

Diploma thesis

Identification of antigen-specific natural killer T cells in patients with antiphospholipid antibody syndrome

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Zusammenfassung

Hintergrund

Das Antiphospholipid Syndrom (APS) gehört zur Gruppe der Autoimmunerkrankungen. Arterielle sowie venöse Thrombosen, Schwangerschaftskomplikationen, wie z. B. rezidivierende Aborte, sind die typischen klinischen Manifestationen, die in Gegenwart von Antiphospholipid-Antikörpern (aPLA) auftreten. Die Forschungsergebnisse der letzten Jahre zeigen, dass aPLA, wie anti-Cardiolipin (aCL) und anti- β 2-Glycoprotein 1, eine entscheidende Rolle in der Pathogenese des APS spielen. Die Ergebnisse einer kürzlich veröffentlichten Studie deuten darauf hin, dass in einem murinen System natürliche Killer-T-Zellen (NKT Zellen), die Lipide als Antigene erkennen, einen Einfluss auf die aCL Produktion haben könnten. In der folgenden Arbeit stellen wir die Hypothese auf, dass solche NKT Zellen auch bei Patienten mit APS existieren.

Methodik

Eine prospektive Studie an 8 Patienten mit APS und 11 gesunden Kontrollen wurde mittels durchflusszytometrischer Verfahren mit CL beladenen CD1d-Tetrameren durchgeführt, um CL-bindende NKT Zellen zu identifizieren. Neben der Bestimmung der Prävalenz der CL NKT Zellen in APS Patienten und in der Kontrollgruppe, wurde die Expression von Oberflächenmarkern analysiert. Anschließend erfolgten Stimulationsversuche der NKT Zellen in vitro. Das intrazellulär vorkommende Protein Ki67 diente dabei als Proliferationsmarker, welches nach Zugabe von CL gemessen wurde. Schließlich wurde mittels Multiplex Immunoassay die Produktion verschiedener Zytokine (GM-CSF, Granzyme B, IFN- γ , IL-4, IL-6, IL-10, IL-17A, IL-21, TNF- α) nach CL-Stimulation bestimmt.

Ergebnisse

Wir identifizierten mit Hilfe von CL beladenen CD1d-Tetrameren (0,028 % [\pm 0.01] der gesamten T Zell Population) CL-bindende NKT Zellen sowohl in Patienten mit APS als auch in gesunden Probanden. Wir können zeigen, dass diese

Subpopulation weder invariante NKT Zellen noch $\gamma\delta$ T Zellen enthält, welche ebenfalls Lipide als Antigene erkennen könnten. Patienten mit APS hatten im Vergleich zu der gesunden Kontrollgruppe, eine signifikant erhöhte Anzahl an CL NKT Zellen im peripheren Blut (0,024 % [\pm 0.006] der gesamten T Zell Population vs. 0,016 % [\pm 0.007]; $p = 0,017$). Die Exposition von CL NKT Zellen mit CL in Stimulationsversuchen steigerte die intrazelluläre Expression von Ki67 Protein. (20,37 % der gesamten CL NKT Zellen [\pm 13,40]) vs. 8.08 % [\pm 4,42] $p = 0,027$) Hinsichtlich der Zytokinproduktion bewirkte die Behandlung mit CL eine Suppression aller gemessenen Zytokinspiegeln (Siehe Tabelle 11), wohingegen die basale Zytokinproduktion der unbehandelten Zellen unbeeinträchtigt blieb.

Schlussfolgerung

In dieser Arbeit beschreiben wir eine neue T-Zell Subpopulation, die in der Lage ist CL als Antigen zu erkennen. Da die Anzahl dieser Subpopulation in Patienten mit APS im Vergleich zu gesunden Probanden erhöht ist, könnte dieser Umstand einen Einfluss auf die Produktion von anti-CL-Antikörpern haben. Die Expression von Ki67 in gesunden Probanden nach CL-Gabe deutet darauf hin, dass CL als Antigen eine Immunantwort einleiten kann. Darüber hinaus zeigt die Suppression der Zytokinausschüttung, dass CL neben seinen Antigen-Eigenschaften auch weitreichende Einflüsse auf übergeordnete immunologische Prozesse haben könnte. Deshalb sind weitere Studien notwendig, um die genauen Funktionen von CL NKT Zellen zu klären.

Abstract

Background

Antiphospholipid Syndrome (APS) is an autoimmune disease, characterized by arterial or venous thrombosis, pregnancy complications such as recurrent pregnancy loss and the occurrence of antiphospholipid antibodies.

Research indicates that antiphospholipid antibodies such as anti-cardiolipin and anti- β 2-glycoprotein 1 play a crucial role in the pathogenesis of antiphospholipid syndrome. However, little is known about their emergence, regulation and pathological effects. Recently published mouse studies suggest that a T cell subset called natural killer T cells (NKT cells) with the capability to recognize lipid antigens could have a decisive influence on the regulation of the anti-cardiolipin antibody production.

In the following work, we hypothesize the existence of such NKT cells in humans particular in patients with APS. Besides the detection of cardiolipin recognizing NKT cells, this study aims at providing a better understanding of their role in APS.

Methods

A prospective study on 8 patients with APS and 11 healthy controls was performed to identify and characterize cardiolipin binding NKT cells via flow cytometry.

Cardiolipin (CL) loaded CD1d tetramer was used to determine the prevalence of CL on patient with APS and healthy group in the peripheral blood. Additionally, the phenotype of the CL-binding NKT cells was analysed by using distinct surface markers. A stimulation assay was performed in cell culture to investigate the capability of CL NKT cell activation. The extent of proliferation was measured by means of intracellular Ki67 staining. The production of cytokines (GM-CSF, Granzyme B, IFN- γ , IL-4, IL-6, IL-10, IL-17A, IL-21 and TNF- α) was measured via a Multiplex Immunoassay upon stimulation with CL.

Results

We identified CL-binding NKT cells in peripheral blood of APS patients and healthy individuals using CL loaded CD1d tetramers (0,028 % [\pm 0.01] of total T cell population) Moreover, we showed that this novel T cell subgroup are neither

invariant NKT cells nor $\gamma\delta$ T cells, which are known for their potential to recognize lipid antigens.

Patient with APS had elevated numbers of CL-binding NKT cells (0,024 % [\pm 0.006] of total T cell population vs. 0,016 % [\pm 0.007]; $p = 0,017$) in peripheral blood compared to healthy control group.

The exposure of CL recognizing NKT cells to CL in stimulation assays caused an up-regulation of Ki67 protein expression in contrast to untreated cells. (20,37 % of total CL NKT Cells [\pm 13,40]) vs. 8.08 % [\pm 4,42] $p = 0,027$). Treatment with CL induced suppression of basal cytokine secretion.

Conclusion

We describe a novel subpopulation of T cells recognizing CL as an antigen. Moreover, this subset occurred more frequently in APS patients than in healthy individuals, which suggests that these NKT cells could play a role in the production of anti-CL antibodies. The up-regulation of Ki67 in healthy individuals upon the substitution of CL also indicates that CL can serve as an antigen eliciting an immune response. The suppression of production of several cytokines suggests that CL not only affects the NKT cells but also has extensive influence on superordinate immunological processes. Therefore, further studies are needed to elucidate the exact role of CL-binding NKT in the pathogenesis of APS.

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Abbreviations

α -GalCer – α -galactosylceramid
APS – Antiphospholipid syndrome
APCs – Antigen presenting cells
aPL – Antiphospholipid antibodies
anti- β 2GP1– anti- β 2-glycoprotein I
ApoE2 – Apolipoprotein E
CL – Cardiolipin
CD – Cluster of Differentiation
CIA – Collagen-induced arthritis
CNS – Central nervous system
DC – Dendritic Cells
dNKT – diverse Natural Killer T cells
EAE – Experimental autoimmune encephalomyelitis
FasL – Fas ligand
FSC – forward scatter
HLA – Human Leukocyte Antigen
HMB-PP – E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate
ICAM – Intracellular Adhesion Molecule
iGB3 – isoglobotrihexosylceramide
IL – Interleukin
IFN γ – Interferon gamma
 $\gamma\delta$ T cells – Gamma delta T cells
GSL – Glycosphingolipid
LDL – Low Density Lipoprotein
LPC – Lysophosphatidylcholine
LPS – Lipopolysaccharide
MIP-1 α – Macrophage Inflammatory Protein 1 α
MHC – major histocompatibility complex
NAC – N-acetylcysteine
NKT cells – Natural Killer T cells
NO – Nitric oxide
NOS – Nitric oxide synthase

PAMPs – Pathogen-associated molecular patterns
PE – phycoerythrin
PL – Phospholipid
PS – Phosphatidylserine
SLE – Systemic lupus erythematosus
SSC – side scatter
TCR – T cell receptor
TFPI – tissue factor pathway inhibitor
TF – tissue factor
Th cells – T helper cells
TLR4 – Toll-like receptor 4
TNF- α – Tumor necrosis factor alpha
Tregs – regulatory T cells
RPMI medium
VCAM – Vasular Cell Adhesion Molecule

Introduction

Antiphospholipid syndrome

Defintion

The Antiphospholipid syndrome (APS) is an autoimmune disease. It is associated with venous and arterial thrombosis, abortion and premature births in conjunction with preeclampsia or placental insufficiency. Thrombosis can affect any organ, although deep vein thrombosis and cerebral strokes are most frequent. (1)(2)

Over many years, the aetiopathogenesis of APS was unknown and considered to be a part of systemic lupus erythematoses (SLE). In the early 1980s, Graham Hughes defined this antibody-associated disorder a separate disease.(3)

In the following years, the classification criteria for APS were improved and the importance of antiphospholipid antibodies for the diagnosis of APS underlined.

