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Inhibition of sphingolipid synthesis impairs cellular activation, cytokine production and proliferation in human lymphocytes☆

Norbert Blank [a,*], *Martin Schiller* [a], *Christoph Gabler* [b], *Joachim R. Kalden* [b], *Hanns-Martin Lorenz* [a]

[a] *Department of Internal Medicine V, Division of Rheumatology, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany*

[b] *Institute of Clinical Immunology and Rheumatology, University of Erlangen-Nuremberg, Erlangen, Germany*

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Abbreviations:
CTB, cholera toxin B subunit
GM1, ganglioside GM1
LAT, linker of activation in T cells
MM, membrane microdomains
PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol
SPT, serine palmitoyl transferase

ABSTRACT

The localisation of the T cell receptor and other signalling molecules in membrane microdomains (MM) is essential for the activation of T lymphocytes. These MM are stabilized by sphingolipids and cholesterol. It was recently shown that the activation of T lymphocytes leads to the confluence of small MM and the formation of an immunological synapse which is thought to be essential for a persistent activation and proliferation. We studied the effects of an inhibition of sphingolipid synthesis on T lymphocyte function. Both sphingolipid inhibitors, PDMP and myriocin, inhibited glucosphingolipids in whole cell lipid extracts and in MM. Both compounds inhibited the proliferation of superantigen-stimulated PBMC without inducing cell death. However, only the ceramide-like compound PDMP inhibited the expression of activation markers and the secretion of IFN-gamma which was not seen with myriocin treatment. The MM localisation of Lck and LAT was not significantly reduced in PDMP-treated cells.

In conclusion, our results show that glucosphingolipids are necessary for cell growth of human T lymphocytes. However, inhibition of glucosphingolipid synthesis itself did not inhibit cellular activation. Our data show that glucosphingolipids – in contrast to cholesterol – are not essential for the stabilisation of MM.

1. Introduction

The localisation of the T cell receptor and other signalling molecules in membrane microdomains (MM) is essential for the activation of T lymphocytes [1,2]. T cell activation by antigen presenting cells leads to the confluence of small MM and the formation of an immunological synapse at the contact site with the antigen presenting cell [3]. This mechanism is essential for the proliferation of T lymphocytes. MM are also referred to as lipid rafts because sphingomyelin, glycosphingolipids and cholesterol were enriched in these MM [4,5]. Because MM can be isolated on basis of their detergent resistance, MM are also named detergent resistant membranes [6].

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* Corresponding author. Tel.: +49 6221 56 39676; fax: +49 6221 56 4920.
E-mail address: Norbert_Blank@med.uni-heidelberg.de (N. Blank).

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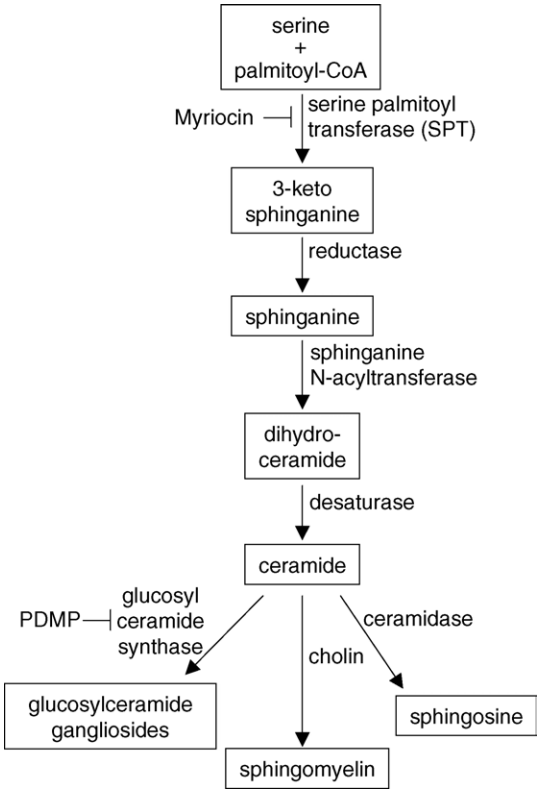

Fig. 1 – Physiology of sphingolipid synthesis, inhibition sites of myriocin and PDMP.

Biochemical analysis of MM proteins showed that MM contain the T cell receptor complex as well as adaptor molecules, integrins, glucosylphosphatidylinositol-anchored molecules, acylated molecules, cholesterol and ganglioside GM1 [1,2]. The initial step of sphingolipid synthesis is catalyzed by the enzyme serine palmitoyl transferase (SPT) [7] which generates 3-keto-sphinganine (Fig. 1). Various other enzymes catalyze reactions leading to the formation of ceramide. Ceramide can be a precursor for sphingolipid synthesis or can be degraded to sphingosine. The key enzyme for the synthesis of gangliosides is glucosylceramide synthase which conjugates the first sugar to ceramide [8]. Finally, a large variety of gangliosides is generated by subsequent addition of various other sugars.

Myriocin is an inhibitor of ganglioside and sphingomyelin synthesis by inhibiting serine palmitoyl transferase (SPT) [7]. Compared to other known SPT inhibitors like β-chloro-L-alanine, L-cycloserine and sphingofungin B, myriocin has both, a high specificity and a high affinity for SPT [7,9]. It was shown that myriocin was able to inhibit the growth of the IL-2-dependent cytotoxic T cell line CTLL and the mixed lymphocyte reaction in mice with a 50% inhibitory concentration (IC_{50}) of about 15 nM [7]. Addition of sphingosine to myriocin treated CTLL-2 cells restored proliferation to 97%, but addition of ceramide or sphingomyelin failed to restore proliferation [7]. This finding indicates that 3-keto-sphinganine, sphinganine, dihydroceramide, ceramide and eventually other metabolites of the glycolipid and sphingolipid synthesis pathways should also be considered for their role on cellular physiology (Fig. 1). Despite the IC_{50} of ~15 nM for inhibition of SPT, some investigators used up to 10 μM myriocin for cell proliferation assays [9], suggesting that cellular models may differ in their sensitivity to myriocin treatment.

