward trend that was interrupted at \(C_{11}\). The nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) signaling was also compromised, but the cutoff appeared earlier at \(C_{10}\). The pattern of immunomodulation and transcriptional dysregulation induced by the \textit{n}-alkanol series suggested the existence of interaction pockets of defined dimensions within intracellular targets that compromise the activation of NFAT and NF-\(\kappa\)B transcription factors and ultimately modulate the effector function of the T lymphocyte.

Aliphatic \textit{n}-alkanols are a family of ubiquitous substances composed of short chain (\(C_1-C_5\)) and long chain (\(C_6-C_{22}\)) alcohols that are used in a variety of domestic and industrial applications (1, 2). Extensive literature has documented the capacity of ethanol to modulate the immune system directly or indirectly by interfering with the function of T lymphocytes, monocytes, macrophages, dendritic cells, neutrophils, and endothelial cells (3–8). Recent results have provided evidence that two other short chain alcohols also possess a discernible immunomodulatory footprint. Thus, isopropyl alcohol was shown to down-regulate the effector function of T cells, NK cells, and monocytes (9, 10). Conversely, methanol enhances the inflammatory cytokine release from activated T lymphocytes (11). A common thread in the mechanisms that underlie the biological effect of the above short chain alcohols is their obvious impact on transcriptional pathways that are important for immune cell function. Many of the effects of ethanol on the immune system have been associated with the dysfunctional activation of the nuclear factor-\(\kappa\)B (NF-\(\kappa\)B)\(^2\) (4–6), and, at least in lipopolysaccharide-activated macrophages, these biological consequences have been suggested to result from a change in the dynamics of protein recruitment into rafts on the cell membrane (6, 12). Instead, the impact of isopropyl alcohol and methanol initiates downstream of the cell membrane and is mediated by the dysregulation of distinct members of the nuclear factor of activated T cells (NFAT) family of transcription factors with or without additional involvement of the activator protein-1 (1).

The work described in this article was set off by two observations: first, unbranched aliphatic \textit{n}-alkanols display general anesthetic properties that correlate with their degree of hydrophobicity (13–16); and second, our own preliminary data show that isopropyl alcohol is substantially more effective than ethanol in down-regulating the effector function of T lymphocytes. We reasoned that because unbranched aliphatic \textit{n}-alkanols are structurally very similar they might have an immunological...
impact that mirrors their anesthetic potency observed within the C7–C12 range. We have chosen the production of interferon-γ (IFN-γ) by primary human T lymphocytes as the readout to test our hypothesis because this cytokine is essential for the innate and adaptive immune response (17). The present article reports that indeed the size of the aliphatic chains determines the immunomodulatory potency of n-alkanols. Furthermore, our results suggest the existence of discrete molecular targets downstream of the cell membrane, which display defined alcohol interaction cutoffs.

EXPERIMENTAL PROCEDURES

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). Written informed consent was obtained from all donors. Monocyte-depleted populations were >95% pure human T cells (CD3+/CD4−) and were kept in complete medium: RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (BioCell Inc., Drummondville, Canada).

T cells were activated with anti-CD3/CD28 antibody-coated magnetic beads (Invitrogen) at 37 °C for 5 h (luciferase assays) or 6 h (ELISA). When indicated, three alternative T cell activation protocols were used: (i) pretreatment for 20 min on ice with 1 μg/ml mouse anti-human CD3 monoclonal antibody (CD3-2; Mabtech, Nacka Strand, Sweden) and 2.5 μg/ml mouse anti-human CD28 (CD28.2; BioLegend, San Diego, CA), followed by incubation at 37 °C for 5 h with a 10-fold excess of goat anti-mouse IgG (Sigma); (ii) treatment in the early signaling step at 37 °C that was performed for 3 min; and (iii) treatment with 10 ng/ml phorbol 12-myristate 13-acetate/ionomycin as described earlier with or without the relevant alcohol at the indicated molar concentration. Nuclear extracts were prepared with the Active Motif kit; samples were separated in 7.5% polyacrylamide gels and blotted onto nitrocellulose filters (Hybond-C, GE Healthcare). The membranes were first probed with mouse anti-human NFATc1 monoclonal antibody (7A6, 1/500; BioLegend) and rabbit anti-human histone deacetylase (HDAC1) polyclonal antibody (H-51, 1/2000; Santa Cruz Biotechnology, Santa Cruz, CA).