Apart from the occurrence of a thrombotic event or pregnancy complication, the persistence of anti-phospholipid antibodies (lupus anticoagulant, anti- β 2-glycoprotein I, anticardiolipin) are obligatory for the diagnosis of APS. (4)

Autoantibodies in patients with antiphospholipid syndrome

Antiphospholipid antibodies (aPL) are a heterogeneous group of antibodies with the ability to interact with anionic phospholipids, plasma proteins or with protein-phospholipid-complexes. In addition to anti- β 2GP1, aCL other antiphospholipid antibodies such as autoantibodies against phosphatidylserine, phosphatidylinositol, prothrombin, protein C and annexin exist.

In this section, I will illustrate and discuss the most important lipid antigens. (1)(2)

Cardiolipin

Cardiolipin (CL) was first isolated from a bovine heart. It is a phospholipid, which is solely located in the inner membranes of eukaryotic mitochondria, and is involved in various mitochondrial processes, such as ATP production. It also acts as a stabilizer for the cell structure. After the synthesis, acyl chains of CL are modified through three different proteins. This alteration gives it a unique structure that plays a significant role in the presentation as an antigen. Furthermore, it is possible to find CL in the membranes of some bacteria.(5)

CL was discovered as an antigen through the VDRL-test (veneral disease research laboratory) in patients with syphilis. (6)

Beta-2-Glycoprotein-1

β 2-glycoprotein 1 (β 2GP1) is a protein produced by liver cells and is composed of five subunits. It can exist in two different forms. β 2GP1 has a high affinity to bind anionic phospholipids.(7)

The results of research about physiological functions of β 2GP1 are contradictory. Coagulation as well as anticoagulation properties were ascribed to it. Additionally, β 2GP1 seems to play a role in the innate immune system, where it acts as an inhibitor on the complement system. (7)(8)(9)

When closely observed, the circular form of β 2GP1 shows that domain I and V bind together. The epitope is located on the first domain and remains hidden in the circulation form. If β 2GP1 binds on anionic phospholipid surfaces using domain V, the epitope can be recognized by anti- β 2GP1 autoantibodies. (1)

However, it seems that only antibodies against the domain I of β 2GPI correlates well with thrombotic events and pregnancy complications.

Lupus anticoagulant

The lupus anticoagulant (LA) assay detects heterogeneous antibodies, binding phospholipid-protein-complexes. They can prolong the coagulation time, in vitro. The misleading name was derived from its in vitro effects, as it prolongs the activated partial thromboplastin time (aPTT). (10) Phospholipids play a crucial role in the coagulation cascade; they can interact with different clotting factors as well as with LA during coagulation. Therefore, LA acts as a competitive inhibitor of clotting factors and prolongs aPTT in vitro.

However, in vivo LA appears to trigger the coagulation cascade by binding on phospholipid membranes of cells (endothelial cells, monocytes, thrombocytes), which are involved in the clotting process. (11)

Pathogenesis of thrombosis formation

Until today, the complex pathogenesis of APS is not fully understood. Antiphospholipid antibodies isolated from APS patients have been shown to induce thrombosis in mice. (12)(13)

Therefore, it is obvious that antiphospholipid antibodies play a relevant role in the pathogenesis, but the detailed mechanisms have not yet been entirely understood. A possible explanation for thrombosis is the “two-hit” hypothesis.(1) The interaction of autoantibodies with endothelial cells, monocytes and thrombocytes and coagulation factors induce the “first hit” and enhance the thrombogenic condition. (1) An inflammatory second hit (such as infection, trauma, SLE exacerbation) has been suggested to trigger a thrombotic formation (see Figure 1). (1) As already mentioned, β 2GP1 plays a crucial role in the pathogenesis of APS. The β 2GP1 antibody complexes can affect several prothrombotic mechanisms, including cellular factors, coagulation cascade, complement activation and increased oxidative stress. All these effects increase the risk of thrombosis formation.(2)

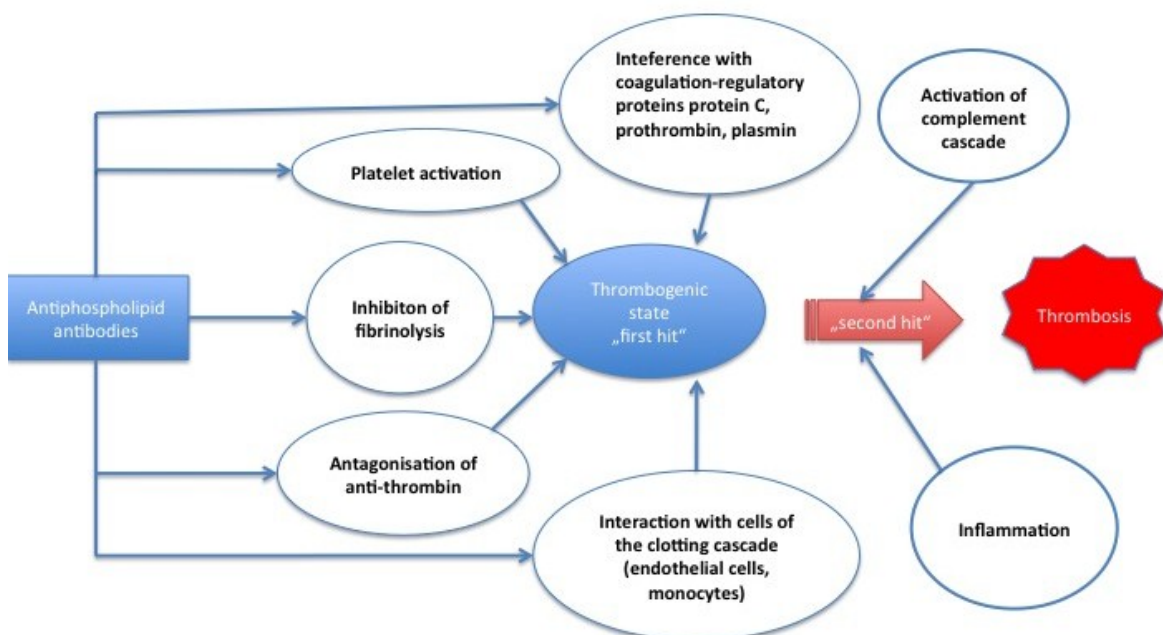


Figure 1 Pathogenesis of antiphospholipid syndrome
(Adapted from Ruiz-Irastorza et al. (2))

Cellular factors

Research from recent years underlines the importance of endothelial cells, thrombocytes, monocytes and the complement system in the development of thrombotic events.

Anti- β 2GP1-complex binds to several proteins such as annexin A2 or TLR4 on the endothelial surface to induce intracellular signals. This, in turn, activates monocytes and endothelial cells. As a result, different adhesion molecules such as ICAM-1, VCAM-2, and E-selectin are released by endothelial cells. In addition, endothelial cells and thrombocytes are able to increase the production of tissue factors. (10) This has a significant influence on the thrombotic formation. (14)

An annexin A2-deficient mouse is resistant against anti- β 2GP1-antibodies from APS patients, whereas annexin A2-positive mice developed thromboembolic events. (15)

Thrombocytes, which are also activated by the anti- β 2GP1-complex increased the amounts of glycoprotein 2b-3a and boosted the synthesis of thromboxan A2. (16)(17) Numerous cases of catastrophic APS indicate the contribution of the complement system, since it was possible to treat the patient with C5 inhibitor eculizumab. (18)

This treatment, based on murine studies, showed that the classical complement cascade is activated in antiphospholipid syndrome.