The synthetic ceramide-like compound PDMP inhibits glucosylceramide synthase [10], which is the key enzyme of the synthesis of gangliosides and a late step in the synthesis of gangliosides (Fig. 1). The IC_{50} of PDMP for glucosylceramide synthesis is 15 μM [10]. PDMP treatment altered cellular growth and the adhesion of an epithelial cell line to fibronectin [11]. PDMP treatment of neuronal cell lines caused apoptotic cell death [12] and an inhibition of neurite outgrowth [13]. Stimulation of mouse splenocytes with mitogenic lectins was strongly inhibited and the IL-2-dependent phosphorylation of tyrosine residues was enhanced upon PDMP treatment [14].

It has been shown, that MM are composed of proteins, glucosphingolipids and cholesterol. Because of the important role of membrane microdomains for T lymphocyte physiology, we investigated whether an inhibition of the synthesis of the sphingolipids ganglioside and sphingomyelin has an influence on the formation of MM and furthermore impacts T cell functions like cellular activation, cytokine production, proliferation and cell death.

2. Materials and methods

2.1. *Cell culture*

Peripheral blood mononuclear cells (PBMC) were isolated from different healthy donors using ficoll gradient centrifugation (Ficoll-Histoprep/BAG, Lich, Germany). Cells were cultured either in serum free medium (RPMI 1640 (Gibco, Eggenstein, Germany)) supplemented with sodium selenite, pyruvate, insulin, transferrin and ethanolamine (SPITE medium supplement, Sigma, Deisenhofen, Germany) or serum containing medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco)) with similar results. The medium was supplemented with 4 mM L-glutamine, 10 mM HEPES buffer, 100 IU/ml penicillin, 0.1 mg/ml streptomycin (Gibco) and 50 nM 2-mercaptoethanol.

PBMC (4×10^6 ml^{-1}) were pre-incubated with inhibitors of sphingolipid synthesis for 24 h to decrease the cellular sphingolipid pools [10]. T lymphocytes were stimulated by addition of 10 ng/ml staphylococcus enterotoxin B (SEB, Sigma) and were cultured for up to 4 days. The viability of cultured cells was determined by 0.1% trypan blue staining (>80%).

2.2. *Reagents*

DL-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol*HCl (PDMP, Calbiochem, Bad Soden, Germany) was dissolved in pure ethanol to obtain a 100 mM stock solution. Myriocin (Sigma) was dissolved in DMSO to obtain a 10 mM stock solution.

2.3. *Proliferation assay*

10^5 PBMC from five healthy donors were pre-incubated with or without inhibitors for 24 h in a 96 well plate (Cellstar, Greiner

BioOne, Frickenhausen, Germany). T lymphocytes were activated with 10 ng/ml SEB and cultured for additional 3 days. Control cells were not stimulated with SEB. Proliferating cells were labelled with 0.5 μCi of ^{3}H thymidine/well for 8 h at 37 °C. Labelled cells were transferred to a glassfibre filtermat, and ^{3}H activity was counted using a 1205 Betaplate counter (Wallac Oy, Turku, Finland). Triplicates of each sample were analyzed from three independent healthy donors.

2.4. Viability

Viable cells were characterized by the exclusion of apoptotic and necrotic cells. Briefly, 10^5 cells from four healthy donors were cultured as described above. After stimulation with SEB for 3 days, cells were incubated with 0.5 ml of a staining solution (25 μl FITC-labeled AxV (FLUOS, Roche, Mannheim, Germany) and 25 μl propidium iodide (10 mg/ml PI, Sigma) in 50 ml Ringer solution) for 30 min at 4 °C in the dark and subsequently analyzed by cytometry (EPICS XL™, Coulter, Hialeah, FL, USA). Viable cells were negative for AxV-FITC and PI staining. A minimum of 10^4 cells were measured. Data analysis was performed with Coulter XL™ software, Version 3.

2.5. Marker analysis

Cell surface expression of CD3-FITC/CD69-PE and CD3-FITC/CD25-PE (Becton-Dickinson, Heidelberg, Germany) were determined after stimulation of 10^5 cells/150 μl with SEB for 24 h (CD69/CD3) or 96 h (CD25/CD3). Only CD3 positive cells were gated.

2.6. Quantification of cytokines

10^6 PBMC/ml from three healthy donors were pre-incubated with or without inhibitors in a 48 well plate (Cellstar, Greiner BioOne), stimulated with SEB and cultured for the indicated time. Each sample was cultured in duplicates. Cytokines in cell culture supernatant were measured after 48 h using IFN-gamma ELISA kits from R&D Systems (Wiesbaden, Germany) according to the manufacturer's recommendations.

