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Effect of sesquiterpene lactones on the expression of the activation marker CD69 and of IL-2 in T-lymphocytes in whole blood

Matjaz Humar[a,1], Alfonso J. García-Piñeres[b,1], Víctor Castro[c], Irmgard Merfort[b,*]

[a]*Department of Anesthesiology, University Hospital, Breisacherstr. 66, 79106 Freiburg, Germany*
[b]*Institute of Pharmaceutical Biology, Albert-Ludwigs-Universität Freiburg, Stefan-Meier-Str. 19, 79104 Freiburg, Germany*
[c]*Universidad de Costa Rica, Escuela de Química, San José, Costa Rica*

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Abstract

We used flow cytometry to investigate the inhibitory effect of sesquiterpene lactones (SLs) on T-cell activation measured by the expression of its early marker CD69, and on interleukin (IL)-2, a mediator of activation, in whole blood. SLs are biologically active compounds found especially in plants from the Asteraceae family. Overnight treatment of blood with these substances led to the inhibition of CD69 and IL-2 expression. Interestingly, bifunctional SLs showed a weaker activity than monofunctional substances, which is in contradiction with the data obtained so far, using other biological test systems. Additionally, SLs did not completely inhibit CD69 or IL-2 expression. We also determined their toxicity and observed only a low effect. Up to now, studies on cytotoxicity have only been performed using cultured cell lines. From these results it may be supposed that these natural compounds preferentially show toxic effects towards transformed cell lines. Altogether, the results demonstrated that SLs effectively inhibit the activation of the T-lymphocyte response in whole blood and proved the utility of a whole blood system in studying their biological effects.

Keywords: CD69; IL-2; NF-κB; NFAT; Sesquiterpene lactones; Flow cytometry

1. Introduction

SLs are the active components of some species from the Asteraceae family. Preparations of these plants are externally used in traditional medicine for the treatment of various inflammatory disorders. A prominent example is the European *Arnica montana*. Its flowerheads are used for the preparation of alcoholic tinctures. The secondary metabolites that are mainly responsible for its anti-inflammatory effects are SLs of the 10α-methylpseudoguaianolide-like helenalin, 11α,13-dihydrohelenalin, and their ester derivatives [1]. A further well-known medicinal plant is *Tanacetum parthenium* with the germacranolide parthenolide as its major secondary metabolite [2].

It has been shown in various assays that these plant extracts, as well as the purified SLs, possess anti-inflammatory properties [1]. Most importantly, we and others have recently demonstrated that they inhibit the central transcription factor, nuclear factor-κB (NF-κB) [3–6]. Additionally, we could show that also DNA binding of the transcription factors, nuclear factor of activated T-cells (NFAT) and activator protein 1 (AP-1), was prevented [7]. We proved that NF-κB inhibition is mainly due to a covalent reaction of SLs with cysteine 38 in the DNA binding domain of the p65 subunit, and that cysteine residues are also probably targeted in NFAT and AP-1 DNA binding domains [7,8]. These transcription factors play a pivotal role in controlling the expression of multiple inflammatory and immune genes involved in toxic shock, asthma, rheumatoid arthritis, or cancer [9,10]. Among the upregulated mediators, cytokines deserve special attention. They control the proliferation, differentiation, and activation of cells of the hematopoietic

* Corresponding author. Tel.: +49-761-203-8373; fax: +49-761-203-8383.
E-mail address: irmgard.merfort@pharmazie.uni-freiburg.de (I. Merfort).
[1] These authors contributed equally to the results of this work.
Abbreviations: RA, rheumatoid arthritis; PMA, phorbol 12-myristate-13-acetate; BFA, brefeldin A; IL, interleukin; CD, cluster of differentiation; TCR, T-cell receptor; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T-cells; AP-1, activator protein 1; SLs, sesquiterpene lactones; EMSA, Electrophoretic Mobility Shift Assay; PBMC, peripheral blood mononuclear cell; 7AAD, 7-amino actinomycin D; A5, Annexin V; TNF, tumor necrosis factor; PE, phycoerythrin; FITC, fluorescein isothiocanyte; PerCP, peridinin chlorophyll protein.

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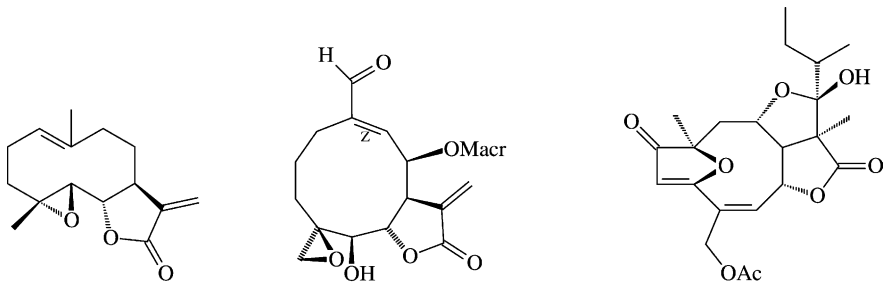
lineage, such as phagocytic granulocytes, lymphocytes, and macrophages [11,12]. As a consequence of NF-κB and NFAT/AP-1 inhibition, downstream effects, such as the release of the cytokines IL-1β, TNF-α, IL-6, and IL-8 are inhibited by SLs [13–16].

IL-2 is a further cytokine whose expression is dependent on the transcription factors, AP-1, NF-κB, NFAT, and Oct-1 [17,18], and is known to induce lymphocyte proliferation among other effects [19]. It has originally been described as an autocrine growth factor for activated T-lymphocytes [19], but it additionally enhances growth and differentiation of other immunocompetent cells, such as B-lymphocytes, monocytes, macrophages, and natural killer cells. Moreover, it stimulates the synthesis of IL-1β. IL-2 is involved in the Th1 cytokine pathway of the immune response which is reported to be involved in inflammation and organ-specific autoimmunity [20].

Abnormal cytokine expression has been reported for diseases, such as rheumatoid arthritis and osteoarthritis, in which high infiltration levels of T-cells to the synovial membrane were found. This was associated to a high expression of antigens of T-cell activation (CD69, CD25, CD38, CD43, CD45RO, and class II HLA) and high Th1 cytokine levels (IL-2 and IFN-γ) [21].

One of the earliest markers of activated $CD4^+$ and $CD8^+$ peripheral T-cells is the antigen CD69. This transmembrane protein is also designated as the activation inducer molecule (AIM), early activation antigen (EA-1), or Leu-23. CD69 is also expressed on activated B and natural killer cells following stimulation by a variety of mitogenic agents, such as IL-2, phorbol esters, phytohemaglutinin, or TCR engagement [22]. CD69 is discussed to be involved in the pathogenesis of diseases, such as rheumatoid arthritis, chronic inflammatory liver disease, asthma, and acquired immunodeficiency syndrome [22]. Besides its properties to mediate cellular activation, CD69 may also be involved in mediating the induction of apoptosis. The transcription of CD69 has been shown to be controlled by NF-κB, Egr-1, and AP-1 [23].