Pierangeli et al. found out that antiphospholipid antibodies induce the activation of C5a, which, subsequently, bind to neutrophils and stimulate them to release tissue factors. (19)

In conclusion, antiphospholipid antibodies activate monocytes, endothelial cells and thrombocytes leading them to an increased release of adhesive molecules and a synthesis of the tissue factors and thromboxane A2. This process contributes to the development of a procoagulant state. Furthermore, antiphospholipid antibodies interact with several key proteins of secondary hemostasis, which include factor X, protein C, plasmin, tissue factor pathway inhibitor and prothrombin.

On the one hand, the interaction of antibodies inhibits the inactivation of coagulatory factors; on the other hand, they hinder the fibrinolysis. (20)(21)

Generally, endothelial cells contain higher levels of tissue factor pathway inhibitor (TFPI) compared to tissue factor (TF) to prevent coagulation. The exact opposite happens in the case of APS patients. The inhibition of TFPI with a simultaneous increase of TF by monocytes and endothelial cells contributes significantly to the pathogenesis of APS. (22)(23)

It was also ascertained that a decreased function of protein C, which is known as an essential activator for fibrinolysis, combined with the effects of antibodies against tissue plasminogen activators and plasmin, have an inhibitory influence on fibrinolysis. (24)

Annexin A5 is known for its anticoagulant properties, as it forms a protective shield over the phospholipid bilayers and, consequently, disturbs their complex formation with another coagulation factor, such as TF and VIIa. Here as well, the aPL antibodies interact with annexin A5 and impede its protective function.(1) (24)

Rand et al. demonstrated that hydroxychloroquine, an antimalarial drug used as a modulatory medication in SLE, inhibits the anti- β 2GP1-complex to interact with annexin A5 on the surface of endothelial cells. (25)(26)

Oxidative stress

Oxidative stress describes the balance disorder between the production of reactive species (free radicals) and body's ability to detoxify their harmful effects by antioxidants. (27)

Several studies suggest that APS patients have an elevated oxidative stress, which is caused by the imbalance or decrease of antioxidant paraoxonase (protects LDL from oxidation) and elevated 8-epi-prostaglandin F, a marker of lipid peroxidation. (1)(28)

This oxidative imbalance has a crucial influence on the structure and function of β 2GP1. As already mentioned, β 2GP1 consists of four domains, which, in turn, can exist in two different forms. A distinction is made between the disulfide bridges and the thiol groups of the second and fourth domain. The β 2GP1, which contains disulfide bridges, is the oxidized form of a β 2GP1, whereas thiol groups represent a biochemically reduced form.

In persons with APS, oxidized forms occur more frequently, which suggests that reduced β 2GP1 might be the physiological form. The increased oxidative stress in patients with APS seems to be the explanation for the oxidation of β 2GP1. (29)

Moreover, it seems that the oxidation uncovers the B-cell epitope, which is in fact important for the autoimmune processes. This presumption is further supported by a research group, which figured out that purified anti- β 2GP1 antibodies have a decreased affinity to a oxidoreductase-treated (thiol form of β 2GP1) β 2GP1. (30) The relationship of circular forms of β 2GP1 to the free thiol forms have not yet been determined.

Clinical studies demonstrated that smoking counts to the exogenous factors of oxidative stress, and that it can significantly increase the risk of an ischemic stroke in the presence of lupus anticoagulants.(31)

Furthermore, oxidative stress can induce the endothelial cells to increase the expression of annexin A2 on their surface. (32)

The treatment with N-acetylcysteine (NAC) prevents the formation of thrombosis (in a mouse model of thrombosis). NAC is also well known for its properties as a scavenger for reactive oxygen types. These results also support the importance of oxidative stress in the pathogenesis of APS. (33)

Endothelial nitric oxide synthase

Nitric oxide (NO) is produced by nitric oxide synthase (NOS) and is a key factor in the regulation of various biological processes, including vasodilation, vessel permeability, leukocyte adhesion, immigration and proliferation of endothelial cells. In patients with APS, measurements showed lower plasma levels of nitrite than in a healthy group. Decreased plasma concentrations of nitrite are directly linked to a nitric oxide synthase activity, which seems to be disturbed in APS patients. (34)(35)

Ramesh et al. demonstrated in a mouse model that anti- β 2GP1-antibodies inhibit the activity of NOS, followed by a decreased level of NO, which, in turn, provokes an endothelial adhesion of monocytes. As a further consequence, the NO-dependent vessel relaxation is impaired, due to anti- β 2GP1-antibodies. (36)

The apolipoprotein E receptor 2 (ApoE2), a member of the LDL receptor family, is known for its activation by dimerized form of β 2GP1, which results as a consequence of anti- β 2GP1-antibodies binding. The inhibition of NOS is mediated by activation of ApoE2. Thus, anti- β 2GP1-antibodies did not trigger thrombus formation in ApoE2- or NOS-deficient mice.(36)

Clinical manifestation

Thrombosis is the most frequent clinical manifestation of APS. It occurs in venous as well as in arterial systems. APS is associated with deep vein thrombosis and pulmonary embolisms in venous vascular beds, whereas arterial thrombosis mainly affects the central nervous system. (37)

Strokes or transient ischaemic attacks, cognitive dysfunctions, chorea, migraine, vascular dementia, epilepsies and multiple sclerosis-like lesions are the cerebral signs that have been reported by APS patients. (38)

Furthermore, it was shown that patients who were positive in all three assays (antiCL, anti- β 2GPI, lupus anticoagulant) have the highest risk to develop a thrombosis. (39) The occurrence of antiphospholipid antibodies is also associated with cardiovascular complications. Heart valves abnormalities are associated with APS, particularly the mitral and aortic valves (irregular nodular excrescences) are affected. The damage to heart valves occurs due to the deposit of autoantibodies (anti-CL) and complement factors in subendothelial tissues. (40) Further important clinical manifestations that affect the cardiovascular system are the occlusion of coronary vessels and pulmonary hypertension. (40)

Indeed cerebral vascular presentations predominate over cardiovascular events. Another crucial clinical feature is the involvement of small vessels, particularly the vessels of kidney, lungs, retinae and the skin. The kidney involvement can be characterized by the thrombotic microangiopathy with the clinical presentation of the acute renal insufficiency. (41)

In addition, the development of renal hypertension and nephrotic syndrome are also possible. (42) APS patients have an increased risk for obstetric complications. The recurrent miscarriage is the most common manifestation, especially before the tenth week of gestation. At the end of gestation the occurrence of preeclampsia and placental insufficiency are further manifestations of APS. (43)(44)The catastrophic antiphospholipid syndrome is an extremely rare observed form of APS. This type is characterized by multi-organ failure as a consequence of numerous vessel thromboses with high lethality. (45)

Diagnosis and classification

The criteria to define the diagnosis of APS were established at the international APS conference in Sapporo in 1999. The simultaneous presence of clinical events

and a positive verification of antiphospholipid antibodies are essential to diagnose APS. (46) Classification criteria were improved in 2006 (see Table 1), and included two important modifications: the time difference between two positive tests of the antiphospholipid antibodies was prolonged to twelve weeks, and both antibody classes (IgG, IgM) of anti-β2-glycoprotein 1 were included in the laboratory criteria. (47)

Table 1 Revised classification criteria of APS

(Adapted from Miyakis et al. (4))

Clinical criteria of APS	Laboratory criteria
<p>Vascular thrombosis: One or more clinical episodes of arterial, venous or small vessel thrombosis in any tissue or organ.</p> <p>Pregnancy morbidity: One or more unexplained deaths of a morphologically healthy fetus at or beyond the tenth week of gestation, with healthy fetal morphology, documented by ultrasound or by direct examination of the fetus. One or more premature births of a morphologically healthy newborn baby before the 34th week of gestation because of eclampsia or severe preeclampsia defined according to standard definitions or recognized features of placental failure. Three or more unexplained consecutive spontaneous abortions before the tenth week of gestation, with maternal anatomical or hormonal abnormalities and paternal and maternal chromosomal causes excluded.</p>	<p>Lupus anticoagulant present in plasma on two or more occasions at least twelve weeks apart; detected according to the guidelines of the International Society on Thrombosis and Hemostasis (Scientific Subcommittee on lupus anticoagulant/phospholipid-dependent antibodies).</p> <p>AntiCL antibody of IgG or IgM, or both, in serum or plasma, present in medium or high titers (ie, > 40 U/ml or greater than the 99th percentile) on two or more occasions, at least twelve weeks apart, measured by a standardized ELISA.</p> <p>Anti-β2GP1-antibody of IgG or IgM, or both, in serum or plasma (in titers greater than the 99th percentile), present on two or more occasions, at least twelve weeks apart, measured by a standardized ELISA, according to recommended procedures.</p>

The treatment of APS

To avoid thrombotic events in patients with APS, it is recommended to initiate anticoagulation. The results of the retrospective studies recommend a therapeutic INR range (international normalized ratio) from 2.0 to 3.0 for patients with thrombosis, whereas patients with recurrent thrombotic manifestations may need an anticoagulation treatment with a target INR range from 3.0 to 4.0. (2)

Patients with lupus and antiphospholipid antibodies without a thrombotic event appear to have a higher risk for the development of thrombosis. However, recent randomized studies demonstrated that a prophylactic treatment with ASS has no beneficial effect in comparison to the placebo group. (48) Thus, prophylactic treatment with low-molecular weight (LMW) heparin and ASS is recommended in the first trimester of pregnancy.