2.7. Isolation of membrane microdomains

10^7 to 10^8 PBMC were pre-incubated for 24 h with or without inhibitors and were subsequently stimulated with SEB for 72 h. The isolation and quantification of lipid microdomains was performed as previously described in detail [15] with some modifications. Briefly, 5×10^7 to 10^8 SEB stimulated cells were centrifuged at $450 \times g$ for 5 min and culture medium was discarded. Cells were resuspended in 100 μl phosphate buffered saline (PBS) and incubated with 2 μl of the CTB-HRP stock solution for 30 min on ice. The CTB-HRP stock solution contained 40 μg CTB and 100 μg HRP (Calbiochem) dissolved in 100 μl of double-distilled water with a final CTB-HRP activity of 191 mU/ul. Cells were washed in 3 ml ice cold PBS to remove unbound CTB-HRP. Thereafter, cells were lysed at 37 °C for 10 min in 1 ml TNEV lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 5 mM Na_3VO_4 (Merck, Darmstadt, Germany), pH 7.4) supplemented with one tablet Complete mini protease inhibitor/10ml (Roche) and with 1% Brij-98 (v/v) (Sigma). The protein content was determined using a Bradford assay (Bio-Rad, Munich, Germany). TNEV buffer without detergent was used for the preparation of the different sucrose solutions. The cell lysate was mixed with 2 ml 80% sucrose, transferred an ultracentrifugation tube (Beckmann Coulter, Krefeld, Germany) and was overlaid with 2 ml 37.5% sucrose, 2 ml 35% sucrose, 2 ml 30% sucrose and 3 ml 5% (w/v) sucrose. The ultracentrifugation (Centrikon T-1170, Kontron Instruments, Neufahrn, Germany) was performed at $150{,}000 \times g$ for 20 h at 4 °C. Twelve fractions were harvested with a 1 ml pipette from top (named F1) down (F12).

2.8. Quantification of ganglioside GM1

Duplicates (25 μl) of each sucrose fraction and triplicates of the diluted standard CTB-HRP solutions (range from 0.5 to 300 μU/25 μl) were transferred into a 96 well flat-bottomed microtiter plate (CellStar, Greiner BioOne) and 100 μl of 2,2′-azino-di-3-ethyl-benzthiazoline-6-sulphonic acid (ABTS, Roche) were added. Absorption was determined at 405 nm (A_{405}) with a Spectra ELISA reader (SLT Labinstruments, Crailsheim, Germany). The correlation of A_{405} values with CTB-HRP standards was determined and the A_{405} of each sample was calculated into CTB-HRP activity (Blank et al. [15]).

2.9. Protein staining and immunoblot detection

Membrane microdomains were isolated and quantified as described above. Proteins were precipitated from each sucrose fraction with trichloroacetic acid 10% (v/v) and precipitation in 1 ml acetone at −20 °C overnight. Protein pellets were dissolved in a modified RIPA buffer (Blank et al. [15]) and were stored at −20 °C until use. Proteins were separated using SDS-PAGE (12.5%, w/v, polyacrylamide gels) and were stained with SYPRO Ruby solution (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's recommendations. The wet gels were photographed under UV light using a light cabinet.

Immunoblot detection was performed by semidry protein transfer onto nitrocellulose membranes which were sequentially incubated with specific antibodies against Lck (Transduction, Lexington, KY, USA) and LAT (Upstate, Lake Placid, NY, USA). Specific bands were visualized using HRP-conjugated goat anti-mouse IgG (or anti-rabbit, both from Jackson, West Grove, PA, USA) followed by enhanced chemiluminescence. Further detections were performed after stripping the membranes by incubation in 62.5 mM NaCl, 100 mM 2-mercaptoethanol, and 2% SDS for 20 min at 55 °C.

2.10. HPTLC for sphingolipids and phospholipids

6×10^6 PBMC from two healthy donors were pretreated with PDMP for 24 h in cell culture flasks (Greiner BioOne) and stimulated with SEB for additional 96 h. During the final 24 h of this incubation 0.75 μCi of L-(U-^{14}C) serine (50 μCi/ml, Amersham Biosciences, Little Chalfont, UK) were added to each culture flask. Lipids were extracted from 4.5×10^6 cells by incubation of the cell pellet in 400 μl chloroform:methanol (2:1). Addition of 200 μl $CaCl_2$ 0.02% resulted in a two phase separation and the upper aqueous phase was discarded. The

lower phase contained sphingolipids and phospholipids and was dried under vacuum. Lipids were redissolved in chloroform and spotted on a HPTLC plate (Silica gel 60, No. 1.05547, Merck, Darmstadt, Germany). HPTLC was performed using two runs of methyl acetate:n-propanol:chloroform:methanol:0.25% KCl (25:25:25:10:9, v/v) [9]. Autoradioactivity was evaluated using a fluorescent image analyzer (FLA-3000, raytest, Straubenhardt, Germany) equipped with AIDA software.

2.11. *Statistical analysis*

Data represent mean ± S.D. Statistical analyses were performed using a two-tailed Student's *t*-test.

3. Results

3.1. *Inhibition of sphingolipid synthesis*

Sphingolipids were metabolically labelled with ^{14}C serine, extracted with chloroform:methanol and separated with HPTLC. Medium cultured PBMC were compared to PDMP- or myriocin-treated cells. The autoradiography show faint bands for ceramide (Cer) and glucosylceramide (Glu-Cer) in cells cultured without inhibitor (Fig. 2A). ^{14}C serine was incorporated by the phospholipids phosphatidylethanolamin (PE) and phosphatidylserine (PS) and by sphingomyelin (Sph) (Fig. 2A). Single sphingolipids were normalized to their levels in medium cultured cells (Fig. 2B and C). As expected, PDMP treatment primarily reduced the amount of glucosylceramide ($p < 0.05$). We had expected an accumulation of ceramide due to inhibition of a ceramide metabolizing pathway by PDMP. However, ceramide levels were inhibited in PDMP-treated cells (Fig. 2B). It is remarkable that even the lowest concentration of PDMP effectively inhibited the glucosylceramide synthesis. Higher concentrations of PDMP (10 μM) also inhibited sphingomyelin (Fig. 2B). Myriocin treatment inhibited the synthesis of sphingomyelin, ceramide and glucosylceramide (Fig. 2C) even at the lowest concentration of 10 nM myriocin. The apparent increase of the ceramide level with high concentrations of myriocin is most likely due to the relatively high variability of low amounts of total ceramide and was not described by other groups [7,9].