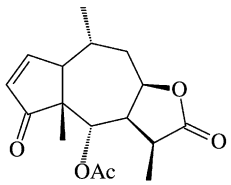
In continuation of our previous investigations on the anti-inflammatory activity of SLs, we have studied whether these substances inhibit the expression of IL-2, and further



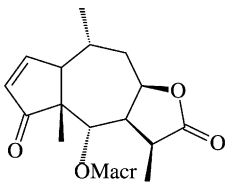
1
Parthenolide

2
4β, 15-epoxy-miller-9*Z*-enolide

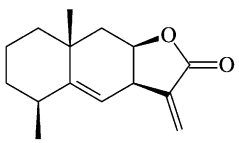
3
15-Acetoxy-eremantolide B



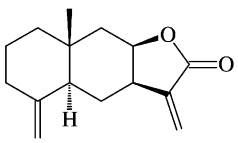
4
11α,13-Dihydrohelenalin acetate



5
11α,13-Dihydrohelenalin methacrylate



6a
Isoalantolactone



6b
Alantolactone

6 Isoalantolactone:Alantolactone (3:1)

Fig. 1. Structures of the investigated sesquiterpene lactones.

events induced by this cytokine, such as peripheral lymphocyte activation, indicated by the appearance of the CD69 ligand on the cell surface. To gain more detailed information about specific lymphocyte subsets responding to particular stimuli and SL treatment, we used a flow cytometric procedure. All studies were carried out with whole blood and not with isolated immune cells in order to use conditions closer to those found *in vivo*. To the best of our knowledge, such studies using whole blood have not been carried out with SLs up to now. Moreover, we investigated the toxic properties of SLs. Altogether, six structurally different SLs were included in theses studies (see Fig. 1). The germacranolide **2** possesses two α,β-unsaturated carbonyl groups which can react following a Michael-type reaction with thiol groups of protein targets [24]. SL **2** can be regarded as a prodrug. It was shown that in acidic medium the epoxide structure of this germacranolide can form a cationic intermediate which easily reacts with nucleophiles [25]. Therefore, both SLs can be considered as bifunctional. The remaining SLs, namely the germacranolide **3**, the pseudoguaianolides **4** and **5**, as well as the mixture of the eudesmanolides (**6**, ratio 3:1), contain only one α,β-unsaturated carbonyl group and are considered monofunctional. These eudesmanolides differ in the position of the double bond which does not influence their reactivity and are therefore regarded as one substance (**6**).

2. Materials and methods

2.1. Test Compounds

Parthenolide and isohelenin were purchased from Sigma. 4β,15-Epoxy-miller-9Z-enolide was isolated from the leaves of *Milleria quinqueflora* [26]. 15-Acetoxy-eremantolide B was isolated from the aerial parts of *Vanillomopsis arborea* [27]. 11α,13-Dihydrohelenalin acetate and 11α,13-dihydrohelenalin methacrylate were isolated from flowerheads of *A. montana*, Spanish chemotype, as previously described [28]. Stock solutions of the SLs were prepared in DMSO. In order to use the same amount of DMSO, a stock solution was prepared for each concentration, so that 1 μL solution was added to each sample.

2.2. Materials

Both the FastImmune assay system for CD69 expression (340365) and the FastImmune cytokine system (340450) for intracellular IL-2 cytokine staining were purchased from BD Biosciences. Additionally, required reagents included the PharM Lyse™ lysing reagent (555899), the FACS™ lysing solution (349202), CellFIX™ (340181), 7AAD (559925), CD2/CD2R (340366), CD3-PerCP (347344), CD3-PE (555340), CD8-FITC (345772), and the A5-FITC apoptosis detection kit I (556547) were also from BD Biosciences. Phorbol 12-myristate-13-acetate (PMA; P1585), ionomycin (I-0634), and BFA (B-7651) were from Sigma-Aldrich. The IntraPrep permeabilization and fixing reagent (IM2388) was obtained from Coulter Immunotech. All reagents for cell culture were ordered from Gibco-BRL.

2.3. Cell culture and stimulation

For whole blood assays heparinized blood was collected from one healthy volunteer in written consent and distributed to round bottomed 96-well plates. One hundred microliters of aliquots of heparinized blood was incubated with or without SLs at the indicated concentrations for 15 hr in a humidified incubator at 37°, 5% CO_2. In all cases, stock solutions were prepared in DMSO in order to add a maximal volume of 1 μL to the samples, and to obtain the desired final concentration. All controls contain 1 μL of the DMSO. Addition of solvent to blood samples did not alter the expression of CD69 or IL-2, nor showed an effect on cell viability.

CD69 expression was stimulated by 70 μL/mL CD2/CD2R whereas cytokine production was induced by addition of 15 ng/mL PMA and 1 μg/mL ionomycin for the last 12 hr. IL-2 secretion was inhibited by addition of 10 μg/mL BFA.

2.4. Measurement of CD69 expression on the surface of $CD4^+$ and $CD4^-$ T-lymphocytes in whole blood by multiparameter flow cytometry

For measurement of T-cell activation CD69 cell surface marker expression was analyzed in whole blood with the FastImmune assay system. Briefly, pretreated blood samples were stained with 20 μL of a three-color direct immunofluorescence antibody cocktail containing CD4-FITC/CD69-PE/CD3-PerCP for 45 min. Red blood cells were lysed by addition of 900 μL 1× FACS™ lysing solution to each reaction. Remaining peripheral blood mononuclear cells (PBMCs) were suspended in 500 μL 1× CellFIX™ fixing solution and prepared for flow cytometry analysis. A gate was set to collect $CD3^+$ cells, and 7000 events were collected for each sample.

2.5. Measurement of IL-2 expression in $CD8^+$ and $CD8^-$ T-lymphocytes in whole blood by multiparameter flow cytometry

For determination of T-cell function, intracellular IL-2 synthesis of activated T-lymphocytes was measured in whole blood by the FastImmune cytokine system according to the manufacturer's description, with minor modifications. After cell surface staining of activated whole blood for CD3 (2.5 μg/mL, clone SK7-PerCP) and CD8 (2.5 μg/mL clone SK1-FITC), red blood cells were lysed by addition of 900 μL 1× FACS™ lysing solution to each reaction. Remaining PBMCs were suspended in 100 μL IntraPrep fixation reagent for 15 min, then in 100 μL IntraPrep

permeabilization reagent with 0.3 μg/mL FastImmune Anti-Hu-IL2PE (clone 5344.11) antibody for 20 min. Stained PBMCs were washed with PBS and prepared in 500 μL 1× CellFIX™ fixing solution for flow cytometry analysis. A gate was set to collect $CD3^+$ cells, and 7000 events were collected for each sample.

2.6. Determination of apoptosis and necrosis by flow cytometry

Early stages of apoptosis and necrotic cells were determined in whole blood by the A5-FITC apoptosis detection kit I. For lymphocyte gating, blood samples were stained for CD3 with 6 ng/mL anti-CD3-PE antibody (clone SK7) for 20 min. Erythrocytes were lysed by addition of PharM Lyse™ ammonium chloride lysis solution for 5 min, as regular lysing reagents contain formaldehyde, which damages the cell membrane. Apoptosis and necrosis were recognized by A5-FITC and 7AAD staining as described elsewhere [29,30]. Briefly, PBMCs were stained with 5 μL A5 per test for 15 min in 100 μL binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$). Cells were then washed in 1 mL binding buffer. After washing, the pellets were resuspended in 400 μL binding buffer and prepared for FACS analysis. For detection of necrotic or late apoptotic cells, 7AAD was added shortly before analysis. A gate was set to collect $CD3^+$ cells, and 10,000 events were collected for each sample.