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

Luciferase Assay—The generation of Jurkat cells carrying the firefly luciferase gene driven by the NFAT synthetic promoter is described elsewhere (9). Jurkat cells containing the above gene under the transcriptional control of the minimal CMV promoter and tandem repeats of the NF-κB response element were obtained by transduction with the Cignal Lenti Reporter system according to the manufacturer’s instructions (Qiagen). The stable Jurkat cell line expressing the constitutive luciferase was produced by transduction with an MFG vector that carries the luciferase gene driven by the Moloney murine leukemia virus long terminal repeat (18). Jurkat cells carrying the inducible or constitutive luciferase were stimulated with anti-CD3/CD28 antibody-coated beads with or without alcohol treatment as indicated in the text. Lysates for luciferase assays were prepared with the passive lysis buffer (E1941; Promega) and analyzed in a Lumat 9501 luminometer (Berthold, Nashua, NH). Relative luciferase units were calculated by normalization to total protein content measured by the Bradford assay (Bio-Rad).

Statistical Analysis—One-way analysis of variance followed by Dunnett’s multiple-comparison post-test was performed with GraphPad Prism (GraphPad Software, San Diego, CA) on data presented in Table 1 and Figs. 3 and 4. Student’s t test was...
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TABLE 1
Correlation between primary alcohol carbon-chain length and T lymphocyte IFN-γ release

| Concentration group | 1-OH | 2-OH | 3-OH | 4-OH | 5-OH | 6-OH | 7-OH | 8-OH | 9-OH | 10-OH | 11-OH |
|---------------------|------|------|------|------|------|------|------|------|------|-------|-------|
| 0                   | 101 ± 2 | 105 ± 7 | 98 ± 4 | 89 ± 2 | 100 ± 7 | 105 ± 1 | 101 ± 2 | 120 ± 8 | 100 ± 3 | 110 ± 4 | 108 ± 8 |
| I                   | 122 ± 8 | 111 ± 8 | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   |
| II                  | 134 ± 3 | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   |
| III                 | 159 ± 14 | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   |
| IV                  | 167 ± 20 | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   | NS   |

*p < 0.05; **p < 0.01 relative to group 0; *p < 0.05; **p < 0.01 relative to group 6; n = 3 for 1-OH, 2-OH, 8-OH, 10-OH, and 11-OH; n = 4 for 4-OH, 5-OH, and 9-OH; n = 6 for 3-OH, 6-OH, and 7-OH.

RESULTS

Primary Alcohols Modulate the Secretion of Interferon-γ by Primary Human Lymphocytes According to the Size of Their Aliphatic Chain—One major effector function of T cells triggered by the engagement of their antigen receptor is the synthesis of IFN-γ (17). In the present study, we have investigated whether exposure in vitro to a panel of primary aliphatic alcohols spanning the C₁–C₁₂ range would have any impact on the ability of primary human lymphocytes to produce IFN-γ once activated by the T cell receptor (TCR). Preliminary assays were performed to determine the optimal concentration window for analysis of each alcohol, which were then tested in three to six independent experiments with cells isolated from different donors. Table 1 illustrates the results obtained with five alcohol concentration points starting from no alcohol (group 0) to the highest concentration (group IV). The mean viability of cells exposed to the highest concentration of each alcohol was always >97% as indicated. Three distinct outcomes were observed. As anticipated, the release of IFN-γ was up-regulated by methanol exposure with about 70% increase measured in the culture supernatants by ELISA. When comparing the data points are shown in Table 1 and were compiled from independent experiments with samples from at least three different donors for each alcohol as detailed in the table footnote.

used for the analysis of the NF-κB luciferase assay. *p values < 0.05 were considered significant.