3.2. *Inhibition of sphingolipid synthesis reduces proliferation*

An inhibition of sphingomyelin and ceramide synthesis may alter cellular function and proliferation since ceramide can induce proliferation and cell death. The proliferation of superantigen-stimulated PBMC was inhibited by PDMP which was significant for 10 μM PDMP (Fig. 3A). The IC_{50} for the inhibition of proliferation was ~15 μM (Fig. 3A) which is consistent with previously published data [8]. Control cells were cultured without superantigen and represent some baseline proliferation of isolated PBMC (Fig. 3A).

However, we observed a more complex pattern when cells were treated with myriocin (Fig. 3C). Small concentrations like 1 nM myriocin significantly increased the proliferation

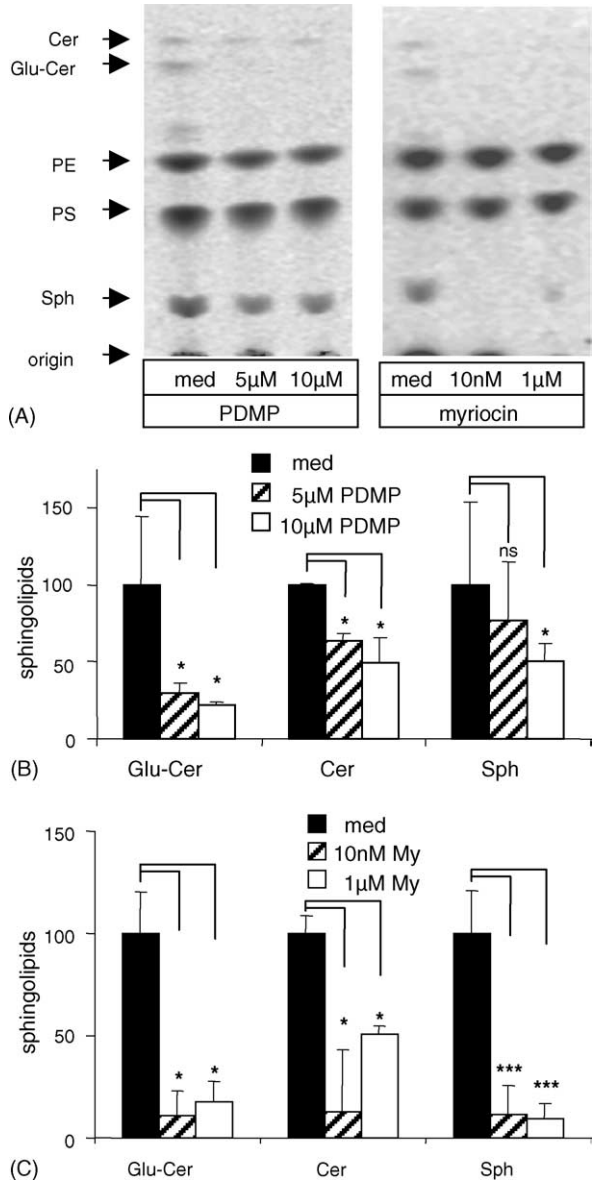

Fig. 2 – PDMP and myriocin suppressed the synthesis of sphingolipids without accumulation of ceramide. Superantigen-stimulated PBMC were cultured with or without inhibitors and were labelled with ^{14}C serine. Autoradiography shows sphingolipids and phospholipids from PDMP or myriocin treated PBMC (A). One representative out of four independent experiments is shown. The sphingolipids glucosylceramide (Glu-Cer), ceramide (Cer) and sphingomyelin (Sph) were quantified and were normalized to their content in medium cultured cells (B and C). Histograms represent mean ± S.D. of four independent experiments from different blood donors. Statistics: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

($p = 0.01$), while 100 nM myriocin inhibited proliferation ($p = 0.04$) (Fig. 3C). Higher concentrations up to 10 μM myriocin did not further decrease proliferation (Fig. 3C). Toxic effects of the inhibitors were excluded because the cellular viability after this 4 day culture was ~65% and was independent of the concentration of PDMP (Fig. 3B) or myriocin (Fig. 3D). There-