2.7. Statistical analysis

All values were obtained from measurements performed from three different samples taken on different days. All data are presented as mean ± SD, and analyzed using an independent *t*-test (two groups). A P value <0.05 is considered statistically significant, and is indicated with an asterisk (∗), a P value <0.005 with a double asterisk (∗∗).

3. Results

3.1. Effect of SLs on T-cell activation measured by the expression of CD69

To study the effect of the SLs **1**–**6** on T-cell activation, we measured the CD69 expression (for structures see Fig. 1). In average, after stimulation 30% of the total lymphocyte population expressed CD69 on their surface. This result is in agreement with previous reports, in which the intracytoplasmic expression of CD69 in PBMCs was measured 18 hr after stimulation with CD2 plus CD28 ligands [31]. Addition of SLs to the sample resulted in a dose-dependent diminishment of CD69 detected on the cell surface. Fig. 2 shows the results obtained for samples treated with isohelenin. No significant difference was observed for the inhibition in $CD4^+$ and $CD4^-$ cells (compare Fig. 3A and B). Therefore, these results are discussed together.

Surprisingly, the bifunctional SLs **1** and **2** showed only a weak inhibition of CD69 expression. A slight, but not significant increase of CD69 was observed at low concentrations (10 μM). The inhibition becomes significant only at much higher concentrations (250 μM). At 500 μM the inhibition was lower than 50%. The monofunctional SLs **3**–**6** inhibit CD69 expression at much lower concentrations, being significant at the lowest concentration tested. In no case a complete inhibition was observed, and the maximal inhibition obtained was 80%. The strongest effect was found for SL **3**, which reaches its half-maximal activity at a lower concentration than 50 μM. The dihydrohelenalin derivatives **4** and **5** showed nearly identical inhibition potentials, leading to a 50% reduction in concentrations between 50 and 100 μM. Isohelenin (**6**) exhibited a weaker inhibitory activity. Here, concentration of about 150 μM was needed for a 50% inhibition.

Activation with monoclonal antibodies against the CD2/CD2R epitopes does not target the entire lymphocyte population, but only those that have this receptor on its surface. In order to assess if the results are applicable to the entire lymphocyte population, the experiments were reproduced for the SLs, 11α,13-dihydrohelenalin acetate and isohelenin, and stimulated with PMA/ionomycin, which represents a general stimulus. The obtained results were in agreement with those for cells stimulated through the IL-2 receptor (data not shown).

3.2. Effect of SLs on the IL-2 expression in $CD8^+$ and $CD8^-$ T-lymphocytes

To investigate whether the SLs **1**–**6** inhibit the intracellular expression of IL-2 in activated $CD8^+$ and $CD8^-$ T-lymphocytes, the production of IL-2 was analyzed using flow cytometry. The amount of IL-2 expressing lymphocytes was observed to be dependent on the amount of PMA/ionomycin added. In average, 26% of the total lymphocyte population expressed IL-2 after the addition of 15 ng/mL PMA and 1 μg/mL ionomycin. This result is also in conformity with reported data [31]. However, the amount of $CD8^-$ cells expressing IL-2 was significantly higher than that of $CD8^+$ cells (18% vs. 8%, respectively, $P = 0.008$; $N = 5$).

Treatment of samples with SL solutions resulted in an inhibition of IL-2 expression. Again, no difference was observed for the inhibition among $CD8^-$ or $CD8^+$ cells, nor a complete inhibition was detected. Fig. 4 shows the results obtained for samples treated with parthenolide and Fig. 5 the inhibition curves of the tested substances.

In all cases inhibition took place at lower concentrations than those needed for CD69 inhibition. Monofunctional SLs, except for **6**, possessed a slightly higher inhibitory activity than those regarded as bifunctional, especially at lower concentrations, but this effect was