FIGURE 1. Individual profiles of the immunomodulatory activity of primary alcohols. Primary alcohols after IFN-γ production in peripheral blood T lymphocytes. Purified T cells were stimulated with anti-CD3/CD28 antibody-coated beads for 6 h in the presence of C₁–C₁₂ alcohols within the molar concentration windows indicated. The IFN-γ levels released in the supernatants were measured by ELISA and used to calculate the percentage alteration in effector function in relation to the positive control activated in absence of alcohols (100%). Resulting data indicating inhibition of IFN-γ release for C₁⁻C₁₂ or enhancement of IFN-γ production for C₁₃ were depicted in the form of curves. C₁₃ did not have any biological activity and is not shown. The corresponding data points are shown in Table 1 and were compiled from independent experiments with samples from at least three different donors for each alcohol as detailed in the table footnote.

measured by the loss of the righting reflex in tadpoles exhibits progressive intensity that parallels the increase in size of their carbon chains. This pattern, however, is abruptly interrupted at C₁₂ with substantial loss of activity at C₁₃ and the observation of a virtual disappearance of activity at C₁₄ and longer alcohols (14). In addition, the potency levels off at C₁₃–C₁₄ in a lipid-free in vitro model of anesthetic-protein interaction (16, 19). To better characterize the profile of immunomodulatory activity of the alcohol series and to verify how close it resembles the pattern of anesthetic potency, the concentration values associated with 50% of the biological effect of each alcohol were calculated from the corresponding curves in Fig. 1 and then plotted against the maximal achievable concentration in aqueous media in Fig. 2. The 50% effect was generally within each experimental curve; in the case of C₁₁, the last experimentally achievable data point corresponded to 30% inhibition, and higher concentrations were beyond its maximal solubility. C₁₁ was totally ineffective. In agreement with the data obtained in the studies on anesthetic potency, examination of the resulting
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The Homologous Series of Primary Alcohols Shares the Lack of Impact on Early Signaling Events Triggered by Activation of the T Cell Receptor—Our previous work has shown that methanol and isopropyl alcohol initiate their biological effect downstream of the cell membrane in lymphocytes (9, 11). It remained possible, however, that the longer n-alkanols would act on the cell membrane in a way reminiscent of the effect of ethanol on ion channels and neurotransmitter receptors (20, 21). In this scenario, longer n-alkanols could plausibly interact with the T cell receptor directly and blunt antigen-dependent signal transduction. We have tested this possibility by checking the phosphorylation status of the ζ-chain-associated protein kinase 70 (ZAP-70), a major player in early T cell signaling. Purified T cells were first activated for 3 min at 37 °C by anti-CD3/anti-CD28/anti-IgG antibodies; then, they were washed.

The Homologous Series of Primary alcohols Reduces the Activation and Nuclear Content of the Nuclear Factor of Activated T Cells in Primary Human Lymphocytes According to the Length of their Aliphatic Chains—We have shown previously that the secondary isopropyl alcohol mediates the attenuation of lymphocyte effector functions, including the capacity to secrete IFN-γ, by down-regulating the activation of NFATc1. As the experiments reported here revealed that primary alcohols with aliphatic chains spanning C2–C11 similarly reduce IFN-γ production in primary human lymphocytes, the underlying dysregulation of the activation of this transcription factor was a plausible assumption. We have set out to examine this possibility by measuring NFATc1 in nuclear lysates from lymphocytes that have been activated in the presence of each one of the various n-alkanols. Moreover, we have chosen to activate the T cells with a stimulus that bypasses the membrane to further corroborate the view that the studied alcohols work without relying on early TCR signaling. Indeed, the Western blot analysis in Fig. 3A shows that ionophore-activated lymphocytes display lower amounts of nuclear NFATc1 when exposed to n-alkanols. The concentrations used were those capable of inducing 75% of the biological effect as measured by IFN-γ secretion and ranged from millimolar amounts for the short chain molecules to micromolar levels for the longer moieties as described in the figure legend. NFATc1 levels in C6–C10-treated samples hovered around half the maximal content of activated T cells (56% for C7, 51% for C8, 54% for C9, 59% for C10). This inhibitory activity on NFATc1 activation was not significantly found in activated lymphocytes exposed to C11, which exhibited close to 75% of the maximal content of activated T cells.