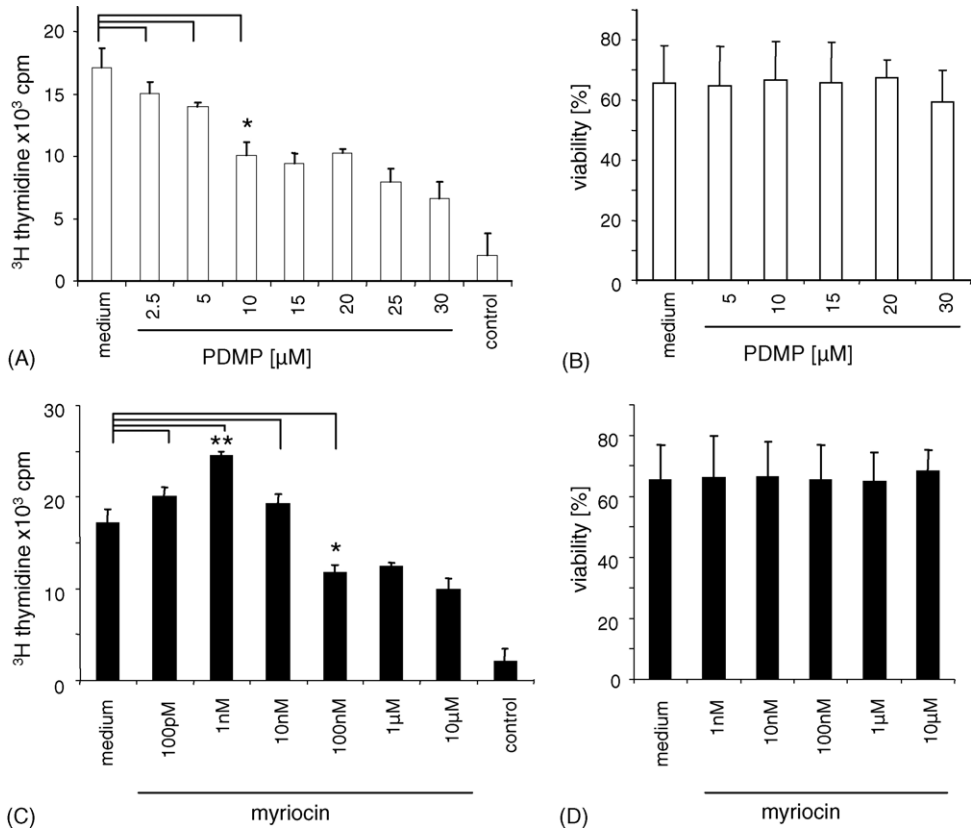

Fig. 3 – Proliferation and viability. 10^5 PBMC/well were pre-incubated with various concentrations of PDMP (A and B) and myriocin (C and D) for 24 h and were stimulated with superantigen for 3 days. Cells were labelled with ^{3}H thymidine for 8 h and proliferation was quantified. Data represent mean ± S.D. of a triplicate from one representative out of three independent experiments (A and C). Control cells were cultured for 4 days without stimulation. Viability was assessed by staining with annexin-V (AxV)-FITC and propidium iodide (PI). Viable cells were AxV-FITC and PI negative. The data represent mean ± S.D. from four different donors (B and D). Statistics: $^*p < 0.05$; $^{**}p < 0.01$.

fore, we can exclude cell death as an explanation for the inhibition of proliferation.

3.3. *PDMP treatment prevents T cell activation and inhibits cytokine production*

Since the inhibition of proliferation is not due to toxic effects of the inhibitors, we investigated the regulation of activation markers in superantigen-stimulated PBMC. The expression of the early (CD69) and late (CD25) activation markers on CD3 gated T cells was ~1% and increased to ~20% upon stimulation with superantigen (Fig. 4A and B). PDMP treatment with 10 μM or higher significantly inhibited the upregulation of CD69 ($p = 0.006$, Fig. 4A) and CD25 ($p = 0.02$, Fig. 4B). Higher concentrations of PDMP nearly abolished the induction of activation markers. In contrast, incubation with up to 10 μM myriocin failed to suppress the upregulation of CD69 (Fig. 4A) and CD25 (Fig. 4B).

The production of IFN-gamma was determined after 48 h of stimulation with superantigen. Treatment with 20 μM PDMP significantly inhibited the production of IFN-gamma (2107 ± 583 pg/ml versus 765 ± 224 pg/ml, $p < 0.05$) (Fig. 4C). In contrast, incubation with 1 μM myriocin had no effect on the IFN-gamma production (Fig. 4C).

These results indicate that the inhibition of T cell activation and the reduced secretion of IFN-gamma by PDMP are not due to the inhibition of ganglioside synthesis.

3.4. *Inhibition of sphingolipid synthesis reduces GM1 ganglioside in MM*

Since PDMP probably interferes with the activation process in T cells, we investigated the composition of MM. CTB is a ligand for ganglioside GM1, and therefore, a good marker for MM. MM were labelled with CTB-HRP, isolated and harvested into fractions 3–5 as described in Section 2. Treatment with PDMP or myriocin inhibited the CTB binding to MM, indicating a reduced expression of the ganglioside GM1 in this membrane compartment (Fig. 5A). The MM-resident GM1 ganglioside was quantified as CTB binding to the MM containing fractions 3–5.

The MM-resident GM1 ganglioside was calculated from four independent experiments without inhibitors and five experiments with various concentrations of PDMP and myriocin (Fig. 5B). The mean values of each group are indicated by horizontal lines and were 99 μU for medium cultured cells, 23 μU for cells treated with PDMP and 29 μU for cells treated with myriocin (Fig. 5B). The inhibition of MM-resident GM1 ganglioside was highly significant for PDMP and