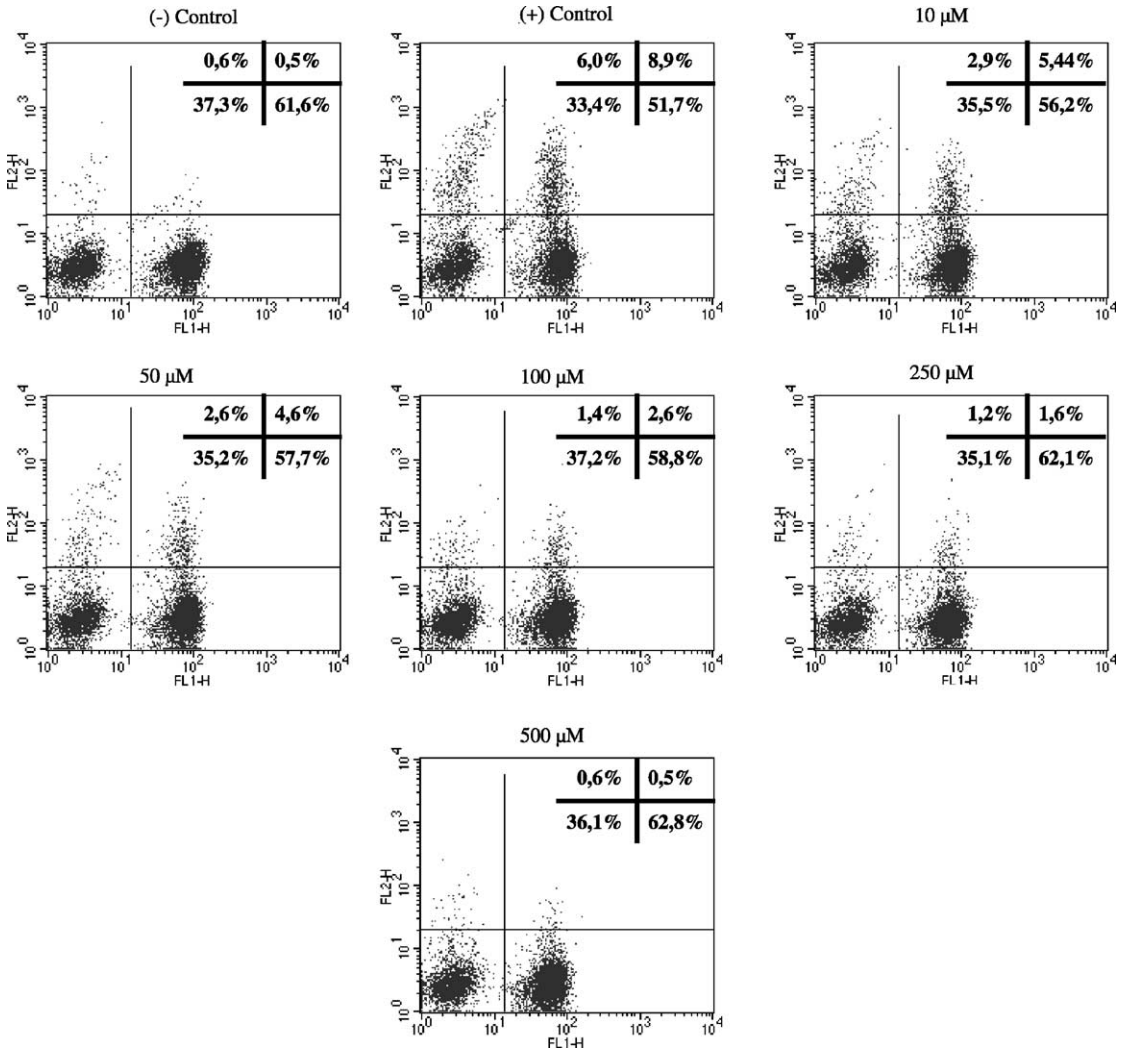


Fig. 2. Inhibition of CD69 expression by the sesquiterpene lactone isohelenin. Whole blood samples were incubated with various concentrations of isohelenin (SL **6**) for 3 hr before stimulating with an antibody against the IL-2 receptor, and further incubated for 12 hr at 37°. The samples were then stained with a three-color direct immunofluorescence antibody cocktail containing CD4-FITC/CD69-PE/CD3-PerCP, and analyzed using flow cytometry. One representative result of three independent measurements is shown for every concentration tested. FL-1H corresponds to the CD4-FITC fluorescence, and FL-2H to the CD69-PE fluorescence.

not so pronounced as with CD69. The germacranolide **1** exhibited the weakest inhibitory activity. A 50 µM concentration only resulted in an IL-2 inhibition of about 20%, in contrast to SLs **3**–**5**. Here, a 70–80% inhibition of IL-2 expression was observed at the lowest concentration tested. Fifty micromoles of the eudesmanolide **6** led nearly to a 50% reduction.

3.3. SLs only show low toxic effects on blood cells at the concentrations used

In order to determine if toxic effects are observed upon incubation of cells with SLs, we measured the amount of apoptotic and necrotic cells after SL treatment using flow cytometry. One of the events involved in apoptotic cell death is the loss of the cell membrane symmetry. In healthy cells, phosphatidylserine (PS) is not detected on the outer cell membrane, due to the activity of membrane remodeling enzymes. These enzymes are inactivated as a consequence of apoptotic processes, and therefore PS can be detected on the outer cell membrane [32]. PS quantification is performed using A5, which binds to PS in the presence of calcium. The viability of cells is indicated by the integrity of their cell membranes, and therefore their ability to exclude dyes, such as 7AAD, which bind to the DNA and in such state are able to fluoresce [30].

Two concentrations (50 or 250 and 500 µM) were tested for every compound, including the highest concentration of 500 µM. The results obtained for 11α,13-dihydrohelenalin acetate are shown in Fig. 6. All results are summarized in Table 1. In general, the SLs had a low or no effect on both necrosis and apoptosis in T-lymphocytes at the highest concentration tested. SLs **1**, **2**, and **6** showed a very weak effect at 500 µM concentration, whereas 13.8 and 20.4% of the cells showed apoptosis after treatment with 500 µM of the dihydrohelenalin derivatives **4** and **5**, respectively, and

29.1% with the SL **3**. The latter SL has to be regarded separately, because it showed the strongest effect, and was already cytotoxic at 250 μM concentration. In summary, it can be concluded that the concentrations of SLs **1** and **2**, as well as **4**–**6**, used in these experiments have low cytotoxic effects.

4. Discussion

SLs are the active secondary metabolites of many medicinal plants of the Asteraceae family, which are used in traditional medicine. They have been shown to influence cellular processes involved in initiation and progression of