Immunological background

The immune system and the pathogenesis of autoimmunity

The immune system is a complex network consisting of several cell types and proteins, which have the task of protecting the human body against viruses, bacteria and parasites.

The innate immune system, which consists of macrophages, granulocytes, dendritic cells and the complement system, allows a rapid unspecific immune response. The adaptive immune system includes B- and T cells and specific antibodies. It represents the delayed specific immune reaction.

However, neither of them acts as a separate system but rather as a well-connected network. In addition to a fast and unspecific immune response, the cells of the innate immune system can activate the adaptive system by presenting antigens on their surface.

Failures in the regulation of immune systems can lead to autoimmune diseases. Besides the autoreactive T and B cells, immune cells with regulating properties play an important role in the pathogenesis of an autoimmune disease.

Immune tolerance, consisting of central and peripheral tolerance, is a powerful mechanism, which is able to eliminate autoreactive T cells. (49)

The central tolerance intervenes during the maturation of the T cells in the thymus. T cells, which have a high affinity to self-antigens are removed by the initiation of apoptosis or remain non-active by the induction of energy. Despite the central elimination, autoreactive cells can be found in the peripheral lymphatic organs, the underlying mechanisms of which are not fully understood. The mechanism of the peripheral tolerance has suppressive properties towards autoreactive cells.

A subpopulation of T cells called Tregs (regulatory T cells) belong to the core elements of peripheral tolerance and are characterized by their ability to down-regulate the proliferation of autoreactive effector T cells. (50) Several studies demonstrated that the absence of Tregs induces autoimmune diseases. (50) (51)

Apart from the already-mentioned factors, the genetic components contribute to the emergence of autoimmune diseases.

The occurrence of distinct HLA-subtypes predestines the emergence of some autoimmune diseases. Mutations in the genes encoding for immunoregulatory

proteins (FOXP3, Caspase 10, AIRE) and transcription factors also induce autoimmune disorders.

FOXP3 has a pivotal role as a transcription factor in the development and function of Tregs. (52)(53) A further important factor is the molecular mimicry, an explanation for a cross-reaction of T or B cells against self-antigens due to structural similarities between foreign and self-antigens. Several viruses and bacteria, such as the Epstein-Barr virus or the bacteria *Campylobacter jejuni*, were identified to have molecular similarities with self-antigens. (54)

Besides Tregs, natural killer T cells (NKT cells) are another exceptional subpopulation of T cells, which are also known for their regulatory properties. They were named „natural killer” T cells due to the expression of CD161, a surface marker on natural killer cells. In contrast to conventional T cells, which recognize MHC molecule-presented peptides, the NKT cells recognize lipids as antigens presented by CD1d molecules. A further hallmark of NKT cells is their ability to produce a range of various cytokines upon antigenic recognition, which gives them immunomodulatory potential. It is assumed that NKT cells have a beneficial role in several autoimmune disorders. (55)

Analogous to mouse studies, in which NKT cells suppress the progression of autoimmune diseases, there are human studies that showed a decreased number of NKT cells in the peripheral blood of humans in various autoimmune diseases. (56) There is also evidence that NKT cells are involved in the pathogenesis of lupus (57)(58). In the following course of my thesis, I will introduce the properties and functions of NKT cells.

CD1 molecules

CD1d and other members of the CD1 family

CD1d proteins have a structural unit that resembles MHC I molecules, but they exhibit a monomorphic conserved form and are part of the big CD1 family, which contains CD1a, CD1b, CD1c, CD1d and CD1e molecules. (59) (60)

The crystal structure analysis of the CD1 molecule reveals that the CD1d complex consists of four domains (α 1- α 3, and β 2-Microglobulin). (61) Lipid antigens presented by CD1d, cannot be recognized by conventional T cells, whereas the

CD1d-restricted T cells can recognize distinct lipids on the surface of the CD1d. (62) (63)(64)

The unique composition of CD1d forms a groove with hydrophobic components, which can provide an appropriate binding site for the lipid antigens. The antigen-binding surface can be divided into two different regions called A' and F' channels. In contrast to MHC, these channels are distinctly larger and can accommodate two alkyl chains of lipids. (65)

One of the first identified lipids that binds to CD1d and is recognized by NKT cells was the glycosphingolipid α -galactosylceramid (α -GalCer). This potent glycolipid was extracted from a marine sponge sample, and can accommodate with both alkyl chains in the channels, whereas the sugar head group protrudes from the surface and can be recognized by the TCR of the NKT cells. The presentation of this glycosphingolipid by the dendritic cells rapidly activates NKT cells. (66)

Since the discovery of NKT cells, several lipids were identified as antigens of NKT cells. (59)

CD1d antigen processing and presentation

Just like MHC molecules, CD1d is expressed by different antigen-presenting cells (APCs) such as dendritic cells, macrophages and B cells. (67)(68) Compared to other APCs, the monocyte-derived DCs seem to be the most potent stimulators of the NKT cells. (69)

The non-covalent binding of β 2-microglobulin and the loading of endogenous lipids take place in the endoplasmic reticulum. The golgi apparatus, subsequently, transports the complex to the surface of the plasma membrane.

Through the tyrosine-based motif on the cytoplasmic tail of the CD1d molecule, it is possible to transport CD1d via clathrin-coated pits back to the cytoplasm. (70)

Afterwards, the CD1d molecules can move into the acid endosome compartment and exchange their endogenous lipid with an exogenous lipid antigen in the presence of saposin proteins. Newly loaded CD1d molecules are transported via lysosomes back to the surface for an antigen presentation. (71)(72)

CD1d-restricted cells

NKT cells

For a long time, peptides were the only well-known antigens for the conventional T cells. The T cells receptor (TCR) of conventional T cells is a heterodimeric protein, which is characterized by its highly variable α - β - chains. Due to the enormous variability in combinations of the $V\alpha$ - and $V\beta$ conventional T cells recognize various peptide antigens presented by MHC proteins. (73)

The discovery of the T cell population, which expresses an $V\alpha 14J\alpha 18$ invariant TCR and a natural killer cell marker CD161 (also known as NK1.1) in C57BL/6 mice marks an important milestone in the research of antigen processing and presentation. Besides their invariant TCR, the discovered subpopulation of T cells recognized lipid antigens. Their T cell receptors are composed of invariant $V\alpha 24J\alpha 18$ chains (in mice $V\alpha 14J\alpha 18$), which paired with a $V\beta 11$ chain. The mice have more varieties of $V\beta$ chains ($V\beta 2$, $V\beta 7$, $V\beta 8.1$, $V\beta 8.2$, $V\beta 8.3$) than humans. (74) Further examination demonstrated that NKT cells were CD1d-restricted and able to produce high levels of various cytokines. (75)(76)

As already mentioned, the recognition of α -galactosylceramid (α -GalCer), extracted from a marine sponge, demonstrated that NKT cells are able to bind on lipid antigens. This discovery was a milestone in the research of NKT cells because it made it possible to load a CD1d tetramer (see Figure 4) with α -GalCer to study the immunological properties of NKT cells. (76)(77)(78) A tetramer is a fluorescently labelled reagent, which is composed of four lipid-loaded CD1d proteins and used to detect NKT cells.

In contrast to invariant NKT cells, which have an invariant TCR α -chain, there is another population with diverse α - and β -chains that can also recognize lipids in a CD1d-restricted manner. This subpopulation, called diverse (dNKT) or type II NKT cells, is an important component of the current research. However, dNKT cells are unable to recognize α -GalCer. (79)

On this basis, it is difficult to study dNKT cells. Moreover, little is known about the functions and their contribution to our immune system.

Table 2 provides an overview of the different CD1d restricted T cells.

Table 2 Classification of CD1d-restricted T cell subgroups.