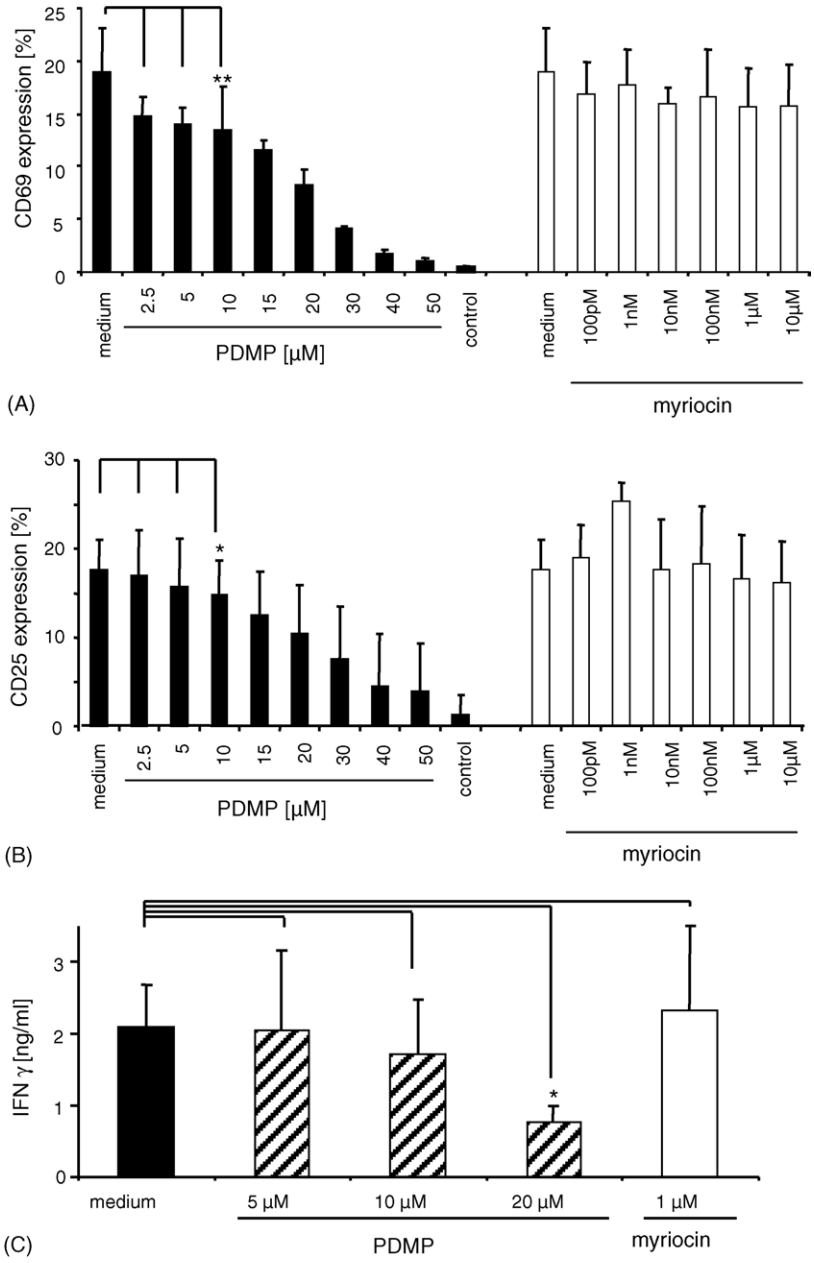

Fig. 4 – Expression of activation markers and cytokine production. Cells were pre-incubated with medium, PDMP or myriocin for 24 h and stimulated with superantigen for another 24 h (A) or 96 h (B). Control cells were cultured without stimulation. Expression of CD69 (A) and CD25 (B) were determined on CD3 positive cells. Production of IFN-gamma was determined in culture supernatant after 48 h (C). Data show mean ± S.D. from three independent experiments. Statistics: $^{*}p < 0.05$; $^{**}p < 0.01$.

myriocin (Fig. 5B). Interestingly, the inhibition of MM-resident GM1 ganglioside was already maximal at concentrations of 5 μM PDMP and 10 nM myriocin, indicating that the inhibition of proliferation requiring high concentrations of PDMP is most probably not mediated by inhibition of ganglioside synthesis.

3.5. *MM resident proteins are not affected by inhibition of sphingolipid synthesis*

Since PDMP treatment interfered with the T cell activation process we tested whether an inhibition of MM-resident GM1 ganglioside leads to changes in the protein composition of MM. Proteins were precipitated from the MM containing sucrose fractions as described in Section 2. We did not detect a significant difference in the Coomassie stained protein pattern of MM from medium cultured (Fig. 6A) or PDMP-treated cells (Fig. 6B).

It is well known that the localisation of signal transducing molecules in MM is critical for their function. Therefore, we investigated the localisation of the MM marker proteins Src-kinase Lck and the adaptor molecule linker of activation in T lymphocytes (LAT) in MM isolated from medium cultured and PDMP-treated cells. Immunoblot detection of Lck showed a strong signal for the MM resident Lck and little Lck in the

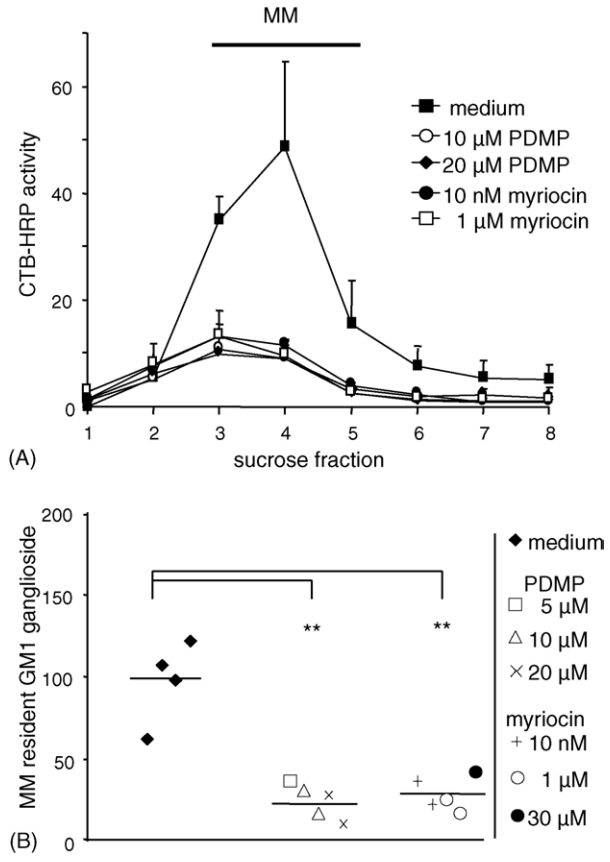

Fig. 5 – CTB-HRP staining reveals inhibition of GM1 ganglioside in MM. PBMC were incubated with PDMP or myriocin and stimulated with superantigen for 72 h. MM were labelled with CTB-HRP and isolated and quantified. Floating MM were harvested in fractions 3–5 containing the highest CTB-HRP activity (A). Data represent mean ± S.D. of two to four independent experiments. MM resident GM1 gangliosides were quantified as CTB-HRP activities from fractions 3–5 and were plotted for each single experiment (B). PDMP and myriocin significantly inhibited MM resident GM1 ganglioside. Statistics: $^{**}p < 0.01$.

detergent soluble fraction 10 (Fig. 6C). We observed a slightly lower Lck expression in PDMP-treated cells. However, Lck was still detectable in cells treated with 10 μM PDMP (Fig. 6C). Similarly, LAT was also detected in MM from medium cultured and PDMP-treated cells (Fig. 6D). These data confirm the presence of Lck and LAT in MM from medium cultured cells. However, treatment with PDMP did not exclude these molecules from MM fractions. These findings indicate that gangliosides play a minor role for the formation and stabilisation of large signalling complexes in the cell membrane.