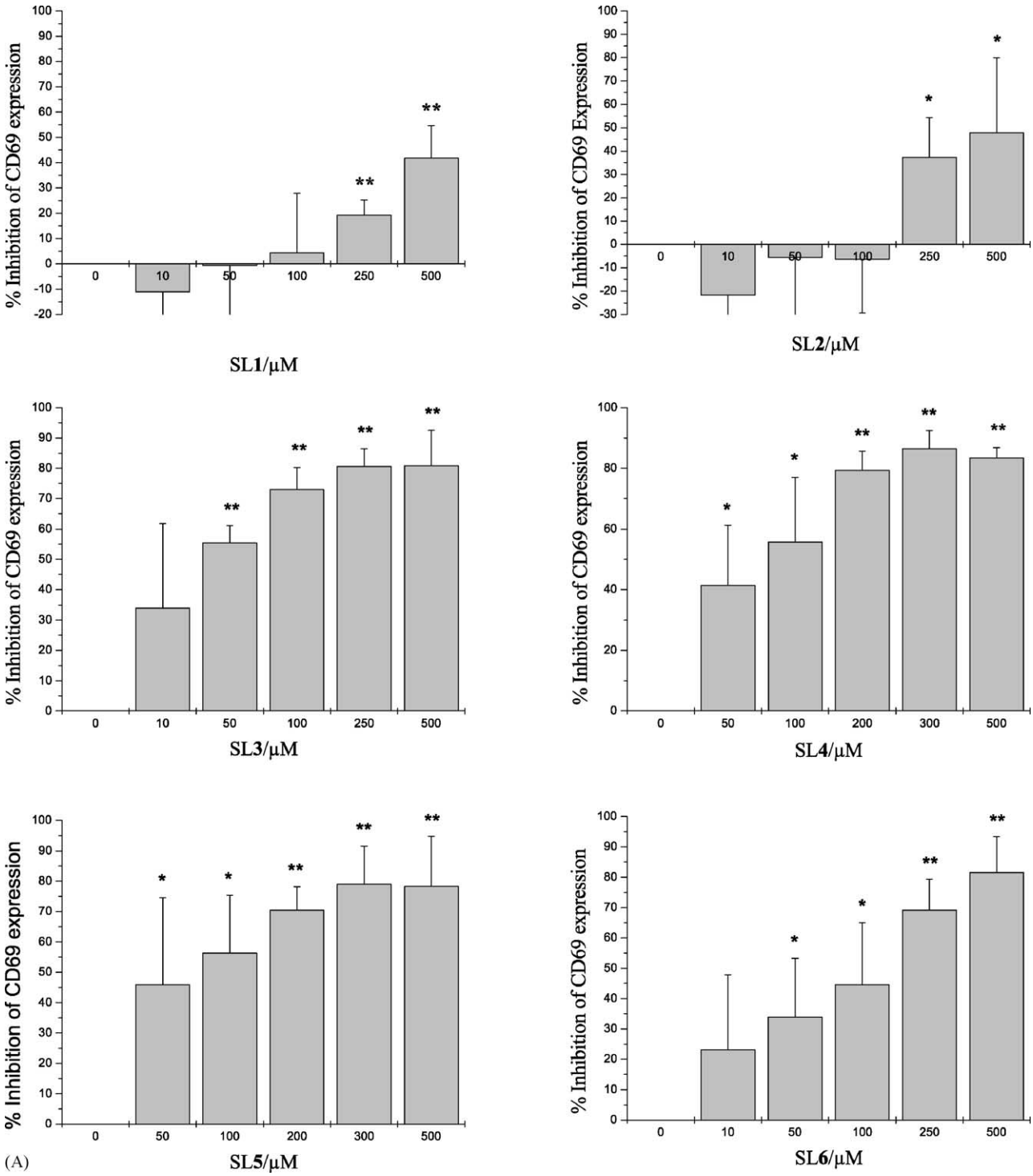


Fig. 3. The effect of sesquiterpene lactones on the expression of CD69. Samples were treated with different concentrations of the SLs **1**–**6** as indicated in Fig. 2. The inhibition of CD69 expression determined using flow cytometry is shown. (A) $CD4^+$ cells. (B) $CD4^-$ cells. Data are expressed as the percent inhibition related to the positive control. The mean ± SD from three separate experiments is presented. A P value <0.05 is considered statistically significant, and is indicated with an asterisk (∗) and a P value <0.005 with a double asterisk (∗∗).

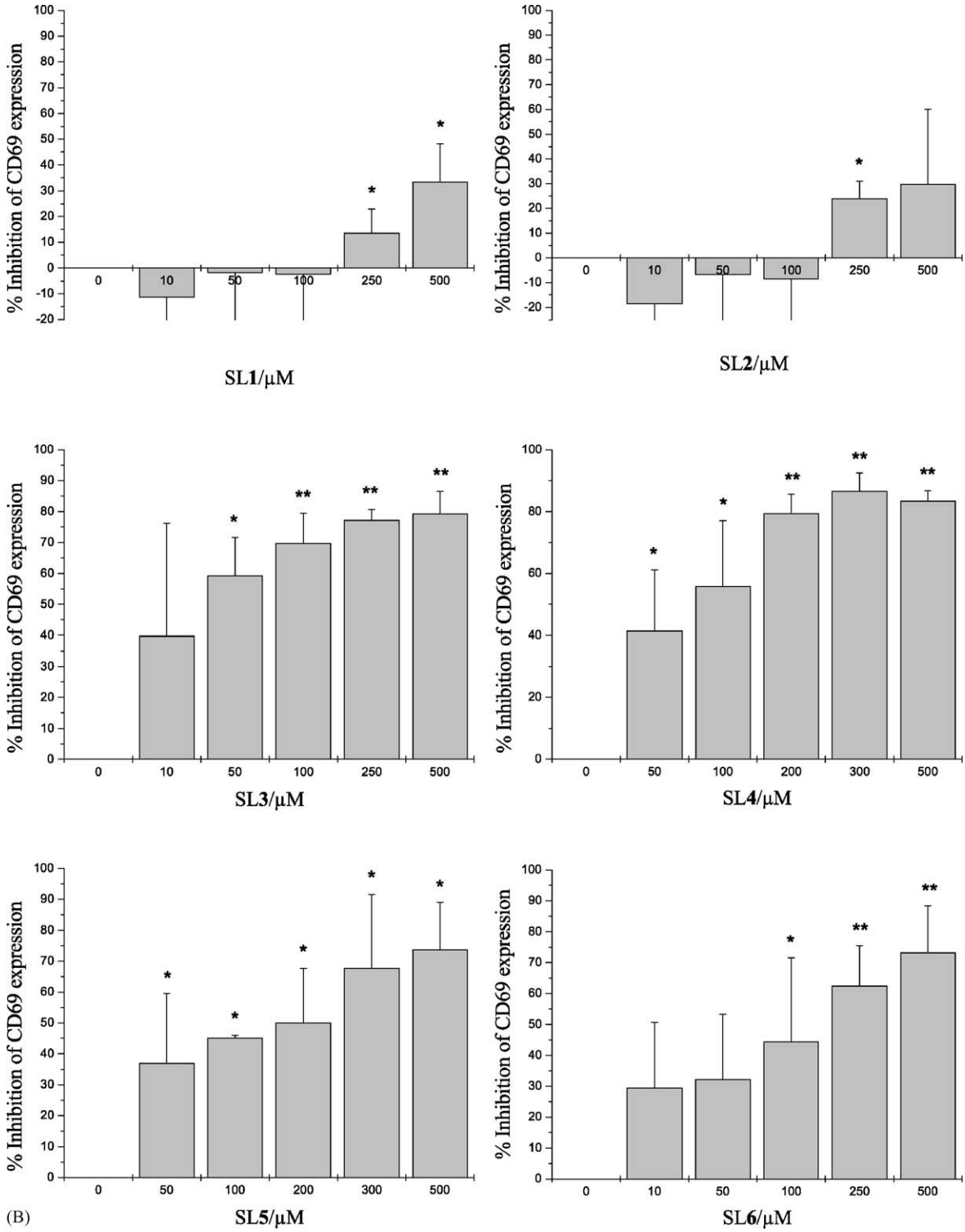


Fig. 3. (*Continued*).

inflammation, such as oxidative phosphorylation, platelet aggregation, histamine and serotonin release, and neutrophil chemotaxis [1].

In previous studies, we have shown that SLs selectively inhibit DNA binding of transcription factors that control the evolution of inflammation, namely NF-κB, NFAT, and AP-1 [4,5,7,8,26,33,34]. We have demonstrated that NF-κB inactivation is mainly due to alkylation of cysteine 38 in the DNA binding domain of its p65 subunit, thus preventing the interaction of the transcription factor with the DNA. This interference occurs because SLs possess α,β-unsaturated structure motifs that react following a Michael addition with thiol groups. In this study, we analyze the effect of mono- and bifunctional SLs on the T-cell