	Type I NKT cells	Type II NKT cells	$\gamma\delta$ T cells
TCR repertoire	Mouse: V α 14J α 18, V β 2, V β 7, V β 8 Human: V α 24J α 18, V β 11	Mouse and human: variable TCR	Mouse and human: $\gamma\delta$ TCR
α-GalCer recognition	all	no	some
Other lipid antigens	Microbial and mammalian glycosphingolipids	Microbial and mammalian phospholipids	Mammalian and pollen phospholipids
Function	Th1 and Th2 cytokines Cytotoxic effects	Th1 and Th2 cytokines Cytotoxic effects	Th1 and Th2 cytokines

The subsets of the NKT cells

The most important difference between type I NKT- (iNKTs) and type II NKT cells is their variability in the TCR expression. It is additionally possible to subdivide them by their expression of CD4 and CD8. In humans, the iNKTs can be either CD4+ or CD4-CD8- (double negative DN) and CD4-CD8+. (77) (80) (81)

Several studies showed that the production of cytokines depends on the expression of CD8 and CD4 markers, and suggests that the subsets of iNKT cells can activate either a Th1- or Th2-immune response. CD4+ iNKTs are able to produce cytokines of Th1- and Th2-cells, which contain IL-4 and IFN- γ , whereas the double negative iNKTs were capable of secreting cytokines of Th1 type. (77) (81)

CD4-CD8+ iNKT cells appear to be able to produce more IFN- γ and have more cytotoxic effects than CD4+ or CD4-CD8-subsets.

The subsets of type II NKT cells (dNKT) are not well described; however, there is a CD4+ type II NKT subpopulation which is able to prevent the development of induced type I diabetes in the mouse model. (82)

Besides the expression of CD4 and CD8 markers, the tissue location influences the function of NKT cells. Crowed et al. demonstrated that liver-derived CD4-iNKT cells were able to reduce the appearance of the methylcholanthrene (MCA)-

induced sarcomas, whereas the thymus or spleen-derived iNKT cells did not display adequate tumor surveillance. (83)

$\gamma\delta$ T cells

Another T cell population, which responds in a Cd1d-restricted manner, is the $\gamma\delta$ T cell. (84) In 1986, the T cells rearranging γ and δ receptor genes were described in humans for the first time. Since this discovery, several studies have indicated that these cells have an array of different functions, including the innate and the adaptive immune system. $\gamma\delta$ T cells appear to be a minor population in lymphoid tissue and in the peripheral blood. However, they are enriched in diverse organs, such as the skin and intestinal mucosa.

In contrast to conventional T cells, which only interact with MHC molecules, the $\gamma\delta$ T cells are able to recognize either MHC ligands or MHC-related molecules, such as Cd1d, which present lipid ligands. (85)

Furthermore, they recognize heat shock proteins and can strongly be activated by non-peptidic phospho-antigens, such as (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP). (61) Approximately two to ten % of the peripheral blood T cells are $\gamma\delta$ T cells and most of them express V γ 9V δ 2-TCRs, a subpopulation with a high affinity to HMB-PP. (86) (87)

Phospho-antigens are also metabolic intermediates of bacteria and parasites, and activate $\gamma\delta$ T cells, which respond with a secretion of proinflammatory cytokines.

Moreover, they have been shown to kill tumor cells. (88)

The findings from current research show that murine $\gamma\delta$ T cells are capable of recognizing phospholipids in a CD1d-dependent manner, including CL. (89)(90)

The activation of the NKT cells.

NKT cells have been shown to have protective as well as harmful effects. These functions include tumor suppression, protection against bacterial and viral infections, prevention of graft-versus-host reaction as well as regulation of autoimmune disease (91) (92) One of the remarkable attributes of the NKT cells is their rapid cytokine production after antigen interaction. Within one to two hours,

NKT cells are able to release Th1-type cytokines, such as IFN γ , TNF- α , as well as Th2-type IL-4 and IL-13. (93)(94)(95)

The activation of the NKT cells can be initiated in different ways.

Besides the activation by TCR-CD1d-lipid interaction, the NKT cells can be activated through the innate receptors, such as NKG2D or by cytokines. (96)

The TCR-CD1d-lipid interaction is the direct way to recognize glucosphingolipid (GSL) and diacylglycerol antigens in a CD1d-dependent manner. These lipid antigens are part of the membrane of gram-negative bacteria, such as *Sphingomonas*, *Ehrlichia* and *Borrelia burgdorferi*. (97)(98)(99)

Due to the lack of lipopolysaccharide (LPS) of some gram-negative bacteria, it has been suggested that the lipid antigens serve as compensation for LPS, to activate the NKT cells. (92) Other LPS-positive bacteria, such as *Salmonella typhimurium*, are able to activate NKT cells by an endogenous glucosphingolipid, isoglobotrihexosylceramide (iGB3), which is expressed by LPS-activated dendritic cells. (97) LPS can modulate the endogenous lipid expression in APC by either increasing their synthesis or inhibiting the downregulation of CD1d. (100)(101) During a bacterial or viral infection, APCs are also able to activate NKT cells by releasing cytokines, including IL-12, IL-18, and IL-23. This kind of activation is independent of the CD1d-lipid-interaction. (102)(103)

NKT cells lipid antigens

The conserved structure of the CD1d molecule allows it to bind to a wide variety of lipid-based antigens, such as glycosphingolipids (GSL) and phospholipids (PL).

The difference between GSLs and PLs is that the GSLs contains sugar head groups, whereas PLs are glycerol-based lipids (see Figure 2). (92)

The further classification of the glycolipid ligands is the linkage between the sugar molecule and the lipid tails. Bacterial lipid ligands always have an α -linkage between the glycolipid and the lipid backbone. In comparison, mammalian ligands are not capable of attaching to the sugar head group in α -orientation. The α -linkage is, therefore, an important structural motif for foreign antigenicity upon the NKT cells lipid antigens. (92)

α -linked microbial lipid antigens were derived from *Sphingomonas*

(α -galactosyldiacylglycerol), *Borrelia burgdorferi* and *Streptococcus pneumonia*

(α -glucosyldiacylglycerol). (97) (98) (99)(104)

NKT cells can also be activated by APCs in the absence of foreign lipid antigens. (101) (102) As this activation cannot be carried out without lipid-CD1d interaction, it is suggested that a self-lipid antigen can promote NKT activation. Also, the activation of NKT cells during viral infections, cancer and autoimmune diseases support the hypothesis of endogenous ligands.

Sulfatide was the first discovered glycolipid that was able to activate type II NKT cells. Moreover, sulfatide promoted a protective reaction against the development of experimental autoimmune encephalomyelitis (EAE). (105)

The next remarkable achievement was the discovery of lysophosphatidylcholine (LPC) that was able to bind to CD1d. Furthermore, Chang et al. demonstrated that LPC-CD1d-restricted cells were predominantly negative for V α 24 V β 11-TCR, which also indicated that these cells belong to type II NKT cells. Additionally, LPC-CD1d-restricted cells were isolated from human peripheral blood and were increased in multiple myeloma patients (106)

Other phospholipids, including phosphatidylethanolamine and phosphatidylcholine derived from pollen, were able to activate CD1d-restricted T cells.(107)

Based on the recent scientific evidence about CD1d ligands, Cox et al. demonstrated that diverse lipid antigens, including CL bind to human CD1d. (89) In contrast to other diacylated lipid ligands, CLs are tetradecyl dimers of phosphatidylglycerol and it seemed unlikely that they will be the appropriate antigen. However, Cox and colleagues displayed, by the use of mass spectrometry, that CL is a natural human CD1d ligand.(89)

The crystal structure analyses of CL by Dieudé and colleagues confirmed the data. They found out that two of the four alkyl chains are located in the A' and F' channels, and that the other two alkyl chains are not particularly important for TCR-binding because these two alkyl chains are exposed into the solvent and have no contact to a CD1d molecule. Similar to glycolipid antigens, the phosphate-glycerolphosphate, the hydrophilic part of CL, is located above the binding groove.(90)

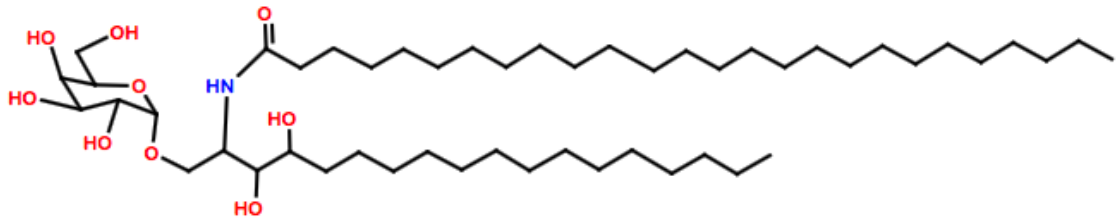
The polar part of CL seems to be important for the recognition by NKT cells. (90)

The findings by Tatituri et al. underline the fact that CL can activate specific T cells in CD1d-dependent manner. They isolated CL and other phospholipids from mycobacterium tuberculosis or corynebacterium glutamicum and presented them

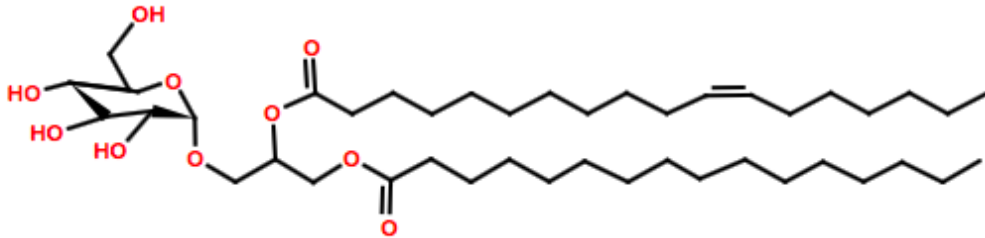
to a different type II NKT hybridoma.