4. Discussion

In the present study, we investigated the effects of an inhibition of sphingolipid synthesis on the activation and proliferation of human T lymphocytes because earlier reports demonstrated an accumulation of gangliosides and sphingomyelin in lipid rafts within the plasma membrane [1]. Two different inhibitors were used to investigate the relevance of sphingolipid synthesis on the function of primary human T cells. The specificity of an inhibitor strongly depends on the concentration used. Therefore, we carefully monitored concentration dependent effects of the used inhibitors to dissect specific pathways addressed by low concentrations of inhibitors and additional metabolic pathways requiring higher inhibitor concentrations. As expected, myriocin treatment inhibited the synthesis of sphingomyelin and glucosphingolipids while PDMP treatment only inhibited the synthesis of glucosphingolipids (Fig. 2). Reduced levels of glucosphingolipids were shown in whole cell lipid extracts and were demonstrated in isolated MM by reduced binding of cholera-toxin to MM. Cellular growth was inhibited in PDMP and myriocin treated lymphocytes. These data are consistent with a reduced proliferation in PDMP-treated and mitogen-stimulated mouse splenocytes and an IL-2-dependent CTLL cell line [14].

Our data show that low concentrations of PDMP inhibit the glucosylation of ceramide (Fig. 2B) but only exert minor effects on T cell activation (Fig. 4). High concentrations of PDMP are required to suppress T cell activation, cytokine release and proliferation significantly. Previous work showed that PDMP can also inhibit other ceramide converting enzymes like 1-O-acylceramide synthase, ceramide deacylase, glucosylceramide galactosyltransferase, lactosylceramide sialyltransferase and two other glucosyl transferases [16], indicating a rather low specificity of high concentrations of PDMP. In addition, even high concentrations of myriocin were unable to suppress T cell function significantly. Therefore, our data indicate that ceramide, gangliosides and sphingomyelin are not required for T cell activation and cytokine secretion.

One could expect an accumulation of ceramide due to inhibition of a ceramide metabolizing pathway by PDMP. However, ceramide levels were inhibited in PDMP-treated cells in our experiments (Fig. 2B). Previous work revealed that PDMP-treated cells might accumulate ceramide as was described by some investigators [11,12,17,18] but was denied by others [13,19]. However, our data exclude an accumulation of ceramide in PDMP-treated lymphocytes. An upregulation of ceramide synthesis in proliferating lymphocytes could explain the inhibitory effects of ceramide on proliferation as a negative feedback mechanism under physiologic conditions. In this scenario, the inhibition of cell growth by PDMP treatment might inhibit an upregulation of ceramide during cellular activation, leading to the observed inhibition of ceramide levels (Fig. 2B). The finding that myriocin can also inhibit cell growth and suppress ceramide levels (Fig. 2C) would be consistent with this hypothesis.

The biphasic effect of low concentrations and high concentrations of myriocin on T cell proliferation (Fig. 3) was observed in three independent experiments and was not seen with PDMP treatment. To our knowledge, this is the first report of a stimulation of T cell growth due to low concentrations of myriocin. We assume that a balance of growth promoting and growth inhibitory sphingolipids might exist and that low concentrations of myriocin might shift this balance towards proliferation by an unknown mechanism.