Next, we have confirmed the above results by using a more sensitive luciferase assay with a stable T cell line carrying an NFAT-responsive promoter. This time we have activated the T cells through the antigen and co-stimulatory receptors with anti-CD3/CD28 antibody-coated beads. Fig. 3B shows a significant downward trend in promoter activity (analysis of variance p < 0.0001) that parallels the increase in size of the alcohol aliphatic chains. The percentage inhibition of NFAT activation measured in this assay was 34, 37, 48, 57, 53, and 57% for C6–C10, respectively. It is noteworthy that alkanols may directly affect the luciferase activity in certain experimental conditions. However, we have conducted the luciferase assays in absence of alkanols with lysates prepared from washed cells. Moreover, to further validate our results, we have tested the effect of each alcohol on a stable T cell line that carries an integrated cassette in which the luciferase gene is driven by a constitutive promoter. Fig. 3C shows that C1–C9 and C12 did not change the constitutive luciferase activity in our experimental conditions. Instead, we noticed that C10 and C11 change the luciferase activity, and C10 did so significantly. Thus, in the specific case of C10 and C11, the luciferase assay is not conclusive as to their impact on NFAT activation. However, the Western blot analysis in Fig. 3A complements these results by revealing that C10 does indeed reduce NFATc1 in the nucleus.

Similarly to the Western blot analysis, the alcohol concentrations used in the luciferase assays were progressively lower so that the longer the alcohol did not show the amount needed to produce the observed effect. Given the higher sensitivity of the luciferase assay, we have chosen the concentrations that are depicted in Fig. 2, which are required to produce 50% of the biological effect as measured by IFN-γ release in lymphocytes. Thus, whereas C6 reduced the NFAT-responsive promoter activity by one third, the dampening effect of C9 reached...
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A

(+) OH

NFATc1

LC

Densitometric units

(+) OH

Luciferase units/

% NFAT activation

B

C

Luciferase units/

% Positivity

FIGURE 3. Primary alcohols affect NFAT activation. A, measurement of NFATc1 in the nucleus was performed by Western blotting. Human peripheral blood T cells were stimulated with phorbol 12-myristate 13-acetate/ionomycin for 5 h in the presence or the absence of the relevant alcohol. The concentrations that produce 75% of the biological effect as measured by IFN-γ release were chosen for C2–C10 (C2, 66 mM; C3, 8.4 mM; C4, 3 mM; C5, 1.9 mM; C6, 2 mM; C7, 839 μM; C8, 314 μM; C9, 201 μM; C10, 114 μM). The highest concentration listed in the group IV of Table 1 was used for C11. The maximal water-soluble concentrations were used in the case of C11 (83 μM) and C12 (22 μM). Nuclear extracts were prepared from each sample and processed for SDS-PAGE; one representative blot of four is shown. Relative quantification in relation to histone deacetylase (HDAC1) is presented underneath as mean densitometric units ± S.E. (error bars); *, p < 0.05; **, p < 0.01 relative to the (+) control group, n = 4. Nonsignificant values, p > 0.05, are not labeled. The relative NFAT1 content in relation to the (+) control group is also presented as percentage values. The mean of the (+) control group, which represents 100%, and the 50% value are indicated by dashed lines. (-), unstimulated cells in the absence of alcohols; (+), TCR-stimulated cells in the absence of alcohols; D, TCR-stimulated cells in the absence of alcohols and in the presence of 0.2% dimethyl sulfoxide; LC, loading control (HDAC1); OH, alcohol. TCR-activated samples treated with alcohols are indicated by the number of carbons of the molecule used in the treatment (1–12). B, primary alcohols modulate the activation of a synthetic promoter containing NFAT binding sites. Jurkat-luciferase cells were stimulated with anti-CD3/CD28 antibody-coated beads for 5 h in the presence or the absence of the relevant alcohol. Gray columns show TCR-activated samples. Black columns represent unstimulated samples. The concentrations used in the experiments were those capable of inducing 50% of the biological effect as measured by IFN-γ release for C2–C10 or those representing the maximal water solubility for C11–C12 (Fig. 2). C1 was used at 94 mM. Ct indicates that the cells were TCR-activated (gray) or left unstimulated (black) in the absence of alcohols. Other symbols are as in A. Samples were lysed and assayed for luciferase activity. Results are presented as mean relative luciferase units/μg of protein ± S.E.; *, p < 0.05; **, p < 0.01 relative to the Ct control group, n = 6. Nonsignificant values, p > 0.05, are not labeled. The relative NFAT activation in relation to the Ct control group (gray) is also presented as percentage values. The mean of the Ct control group, which represents 100%, and the 50% value are indicated by dashed lines. C, impact of primary alcohols on the enzymatic activity of constitutively expressed luciferase. A stable Jurkat subclone carrying the luciferase gene driven by the Moloney LTR was stimulated by anti-CD3/CD28 antibody-coated beads for 5 h in the presence of the same alcohol concentrations used in B. Ct indicates that the cells were TCR-activated in the absence of alcohols. Other symbols are as above. Samples were lysed and assayed for luciferase activity. Results are presented as mean relative luciferase units/μg of protein ± S.E.; *, p < 0.05 relative to the Ct control group, n = 3. Nonsignificant values, p > 0.05, are not labeled. The relative luciferase activity is also presented as percentage values in relation to the Ct control group. The mean of the Ct control group, which represents 100%, is indicated by a dashed line.