Upon the interaction with CL-stimulated APCs, distinct hybridomas were able to secrete IL-2. (108) Interestingly, the 14S.10 type II NKT hybridoma recognized polar lipids, such as CL, and showed no reaction to apolar lipids, whereas 431.A11 type II NKT hybridoma did not respond to polar, but to apolar lipids. This suggests that the variety of the TCR receptors plays an important role in the recognition of diverse phospholipids. (108)

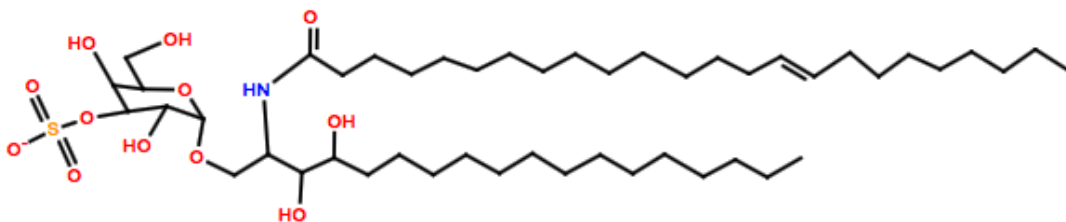
A



B



C



D

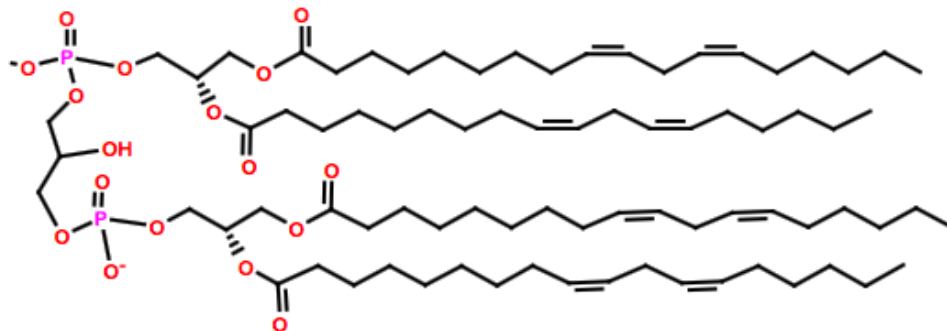


Figure 2 Lipid antigens of NKT cells

Shown are the chemical structures of various antigens for NKT cells. Similar for all antigens are their structure, which consists a hydrophobic lipid tail and a hydrophilic head.

A) α -galactosylceramid is the prototypical antigen of iNKT cells consisting of a lipid backbone (ceramide) and α -linked sugar head group.

B) α -galactosyldiacylglycerol is a microbial lipid antigen and also stimulates iNKT cells.

C) Sulfatide is an endogenous lipid ligand of type II NKT cells and occurs in mammalian CNS.

D) CL is a further lipid antigen, which can be recognized by type II NKT cells and $\gamma\delta$ T cells.

Functional properties of the NKT Cells

The remarkable properties of NKT cells are their ability to produce large amounts of cytokines, including IFN- γ , IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, as well as TNF- α , TGF- β and GM-CSF. (77) (92)(109)(110)(111)

Apart from the production of cytokines, they can also produce high levels of different chemokines, such as RANTES, Eotaxin, LT, MIP-1 α .(112)

Interestingly, the activation of the NKT cells with α -GalCer induces them to secrete both types of Th1- and Th2-type of cytokines simultaneously, such as IFN- γ and IL-4. (78)

In contrast, IL-12 or TLR ligands can promote the NKT cells to stimulate the Th1-helper activation without the activation of the Th2-helper. (102)(113)

Furthermore, different lipid antigens can also modulate a polarized NKT activation. Structurally modified α -GalCer varieties activate NKT cells and lead them to produce either Th1- or Th2-type cytokines. (114)(115)

The creation of J α 18 (a part of the type I NKT TCR receptor) and CD1d-deficient mice (both types affected) allows for a study of the effects and the differences between type I and type II NKT cells. In summary, it can be demonstrated that in some cases, both types of NKT cells have comparable functions, and in the other settings they have strong antagonistic properties. Furthermore, they can influence each other. (116) (117)(118)

Besides the cytokine production, they exert cytotoxic effects, which give them the ability to kill tumor or infected cells directly. The production of high levels of perforin, granzyme B and FasL confirmed their cytotoxic properties. (119)

NKT cells also play a crucial role in autoimmune diseases where they can have protective as well as pathologic functions.

Chiba et al. showed in the collagen-induced arthritis (CIA) that NKT-deficient mice (J α 281- and CD1d knockout mice) developed less severe rheumatoid arthritis than the wild type. (120) In another study, it was observed that an addition of α -

GalCer aggravated rheumatoid arthritis, whereas CD1d blocking alleviated the symptoms. (121)(122)

However, a study in humans revealed contradictory results with a decreased number of blood circulatory iNKT cells. (123)

Therefore, further research is needed to clarify the role of NKT cells in RA.

The involvement of the type II NKT cells in autoimmune diseases was first described in the mouse model of experimental autoimmune encephalomyelitis (EAE). Jahng et al. found out that sulfatide, a myelin-derived lipid, can activate non-invariant NKT cells in a CD1d-dependent manner. Further, they showed that these sulfatide-reactive T cells were present the CNS during the inflammatory reaction. Surprisingly, the sulfatide substitution completely prevented the mice from developing an EAE in contrast to CD1d-deficient mice. (105)

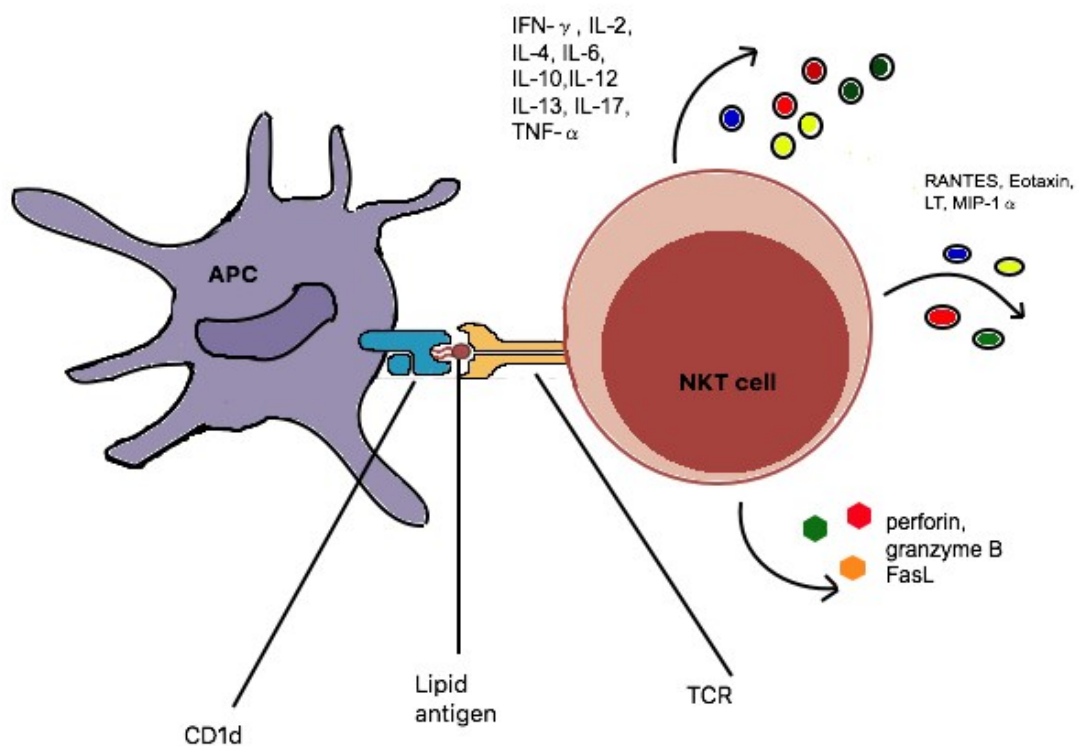


Figure 3 CD1d-mediated activation of NKT cells

The role of NKT cells in SLE and in the antiphospholipid syndrome

Systemic lupus erythematosus (SLE) is a complex autoimmune disease associated with an uncontrolled formation of antibodies by autoreactive B cells against self-antigens, followed by an immune-mediated destruction of several organs, including nephritis, vasculitis, serositis or vasculopathy, such as the antiphospholipid syndrome. (124)

It is well known that several T cell subsets (CD4+, TCR $\alpha\beta$ + DN or $\gamma\delta$ + T cells) can promote the activation of auto-reactive B cells.(125) (126)(127)

Until today, the exact pathogenesis of SLE is unknown.