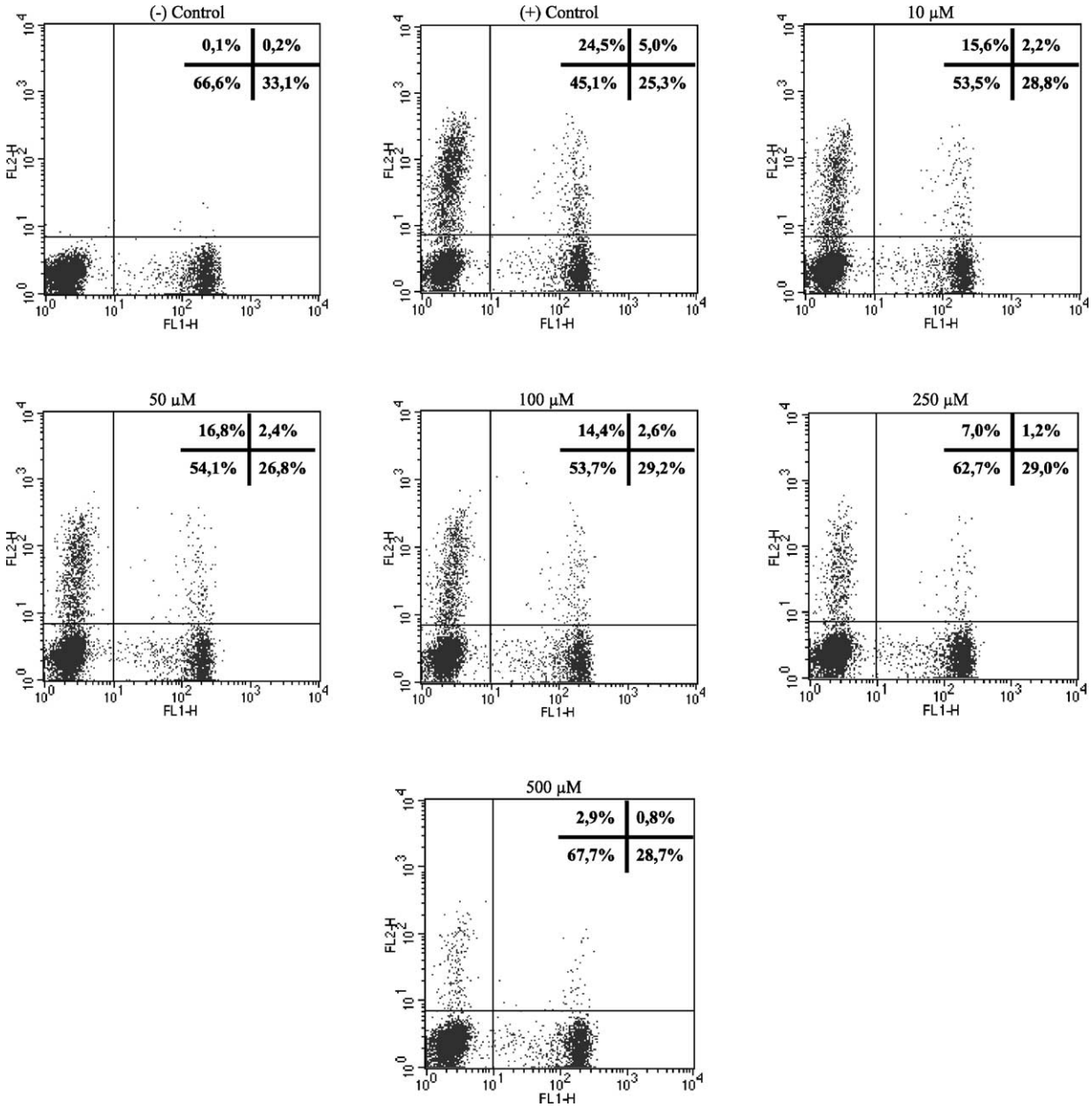


Fig. 4. Inhibition of IL-2 expression by the sesquiterpene lactone parthenolide. Whole blood samples were incubated with various concentrations of parthenolide (SL **1**) for 3 hr before stimulating with PMA/ionomycin, and further incubated for 12 hr at 37°. The samples were then stained with CD3-PerCP, CD8-FITC, and IL-2-PE antibodies, and analyzed using flow cytometry. One representative result of three independent measurements is shown for every concentration tested. FL-1H corresponds to the CD8-FITC fluorescence, and FL-2H to the IL-2-PE fluorescence.

activation, indicated by the expression of CD69, an early lymphocyte activation marker, and IL-2.

All the studied SLs inhibited the expression of CD69 on the lymphocyte surface in a dose-dependent manner. Interestingly, the activity of the analyzed compounds differed from the inhibition data previously obtained for NF-κB and NFAT with EMSA (see Table 2). Using this method, we have established that bifunctional SLs inhibit the activity of NF-κB at a concentration at least 10 times lower than monofunctional [5]. According to these results, this pattern was also observed for the inhibition of the transcription factor NFAT. However, the monofunctional dihydrohelenalin acetate (SL **4**) required a lower concentration to inhibit NFAT than for NF-κB.

In a recent study with peripheral T-lymphocytes, the SL parthenolide inhibited IL-2, IL-4, and IFN-γ by blocking NF-κB-DNA binding, and consequently mRNA synthesis [35]. Based on this evidence, it can be assumed that the inhibition of CD69 expression is related to the inhibitory effect of SLs on NF-κB and NFAT [17,18,22]. However, it cannot be excluded that the observed differences are because SLs inhibit CD69 and IL-2 expression in whole blood by affecting a further target.

Monofunctional SLs inhibited CD69 expression to a higher extent than bifunctional ones. The higher reactivity of bifunctional SLs compared to monofunctional could possibly explain this observation. It has been already shown that SLs are able to react with numerous protein targets (reviewed in [36]). Thus, in a complex sample as whole blood, SLs might bind to proteins in the sample matrix, therefore, not being available to react in the same