approximately 60%. Nevertheless, 1.2 mM C6 and only 106 μM C9 were needed to produce this outcome, thereby stressing the higher potency of the long chain molecules.

Nuclear Translocation of the Nuclear Factor-κB Is Affected by n-Alkanols in Activated T Cells—Although the dysregulation of the NF-κB signaling cascade seems to play an important part in the immunological effects of ethanol, we have previously found that this pathway is not altered in the case of two other short chain alcohols, methanol and isopropyl alcohol. To dissect further the immunomodulation mechanism of the homologous series of primary alcohols, we have measured the nuclear translocation of p65 in TCR-stimulated purified human T cells exposed to the same alcohol concentrations previously used for Western blot analysis of NFAT. The p65 protein is the Rel-transactivating component of the major and most common NF-κB heterodimer.

Fig. 4 shows that lymphocyte activation by anti-CD3/CD28 antibodies led to a 1.6-fold increase in the amount of p65 in the nucleus. Conversely, the same stimulation in the presence of 839 μM C7 barely moved the p65 nuclear content, leading to a small increase that represented 18% of the maximal p65 nuclear content above the unstimulated cell baseline. The effect of the
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DISCUSSION

This article reports three novel findings: (i) The homologous series of n-alkanols displays a clear correlation between immunomodulatory capacity and hydrophobicity that is reminiscent of the well established link of the general anesthetic potency to the size of the aliphatic chain. (ii) The immunomodulatory targets of n-alkanols are located downstream of the cell membrane within the NFAT and NF-κB activation pathways. (iii) The pattern of immunomodulatory activity of the alcohol series suggests the existence of interaction pockets of defined dimensions in these targets.

Most of the research on the mechanism of action of alcohols and other general anesthetics had first been focused on the cell membrane and finds its roots in the independent contributions of Hans H. Meyer and Charles E. Overton more than a century ago (22, 23). Their seminal work established the basis for one of the most tested correlations in biomedicine, namely, that between anesthetic potency of a given compound and its oil:water partition coefficient. In face of the strong experimental evidence in support of this correlation, it was natural to assume that anesthetics (and by extension alcohols) would work under a unifying theory in which the dissolution of lipophilic molecules in the lipid bilayer could modify its physical properties and compromise indirectly the function of embedded proteins (24, 25). Nevertheless, several incongruities have cast doubts about the validity of the different flavors of the lipid theory to explain the mechanism of action of alcohols and anesthetics (26).

On the one hand, our results indicate that the Meyer–Overton correlation do apply to the alcohol immunomodulatory activity, but on the other hand, they do not lend support to the interpretation that membrane alterations are the major underlying process. First, there is a clear cutoff effect, and second, the inability of these molecules, including the longer more hydrophobic C9–C10 alcohols, to change ZAP-70 phosphorylation strongly suggests that they operate downstream of the cell membrane.