Yet, several studies demonstrated that type I NKT cells seem to have a regulatory role in the pathogenesis of SLE. Of particular interest is their inhibition of auto-reactive B cells to produce antibody against self-antigens. (128)

Yang et al. showed that the genetically susceptible lupus mice (BWF1 mouse) with CD1d deficiency have more severe nephritis than BWF1 mice without CD1d deficiency. (57)(58) Additionally, another study revealed that aged iNKT cell-deficient mice ($J\alpha 18^{-/-}$) spontaneously developed lupus-like nephritis. These aged mice produced antibodies, such as anti-ds DNA and have proteinuria. (129)

These studies support the regulatory properties of type I NKT cells in SLE, which can suppress the production of autoantibodies, such as anti-ds DNA. Interestingly, Yang et al. found out recently that CD1d-deficient as well as $\beta 2$ microglobulin-deficient ($\beta 2m^{\circ}$) BWF1 mice have increased serum levels of autoantibodies. In contrast, anti-CL antibodies were significantly reduced compared to the CD1d and the $\beta 2m$ -positive BWF1 mice. (58) Further experiments revealed that type I NKT cells have no influence on the production of anti-CL antibodies. (58)

The decreased levels of anti-CL antibodies might be explained by the absence of anti-CL antibody-producing B cells. However, Yang et al. displayed that CD1d-negative BWF1 mice still have autoreactive B cells with the ability to produce anti-CL antibodies, which was demonstrated by the addition of LPS. (58) This suggests that CD1d-reactive T cells might promote the activation of anti-CL antibody-producing B cells, since it is a known fact that NKT cells have the competence to regulate the activation of autoreactive B cells in a CD1d-dependent manner. (128)(130)

Aim and hypothesis

This study aims at providing a better understanding of the role of NKT cells in the pathogenesis of antiphospholipid syndrome.

Recently published mouse studies suggest that CL-binding T cells could have a relevant role in the anti-CL antibody production. However, there have not yet been any studies, which identified CL-binding T cells in humans.

Accordingly, we formed the hypothesis that CL-binding T cells could also occur in humans. Therefore, we will examine human peripheral blood to identify and characterize such CL-recognizing T cells.

Further, we will investigate, whether there are differences between APS patient and healthy individuals with respect to the presence or frequency of CL-binding T cells. In addition, we will compare the phenotypic properties between the two study groups.

Compared to Type II NKT cells, Type I NKT cells are nowadays relatively well researched and known for their activation upon antigen stimulation. Similar results were described in mouse studies with regard to CL-binding T cells.

For that reason, we hypothesized, that CL could serve as an antigen and activates these Type of NKT cells.

Materials and methods

Patients

In a prospective study, eight female patients with the diagnosis of an antiphospholipid syndrome, based on the 2006 Sydney classification criteria, and eleven healthy females were included to investigate the involvement of CD1d restricted T cells in the pathogenesis of an antiphospholipid syndrome.

Official permission to perform this study was given by the Institutional Review Board of the Medical University of Graz, and a written consent to participate was obtained.

Table 3 Patients characteristics

	APS (n=8)	HC (n=11)
Age [years]	53,38 (\pm 9,59)	43,18 (\pm 16,25)
Sex, Female, n (%)	100	100
Anti-CL antibody IgG	IgG: 51,36 (\pm 44,49)	n.d.
Anti-CL antibody IgM	IgM: 24,36(\pm 37,78)	n.d.
CumarineTherapy n (%):	100	n.d.

Cell preparation

Separation of peripheral blood mononuclear cells (PBMCs)

This method serves primarily for the isolation of peripheral blood mononuclear cells PBMCs from the peripheral venous blood. Histopaque (Sigma-Aldrich) was used as a separation medium during the centrifugation. Cells with the density lower than 1077 g/ml (lymphocytes, monocytes) cannot pass the Histopaque medium and form a thin layer (buffy coat) between the erythrocytes and the blood plasma.

As a first step, 15 ml of Histopaque were filled in a 50 ml tube, and were gently overlaid with 35 ml of in blood diluted in PBS (phosphate buffer saline).

The lymphocytes formed a buffy coat after the centrifugation (1870 RPM, 25 minutes, no break) and were collected. Thereafter, PBMCs were washed twice

with PBS (1500 RPM for five min.) to remove the remaining Histopaque. Finally, the number of cells was determined with the help of a cell counter. (Beckmann Coulter, Brea, California, USA)

Antibody staining and flow cytometry analysis

Table 4 Antibodies for surface and intracellular staining

Antibodies	Antibody-dye	Order number	Company	volume [μ l]
CD3	APC Cy7	341110	BD*	5
CD4	V500	560768	BD	5
CD8	V450	560347	BD	5
CD19	APC	560353	BD	5
CD19	AF488	530199	BD	5
CD25	APC	555434	BD	20
CD28	PerCP	560347	BD	5
CD38	PE Cy7	335825	BD	5
CD45RO	APC	559865	BD	20
CD69	PE Cy7	335792	BD	5
CD161	FITC	556080	BD	20
Ki67	AF488	558616	BD	5
Anti iNKT	APC	130094839	Miltenyi*	10
$\gamma\delta$ TCR	PE Cy7	655410	BD	5

*Becton Dickinson, Franklin Lakes, New Jersey, USA

*Miltenyi Biotec, Bergisch Gladbach, NRW, Germany

Detection of CD1d-tetramer-positive cells

CD1d tetramer is a synthesized protein complex with four CD1d interconnected and fluorescently labelled molecules, which can be loaded with a lipid antigen to identify and characterize the CD1d-restricted T cells (see Figure 4). Several

studies used α GalCer-loaded tetramers to identify iNKT cells, however, we used CL-loaded CD1d proteins, generously provided by Dirk M Zajonc. (La Jolla Institute for Allergy and Immunology, CA, United States)

The tetramerization of CL-loaded CD1d molecules and the labelling of phycoerythrin (PE) were performed in our laboratories.

Additionally, unloaded-CD1d tetramers were used as a negative control.

To detect and to describe the phenotypic properties of NKT cells, isolated PBMCs of APS patients, as well as healthy persons, were stained with fluorochrome labelled antibodies and CD1d-tetramers.

The first staining was performed to identify CD1d-restricted cells, and contained, besides CD1d-CL-tetramers (PE), two unloaded CD1d-tetramers (PE Cy7, V450) to demonstrate that CD1d-CL-tetramers work antigen-specific. The purified lymphocytes (3×10^6 cells) were re-suspended in 100 μ l PBS, and were treated with the CD1d-tetramers. After ten minutes of incubation at room temperature, the cells were stained with further antibodies (CD19 (AF488), CD45RO (APC), CD3 (APC Cy7), CD4 (V500) for 20 minutes at 4° C.

As the next step, the cells were washed twice with 1 ml PBS and re-suspended in 300 μ l PBS, and were analysed by a flow cytometer (FACS Canto II, BD Bioscience).

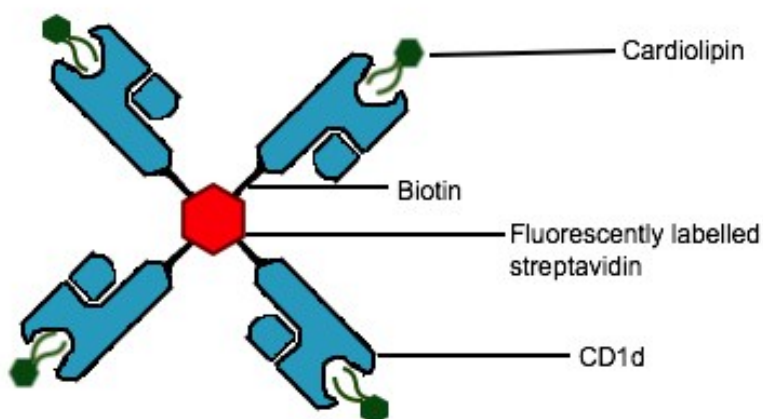


Figure 4 Structure of CL-loaded CD1d tetramer

Phenotypic description of CD1d-restricted T cells

The second staining was performed to describe the cell surface markers of CD1d-restricted cells. Therefore, in addition to CD1d-CL-tetramers (PE), the lymphocytes (3×10^6 cells) were stained with several surface antibodies: CD3 (APC Cy7), CD4 (V500), CD19 (APC), CD8 (V450), CD69 (PE Cy7), CD161 (FITC), CD28 (PerCP).