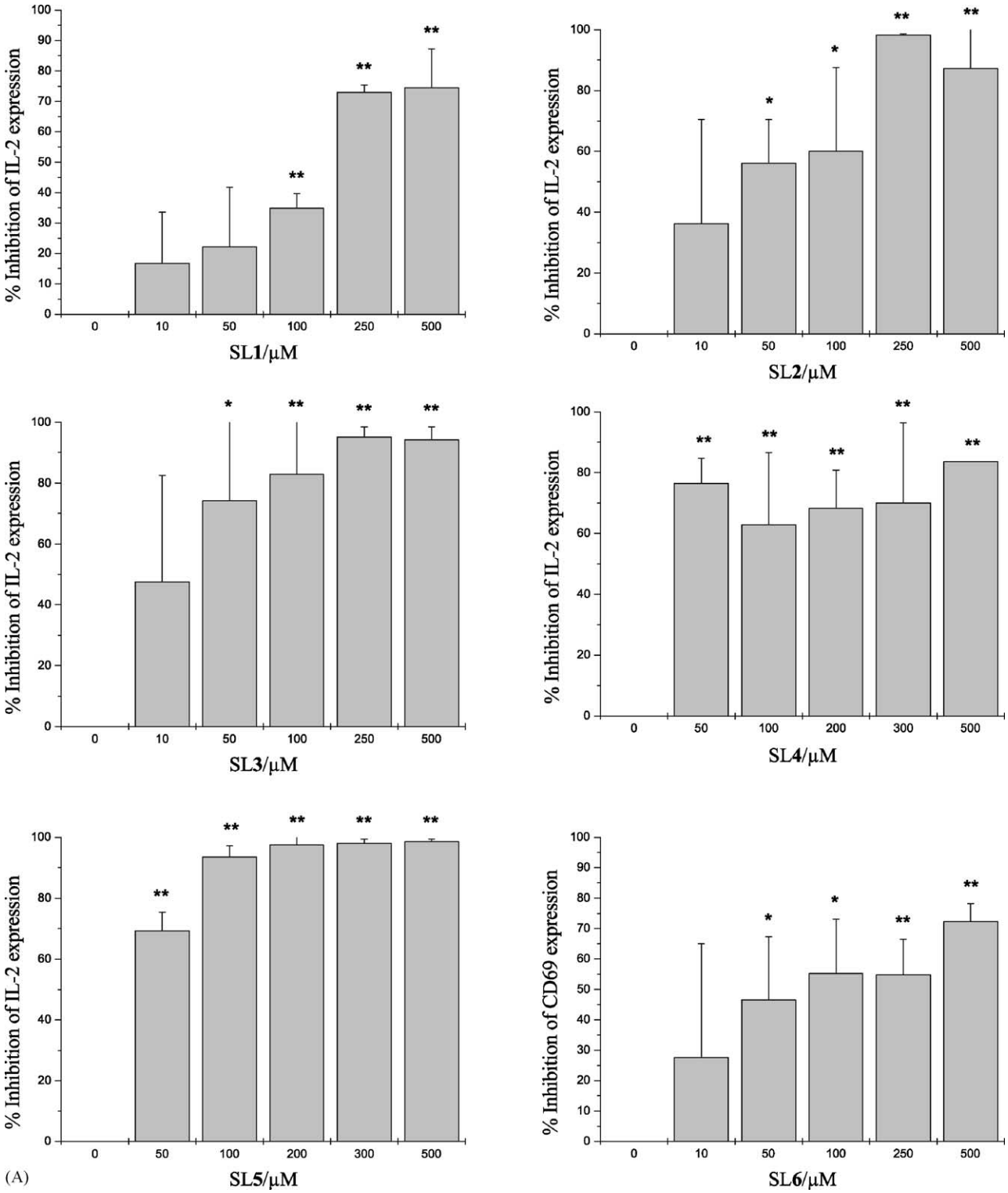


Fig. 5. The effect of sesquiterpene lactones on the expression of IL-2. Samples were treated with different concentrations of the SLs **1–6** as indicated in Fig. 4. The inhibition of IL-2 expression determined using flow cytometry is shown. (A) $CD8^-$ cells. (B) $CD8^+$ cells. Data are expressed as of the percent inhibition related to the positive control. The mean ± SD from three separate experiments is presented. A P value <0.05 is considered statistically significant, and is indicated with an asterisk (∗), a P value <0.005 with a double asterisk (∗∗).

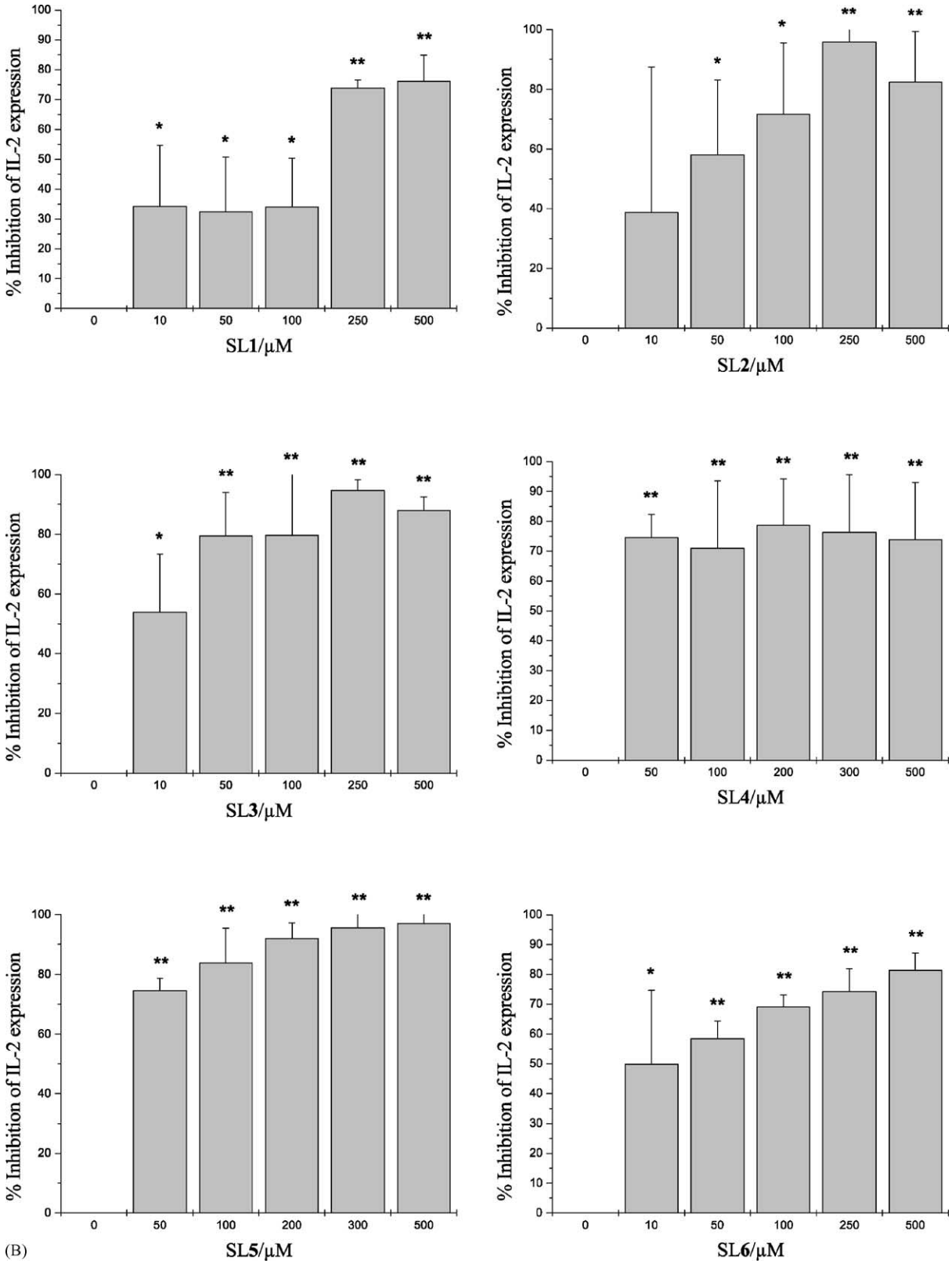


Fig. 5. (*Continued*).

proportion with the desired target. A less reactive SL would not be "trapped" by matrix proteins, and would only react with targets for which it has the highest affinity. Preliminary results indicate that the bifunctional SL parthenolide (**1**) reacts almost entirely with blood serum protein, whereas isohelenin (**6**) can still be detected after incubation with whole blood (data not shown). More detailed studies that confirm this observation are being currently carried out.