Previous studies reported putative alcohol binding sites in several proteins, and there are now crystallographic data for some of them, including ion channels, enzymes, and the odorant-binding protein LUSH (29–33). A feature that was consistently found in these structural analyses of the alcohol binding site was the identification of hydrogen bond acceptor site(s) and of a hydrophobic groove in close vicinity (1). Thus, it is plausible to interpret the Meyer-Overton correlation without resorting to lipid solubility if one applies their concept to the context of hydrophobic protein subdomains. In this scenario, alcohols or similar molecules would dislodge water from hydrophobic pockets and use hydrogen bonds and van der Waals forces to stabilize their binding with potential conformational and functional consequences (34). The hydrophobic nature of the molecules would still dictate the outcome of the interaction, and the volume constraints of the relevant cavities would account for the cutoff effect. It is noteworthy that about half of the molecular mass of the cell membrane is in fact protein (24). Nevertheless, at least in principle, all cellular compartments could provide suitable protein targets for alcohols.
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One of the first and arguably most convincing pieces of evidence that proteins may be the actual mechanistic targets of alcohol action is the demonstrated ability of the homologous series of primary alkanols to inhibit luciferase activity in vitro in absence of a lipid context (15, 16). These experiments revealed an almost linear correlation between luciferase inhibition and general anesthetic potency. Alcohols were shown to inhibit luciferase activity progressively better from C₁ to C₆ and then from C₆ to C₁₂; there was a clear cutoff effect at C₁₆ and also two identifiable activity plateaus between C₆ and C₁₀ and between C₁₂ and C₁₆, in which the binding affinity was nearly the same. These findings implied the existence of a hydrophobic binding pocket in the enzyme with sufficient volume to lodge two 1-hexanol molecules or a single 1-dodecanol molecule (19). Subsequent x-ray structural studies confirmed the existence of specific binding sites that coincide with the substrate binding sites (29). There is a striking similarity between the overall profile of the immunomodulatory activity of primary alcohols reported here and that associated with their ability to inhibit luciferase (16). In both situations, there was a first inflection in the response curve, which leveled off in the mid range at C₅–C₆ luciferase (16). In both cases, however, the second inflection led to the second inflection points for IFN-γ production and luciferase activity have been experimentally determined to occur, respectively. The analysis of the results reported here and the analogy to the luciferase inhibition data permit us to predict that the alcohol immunomodulatory effect is mediated by the alteration of one (and conceivably more than one) intracellular protein target via interaction to a hydrophobic pocket. This cavity is likely to be sufficiently big to accommodate two molecules of 1-pentanol, as suggested by the leveling off of IFN-γ release in the mid range of the curve depicted in Fig. 2, or a single molecule of 1-decanol. Additional methyl groups are likely to contribute to the binding energy by augmenting the van der Waals interactions up to C₅. From this point on, two molecules would be too big to fit simultaneously, and part of their aliphatic chains would remain exposed to the external polar environment. Efficient binding of single molecules would drive the increase in potency from C₇ to C₁₀, but steric constraints would resume at C₁₁. It is noteworthy that the amount of C₁₁ required to produce 30% inhibition of the IFN-γ production, which is within the soluble range, is comparable with the concentration of C₁₀ that induces 50% of the biological effect as can be inferred from Table 1 and Fig. 1. The complete loss of activity at higher concentrations, however, is not necessarily due to molecular size but to solubility. The immunomodulatory cutoff is lower than that observed for anesthetic potency or for luciferase inhibitory activity (14, 16), suggesting that the interaction occurs within a somewhat smaller cavity.

Given that early signaling (as measured by ZAP-70 and LAT phosphorylation) seems to be preserved in the presence of all alcohols tested here and that our previous work on short chain alcohols has shown their exquisite specificity in inducing the dysregulation of transcription factors that play a role in the activation of immunologically relevant genes (9, 11), we favor the hypothesis that the putative intracellular targets are the transcription factors themselves or molecules placed immediately upstream in their signaling cascades. The linear correlation between the size of the aliphatic chain and NFAT inhibition within the C₅–C₁₀ range and the loss of activity at C₁₁ are reminiscent of the findings obtained with IFN-γ release and support the previous assumption of the existence of a pocket sufficiently large to accommodate C₁₀. In the case of NF-κB, the results obtained in the p65 activation experiments are compatible with the existence of an additional alcohol binding site of smaller dimensions within a member of this activation pathway with optimal capacity to fit C₇ but still capable to accommodate C₉–C₁₀. The reduced NF-κB activation would synergize with NFAT inhibition, notably between C₆ and C₁₀. The combined end result of n-alkanol action on these two transcriptional pathways would be the progressive disablagement of immune cell function between C₇ and C₁₀.