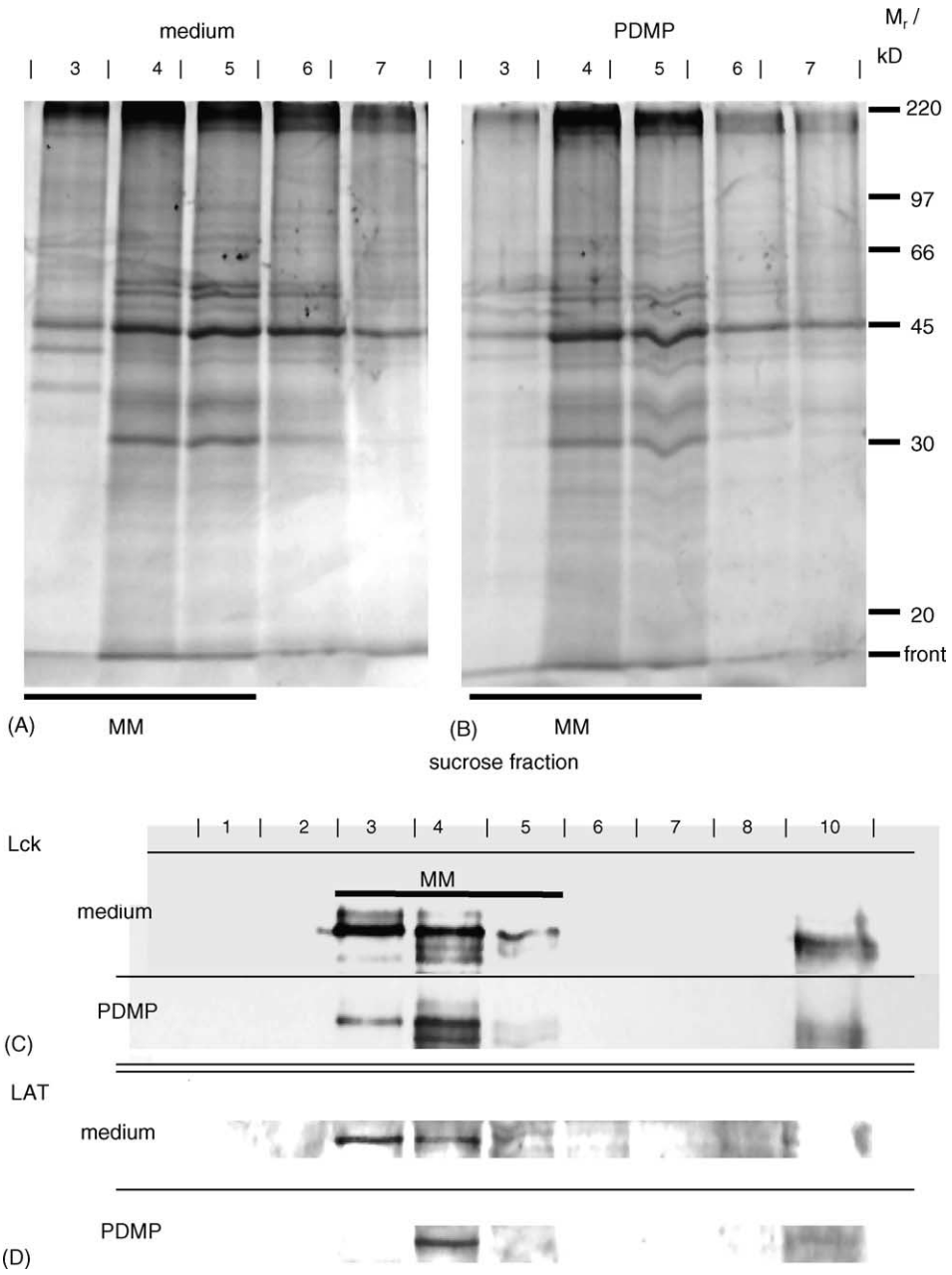

Fig. 6 – PDMP treatment does not alter the major MM resident protein composition. MM resident proteins were isolated from medium cultured cells (A) or PDMP-treated cells (B) and were stained with SYPRO ruby. MM resident proteins were predominantly detected in sucrose fractions 3–6. Immunoblot detection revealed MM resident Lck (C) and LAT (D) in fractions 3–5. Lane 10 represents proteins from detergent soluble membranes (fraction 10). One representative out of two independent experiments is shown.

The analysis of sphingolipid species (Fig. 2) shows that even low concentrations of myriocin inhibited the synthesis of ceramide, glucosylceramide and sphingomyelin. Therefore, it can be suspected that high concentrations of myriocin could be able to inhibit additional enzymes other than serine palmitoyl transferase (SPT). This view is confirmed by an earlier report in this journal [9] showing that the major effect of high concentrations of myriocin on cell growth was actually dependent on SPT inhibition but a minor part of the effect appeared to be independent of SPT inhibition [9]. Given the structural similarity of myriocin with the sphingoid bases sphinganine and sphingosine, it can be speculated that the metabolism of sphinganine and/or sphingosine might be affected by other than SPT mediated mechanisms. High concentrations of myriocin might inhibit sphinganine *N*-acyltransferase activity, which would result in an additional inhibition of the ceramide synthesis pathway. However, our results indicate that cellular ceramide levels were already suppressed with low concentrations of myriocin (Fig. 2A and C). Alternatively, an inhibition of sphingosine-1-kinase activ-

ity could decrease sphingosine-1-phosphate levels. It was shown recently that sphingosine-1-phosphate can suppress the antigen-stimulated proliferation of T cells by interaction with spingosine-1-phosphate receptor type 1 [23] and that myriocin treated mice show decreased sphingosin-1-phosphate levels [24]. In addition to the metabolism of sphingolipid precursors, sphingosine appears to be a potent inhibitor of protein kinase C (PKC) activity which is an important mediator of cellular activation and proliferation [25]. Therefore, high concentrations of myriocin might also exert additional effects by inhibition of PKC activity. Further experiments might consider the use of siRNA to knock down SPT activity. This strategy could avoid potential problems associated with myriocin specificity.

We show that PDMP exerts only a minor inhibition of sphingomyelin levels (Fig. 2A) and the possibility remains that sphingomyelin might be relevant for MM stability. However, our experiments show a similar MM protein pattern in medium cultured and PDMP-treated cells. In addition, previous experiments used exogenous sphingomyelinase treatment of cultured Madin–Darby canine kidney cells to convert sphingomyelin into ceramide [20]. These experiments failed to reduce the association of proteins with MM [20]. Our data show that low concentrations of myriocin significantly inhibited sphingomyelin levels (Fig. 2C) but even high concentrations of myriocin failed to inhibit T cell activation and cytokine secretion (Fig. 4). Therefore, our experiments support the view of Schuck et al. that sphingomyelin is not essential for MM stability [20].

A previous review suggested that MM are essential for the activation of T cells [1]. It was shown that MM formation may serve as a potent costimulatory signal and is essential for a long lasting activation signal committing T cells to proliferation [21]. Another group showed that an increased expression of GM1 ganglioside was associated with a lower signalling threshold of CD4/CD8 double-negative thymocytes compared with to CD4/CD8 double-positive thymocytes which enabled a ligand independent activation of CD4/CD8 double-negative thymocytes and resulted in a more robust Ca^{2+} entry into T cells [22]. These published data promote the role of GM1 ganglioside for T cell function. However, in our experiments myriocin inhibited glucosphingolipid synthesis but failed to interfere with T cell activation and cytokine release. Therefore, our data suggest that sphingolipids are relevant for T cell growth but are not essential for T cell activation and cytokine release.

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