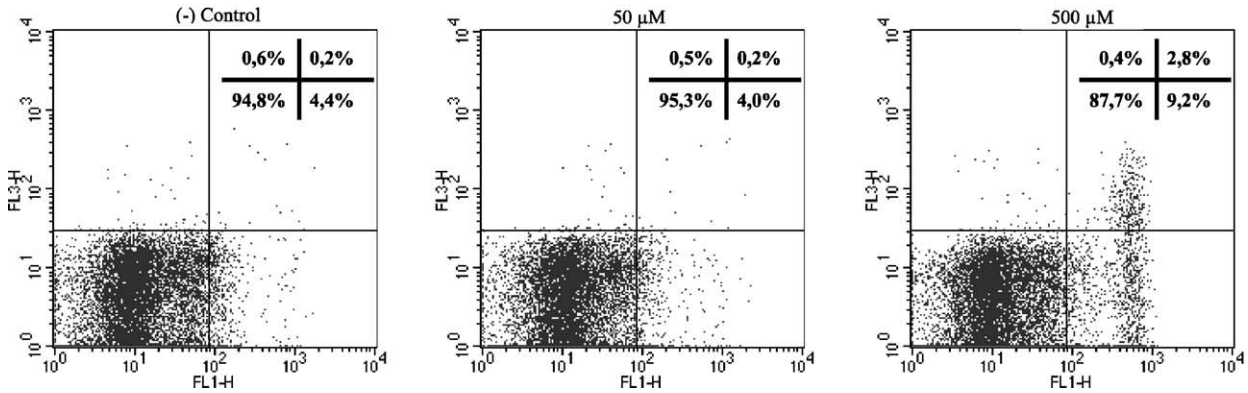


Fig. 6. Toxic effects of the sesquiterpene lactone 11α,13-dihydrohelenalin acetate. Whole blood samples were incubated overnight with various concentrations of 11α,13-dihydrohelenalin acetate (SL **4**). The samples were then stained with Annexin V-FITC and 7AAD, and analyzed using flow cytometry. One representative result of three independent measurements is shown for every concentration tested. FL-1H corresponds to Annexin V-FITC fluorescence, and FL-2H to 7AAD fluorescence.

An alternative explanation to this inversion in reactivity towards cell activation could be based on the fact that bifunctional SLs show a slight T-cell activation at low concentrations. The observed curve would be the sum of two opposing processes, and therefore only a weaker effect can be observed. In contrast, monofunctional SLs do not activate T-cells, and consequently show only the expected inhibition. Moreover, both effects, the binding to serum protein and the activation of lymphocytes, could be two related phenomena.

Regarding the effect of SLs on T-lymphocyte subpopulations, no difference was observed on the inhibition curves of $CD4^+$ and $CD8^+$ cells, which indicates that SLs act on a common step in the activation of both cell types, that is, a common central step is required for the activation of both cell populations, such as the activation of transcription factors.

The effect of SLs on the IL-2 expression led to similar results as those obtained for lymphocyte activation. Again, monofunctional SLs inhibited IL-2 expression at lower concentrations than bifunctional substances. However, bifunctional SLs showed an effect at lower concentrations compared to those required for CD69 inhibition. This divergence could be due to the fact that CD69 is stored in the cytoplasm of non-active cells, and is rapidly transported to the cell surface upon stimulation [37]. Therefore, immediate expression is not dependent on transcription or translation. Higher and sustained CD69 appearance on the lymphocyte surface is posterior to this initial process, and is the result of gene expression. The initial appearance of CD69 on the cell surface is most probably not influenced by SLs. As a consequence, activation of cells would lead to a higher concentration of CD69 detected in comparison to IL-2, because only *de novo* synthesis of these proteins was inhibited.

A further interesting result is that no complete inhibition of lymphocyte activation or IL-2 production is observed at the highest concentration tested, in contrast to EMSA data, in which incubation with SLs leads to a complete blocking of the protein–DNA interaction. This phenomenon may be due to the fact that the inducible transcription of CD69 and IL-2 are controlled by many transcription factors [38,39] and that some of these, such as Oct-1 in IL-2, are not inhibited by SLs [4]. Transcription factors that have not been blocked by SLs could maintain a basal expression of the target gene, at a lower efficiency than in untreated cells. This is consistent with experiments performed with reporter genes under the control of the full-length promoter of TNF. Transfection of cells with a super-repressor for NF-κB causes a drastic, but not full reduction of reporter gene expression [38,39].

To determine the toxic effect of SLs in whole blood samples, we quantified the amount of apoptotic and necrotic cells after overnight incubation. Surprisingly, the tested

Table 1
Toxic effects of the sesquiterpene lactones

Sesquiterpene lactone	$7AAD^+/A5^-$ (necrosis)			$7AAD^-/A5^+$(apoptosis)			$7AAD^+/A5^+$		
	50 µM	250 µM	500 µM	50 µM	250 µM	500 µM	50 µM	250 µM	500 µM
1	–	0	0	–	0.08	0.3	–	0	1.3
2	–	0	0	–	0.4	6.4	–	0	1.9
3	–	0	0.4	–	11.4	20.1	–	7.7	9
4	0	–	0	0.07	–	9	0.4	–	5
5	0	–	0	0	–	9	0	–	11.4
6	–	0	0.2	–	1.3	2.6	–	0.3	2.9

The values are given as percentage of positive cells.

Table 2
Concentration of sesquiterpene lactones required for a full inhibition of the transcription factors NF-κB and NFAT, determined using EMSA

Sesquiterpene lactone	NF-κB	NFAT
1	20[a]	20[b]
2	10[a]	10[b]
3	5[a]	N.D.
4	100[c]	40[d]
5	100[c]	N.D.
6	50[d]	N.D.

The values are given as micromolar concentration. N.D.: not determined.
[a] [5].
[b] [7].
[c] [14].
[d] Unpublished observation.

substances did not show a high toxicity (see Table 1). These results were unexpected considering previous reports. Former analyses of SL cytotoxic potential have shown that these substances are highly toxic, with appreciable effects at low micromolar concentrations, comparable to the concentrations that inhibit the activity of transcription factors. Further studies have shown that SLs trigger apoptosis on Jurkat T-cells, in a mechanism independent of the CD95 death receptor [40].

In our results only low cell death levels can be detected in whole blood after a 15 hr incubation. The SL **3** showed a different behavior, with a higher toxicity. At 250 μM 19% and at 500 μM 29% of the T-lymphocytes exhibited necrotic or apoptotic effects. The SLs **4** and **5** also showed 13.8 and 20.4% toxicity at 500 μM. These concentrations are much higher than those needed to observe an effect on lymphocyte activation or IL-2 expression. All three substances were significantly active at the lowest concentration tested (10 μM for **3**, and 50 μM for **4** and **5**).

One possible explanation for the discrepancy among our study and previous ones is that toxic effects are dependent on the cell type. To date, most of the reported studies have been performed with cultured cell lines [36,41], which are known to be very sensitive to treatment with SLs. Only two studies have been performed with primary cells. In one case, a parthenolide concentration of 30 μM led to a 23% reduction in viability of cultured rat aortic smooth muscle cells [6]. In a further study, Dirsch *et al.* [40] tested the proapoptotic effect of helenalin on PBMCs, and found that they were resistant at 50 μM concentration. Therefore, the observed lack of toxicity could be a consequence of a differential cytotoxic effect of SLs towards specific cell lines. On the other hand, SLs might be bound to plasma proteins, as it was already discussed earlier. Further studies should be undertaken to gain insights into the toxic properties of SLs, and come to a general conclusion. To date, some SL-containing drugs, such as Arnica, are only recommended for external use, due to adverse effects observed upon oral use [41]. In contrast, the parthenolide-containing extract from feverfew has been approved in the French market for the oral treatment of migraine [42].

In conclusion, the data presented here show that SLs inhibit the activation of T-lymphocytes, and its IL-2 production in whole blood. Bifunctional SLs surprisingly showed a lower activity than monofunctional, and the toxic effect of SLs was lower than expected. These results are an indication of the advantages of an experimental setting using whole blood, a system similar to the actual *in vivo* conditions.

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