The analysis of Fig. 1 shows a clear dichotomy between the pattern of response elicited by methanol and that triggered by all the other n-alcohols. Either methanol binds to the same pocket but produces a different conformational change or, perhaps, its smaller size and lower hydrophobicity allow binding to different molecular structures in the same or in a distinct set of targets. In any case, the crystal structure of bromobound form to luciferase demonstrates that a molecule not much bigger than methanol with a single carbon atom is capable of binding the same cavities that accommodate longer alcohols (29).

The biological effect of alcohols, encompassing the anesthetic and immunomodulatory properties, may result from interactions with many potential molecular targets that display suitable hydrophobic cavities. It is also likely to depend on cell type- and activation state-dependent protein expression profiles. Thus, in principle, our results should be interpreted within the context of the activation of human primary T cells through their antigen receptor. The data reported in this article have confirmed our original hypothesis and revealed that straight chain primary alcohols have an immunological impact that mirrors their anesthetic potency. This effect correlates well with hydrophobicity but the site of their action does not involve the membrane. The discrete alcohol modulation cutoff and the general profile of the IFN-γ release curve suggest the binding to a hydrophobic pocket in intracellular protein targets within the NFAT and NF-κB pathways. Altogether, our work contributes to a better understanding of the biological activity of the ubiquitous family of n-alkanols and provides additional insight into the mechanism that underlies their impact on the function of T lymphocytes in particular.

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REFERENCES
1. Désy, O., Carignan, D., and de Campos-Lima, P. O. (2012) Short-term immunological effects of non-ethanolic short-chain alcohols.

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1. Billi, A., and Matthys, P. (2009) Interferon-γ: a historical perspective. Cytokine Growth Factor Rev. 20, 97–113
2. Franks, N. P., and Lieb, W. R. (1982) Molecular mechanisms of general anaesthesia. Nature 288, 1142–1147
3. Overton, E. (1901) Studien über die Narkose, Zugleich ein Beitrag zur allgemeinen Pharmakologie. Gustav Fischer, Jena, Germany
4. Eckenhoff, R. G. (2001) Promiscuous ligands and attractive cavities: how do the inhaled anesthetics work? Mol. Immunol. 38, 257–268
5. Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. 24, 583–655
6. Franks, N. P., and Lieb, W. R. (1984) Do general anaesthetics act by competitive binding to specific receptors? Nature 310, 599–601
7. Franks, N. P., and Lieb, W. R. (1985) Mapping of general anaesthetic target sites provides a molecular basis for cutoff effects. Nature 316, 349–351
8. Veenstra, G., Webb, C., Sanderson, H., Belanger, S. E., Fisk, P., Nielsen, A., Kasai, Y., Willing, A., Dyer, S., Penney, D., Certa, H., Stanton, K., and Sedlak, R. (2009) Human health risk assessment of long chain alcohols. Ecotoxicol. Environ. Safety 72, 1016–1030
9. Pringle, M. J., Brown, K. B., and Miller, K. W. (1981) Can the lipid theories of anesthesia account for the cutoff in anesthetic potency in homologous series of alcohols? Mol. Pharmacol. 19, 49–55
10. Franks, N. P., and Lieb, W. R. (2004) Seeing the light: protein theories of general anaesthesia. Anesthesiology 101, 235–237
11. Jung, S., Akabas, M. H., and Harris, R. A. (2005) Functional and structural analysis of the GABAA receptor α1 subunit during channel gating and alcohol modulation. J. Biol. Chem. 280, 308–316
12. Dai, Q., Zhang, J., and Pruett, S. B. (2005) Ethanol alters cellular activation and lipid rafts: a new mechanism of ethanol action with implications for other receptor-mediated effects. J. Immunol. 176, 1243–1249
13. Taieb, J., Delarche, C., Ethuin, F., Selloum, S., Poynard, T., Guogerot-Pocidalo, M. A., and Chollet-Martin, S. (2002) Ethanol-induced inhibition of cytokine release and protein degranulation in human neutrophils. J. Leukoc. Biol. 72, 1142–1147
14. Zhao, X. J., Marrero, L., Song, K., Oliver, P., Chin, S. Y., Simon, H., Schurr, J. R., Zhang, Z., Thoppil, D., Lee, S., Nelson, S., and Kolls, J. K. (2003) Acute alcohol inhibits TNF-α processing in human monocytes by inhibiting TNF/TNF-α-converting enzyme interactions in the cell membrane. J. Immunol. 170, 2923–2931
15. Désy, O., Carignan, D., Caruso, M., and de Campos-Lima, P. O. (2008) Immunosuppressive effect of isopropanol: down-regulation of cytokine production results from the alteration of discrete transcriptional pathways in activated lymphocytes. J. Immunol. 181, 2348–2355
16. Carignan, D., Désy, O., and de Campos-Lima, P. O. (2012) 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. Toxicol. Sci. 125, 144–156
17. Désy, O., Carignan, D., Caruso, M., and de Campos-Lima, P. O. (2010) Methanol induces a discrete transcriptional dysregulation that leads to cytokine overproduction in activated lymphocytes. Toxicol. Sci. 117, 303–313
18. Daid, Q., Zhang, L., and Pruett, S. B. (2005) Ethanol alters cellular activation and CD14 partitioning in lipid rafts. Biochem. Biophys. Res. Commun. 332, 37–42
19. McCreery, M. J., and Hunt, W. A. (1978) Physico-chemical correlates of alcohol intoxication. Neuropharmacology 17, 451–461
20. Pringle, M. J., Brown, K. B., and Miller, K. W. (1981) Can the lipid theories of anesthesia account for the cutoff in anesthetic potency in homologous series of alcohols? Mol. Pharmacol. 19, 49–55
21. Aryal, P., Dvir, H., Choe, S., and Slesinger, P. A. (2009) A discrete alcohol pocket involved in GIRK channel activation. Nat. Neurosci. 12, 988–995
22. Meyer, H. H. (1899) Zur theorie der alkoholnarkose. Erste mittheilung. Welche eigenschaft der anästhetica bedingt ihre narkotische wirkung? Arch. Exp. Pathiol. Pharmacol. 42, 109–118
23. Overton, E. (1901) Studien über die Narkose, Zugleich ein Beitrag zur allgemeinen Pharmakologie, Gustav Fischer, Jena, Germany
24. Eckenhoff, R. G. (2001) Promiscuous ligands and attractive cavities: how do the inhaled anesthetics work? Mol. Immunol. 1, 258–268
25. Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. Pharmacol. Rev. 24, 583–655
26. Franks, N. P., and Lieb, W. R. (1982) Molecular mechanisms of general anaesthesia. Nature 300, 487–493
27. Dickinson, R., White, I., Lieb, W. R., Franks, N. P. (2000) Stereoselective loss of righting reflex in rats by isoflurane. Anesthesiology 93, 837–843
28. Koblin, D. D., Chortkoff, B. S., Laster, M. J., Eger, E. I., 2nd, Halsey, M. J., and Ionescu, P. (1994) Polyhalogenated and perfluorinated compounds that disobey the Meyer-Overton hypothesis. Anesth. Analg. 79, 1043–1048
29. Franks, N. P., Jenkins, A., Conti, E., Lieb, W. R., and Brick, P. (1998) Structural basis for the inhibition of firefly luciferase by a general anesthetic. Biophys. J. 75, 2205–2211
30. Kruse, S. W., Zhao, R., Smith, D. P., and Jones, D. N. (2003) Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from Drosophila melanogaster. Nat. Struct. Biol. 10, 694–700
31. Nury, H., Van Renterghem, C., Weng, Y., Tran, A., Baaden, M., Dufresne, V., Changeux, J. P., Sonner, J. M., Delarue, M., and Corringer, P. J. (2011) X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. Nature 469, 428–431
32. Pegan, S., Arrabitt, C., Slesinger, P. A., and Choe, S. (2006) Andersen’s syndrome mutation effects on the structure and assembly of the cytoplasmic domains of Kir2.1. Biochemistry 45, 8599–8606
33. Ramaswamy, S., Eklund, H., and Plapp, B. V. (1994) Structures of horse liver alcohol dehydrogenase complexed with NAD⁺ and substituted benzyl alcohols. Biochemistry 33, 5230–5237
34. Klemm, W. R. (1998) Biological water and its role in the effects of alcohol. Alcohol 15, 249–267
35. Bell, G. H. (1973) Solubilities of normal aliphatic acids, alcohols and alkanes in water. Chem. Phys. Lipids 10, 1–10