Stimulating of CD1d-restricted cells

Preparation and dissolving of CL

CL (25 mg/mL, Avanti Polar Lipids, Alabaster, USA) derived from a bovine heart was dissolved in chloroform, and, subsequently, evaporated in a current of nitrogen.

At first, 100 μ L of CL (25 mg/ml) were pipetted into a glass tube. After that, the CL-containing tubes were put into the evaporator, and were exposed to a current of nitrogen for 15 minutes. The crystallized CL can be used for stimulation assays and was stored at -20° C until the date of utilisation.

The crystallized CL was resuspended in 2.5 ml RPMI 1640 medium (Roswell Park Memorial Institute medium, Sigma-Aldrich) to get the desired concentration of 1 mg/ml. As a second step, the tube with re-suspended CL was treated in an ultrasonic bath at 37° C for 20 minutes.

Stimulation of PBMCs with CL

5×10^6 PBMCs cells (4×10^5 cells/200 μ L/well) were cultured in a 96 well plate with three different approaches. RPMI 1640 (Sigma-Aldrich) including 2mM L-glutamine, 10 % FBS (fetal bovine serum), streptomycin (100 μ g/ml) and penicillin (100 IE/ml) were used as culture medium. Furthermore, the PBMCs were cultured for 24, 48 and 72 hours.

The cells were treated with IL-2 only or with IL-2 and 10 μ L (1 mg/mL) of CL dissolved in RPMI 1640. PBMCs stimulated with anti-CD3/CD28 antibody-coated beads (MoAb) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) served

as a positive control, to demonstrate the activation of T cells. After the treatment, the cells were cultured for 24, 48 and 72 hours in the incubator (5% CO₂) at 37° C. For 48- and 72-hour cultures the supplementation of the reagents were repeated every 24 hours.

Intracellular staining

After the respective cultivation, the cells were harvested and prepared for intracellular staining. The supernatants were also collected and stored for cytokine measurements at -20° C.

Before the intracellular staining, the harvested cells were washed twice, subsequently re-suspended in 100 µL PBS and were treated with the CD1d-tetramers. After 10 minutes of incubation with CD1d-CL-tetramer (PE) at room temperature, the cells were stained with following surface markers: CD38 (PE Cy7), CD25 (APC), CD3 (APC Cy7), CD19 (V450), CD4 (V500). After 20 min of incubation at 4° C, the fixation/permeabilization solution (Intracellular Protein Staining Buffer Set, eBioscience) was used to fix and permeabilize the cells, which, in turn, facilitates the staining of intracellular proteins, such as Ki-67. After the supplementation of 1 ml fixation/permeabilization solution, cells were incubated for further 30 minutes at 4° C.

As the next step, the cells were washed twice with a permeabilization buffer, followed by a resuspension in 100 µL perm buffer (eBioscience).

Anti-Ki-67 (AF488) was used to stain the intracellular protein Ki-67, which is associated with cell proliferation.(131)

After a further 30 minute incubation at room temperature and the next washing step, the cells were resuspended in a 250 µl flow cytometry staining buffer (eBioscience).. Finally, the cells were analyzed by a flow cytometer (FACS Canto II, BD Bioscience).

Measurement of cytokine secretion

ProcartaPlex Multiplex Immunoassay (eBioscience, San Diego, USA) was performed to analyze the cytokine levels in supernatants. The multiplex immunoassay allows the detection of several different reagents (antigens, receptors, antibodies) simultaneously from a single sample.

This method involves up to 100 different colored magnetic beads, based on two various fluorescent colors (red and infrared). The different concentration of these colors allows the generation of 100 various beads.

The multiplex immunoassay is based on a double antibody sandwich method. Each bead can be conjugated with an antigen-specific antibody. Subsequently, these antibody-coupled beads are incubated with standard antigens and the supernatants' samples. The biotinylated antigen-specific antibodies are added to detect the antigens. Streptavidin-conjugated phycoerythrin (PE) is attached to the detecting antibody and serves as second fluorescent dye.

Thus, it is possible to determine various analytes and their concentration simultaneously from a single sample.

The detection of the prepared antigen-antibody sandwich complexes was performed with MAGPIX® system (Luminex Corporation, Austin, USA), which is based on the procedure of fluorescence imaging. MAGPIX® uses light-emitting diodes and a CCD camera to detect the analytes, instead of a flow cytometer-based method.

In our approach, we examined the levels of distinct cytokines (Table 2) in the supernatants of the CL-treated and the untreated cells. The supernatants of the cells, which were treated with anti-CD3/CD28 antibody-coated beads, served as a positive control.

Table 5 Determined cytokines

Measured Cytokines	ULOQ = upper limit of quantification (pg/ml)	LLOQ = lower limit of quantification (pg/ml)
GM-CSF	73300	18
Granenzyme B	7700	30
IFN-gamma	11075	11
IL-2	17800	4,35
IL-4	45200	11
IL-6	10850	11
IL-10	1200	2,49
IL-17A	9300	2,27
IL-21	43200	11
TNF- α	28600	6,98

Determination of apoptosis upon CL treatment via annexin V staining

Annexin V staining (eBioscience, San Diego, CA, USA) protocol was performed to determine, whether exposure of CL to PBMC's can induce cell apoptosis or necrosis. Annexins are a group of proteins characterized by their calcium-dependent affinity to membrane phospholipids such as phosphatidylserine (PS). Under physiological conditions, PS is located on the inner side of the cell membrane. Apoptosis provokes a translocation of PS from the cytosolic side to the extracellular surface. By means of fluorescently-labeled annexin V, it is possible to detect extracellular located PS and hence the determination of apoptosis.

At first, 12 wells with 4×10^5 cells/200 μ L/well were cultured in a 96 well plate for 24 hours using RPMI medium. To induce apoptosis, PBMCs were treated with heat (at 60° C for 5minutes), which serves as a positive control, whereas remaining cells were either treated with 10 μ L and 20 μ L CL (1 mg/mL) or stayed untreated (negative control).

After 24 hours of cultivation in the incubator (5% CO₂) at 37°, the harvested cells were washed with PBS, subsequently re-suspended in 100 μ L PBS and were stained with anti-CD3 antibodies (APC Cy7). After 20 minutes of incubation at 4°

C, the cells were washed once in PBS (2ml), then once in binding buffer (eBioscience) (1ml), subsequently the cells were re-suspended in 200 µL binding buffer and were treated with 8 µL of fluorochrome-labelled Annexin V (FITC) , followed by light-protected incubation for 10 minutes. After the incubation, cells were washed with 2ml binding buffer and were re-suspended in 200 µL of binding buffer. Finally, cells were treated with 4 µL of Propidium Iodide Staining Solution (Pe Cy7) and analyzed via flow cytometer.

Software

All flow cytometer results were analysed with FlowJo Version 10.0.7r2 (Ashland, OR, USA) and BD FACSDiva (Becton Dickinson). Determination of cytokine levels were performed with xPONENT Software (Luminex Corporation, Austin, Texas, USA).

Statistics

SPSS (V.23 (Chicago, IL, USA) were performed to analyze the statistical outcomes.

The statistical results were presented either as range and median or mean and standard deviation. Mann-Whitney-Wilcoxon-Test (non-parametric test) and Two-sample T- test were performed for unpaired samples. Paired samples were compared with paired t-test or the Wilcoxon test.

Spearman's rank correlation coefficient was used to evaluate the correlation between variables.

Results

Establishment of staining

During the establishment and characterization of CL-binding NKT cells, we used healthy test persons as well as APS patients.

At first, it was important to identify and characterize the CL-binding T cells. Therefore, the cells were simultaneously stained for CL-loaded CD1d-tetramers and unloaded CD1d tetramers.

Unloaded CD1d-tetramer served as a negative control, since it does not contain a ligand and cannot bind to NKT cells

At the beginning, the lymphocyte populations were selected in the side scatter (SSC) versus the forward scatter (FSC) density plot, followed by the gating out of, doublets and cellular debris. As a next step, CD3-positive cells (T cell marker) were gated and examined with regards to their affinity to a CL-loaded CD1d-tetramer (CL-tetramer) and an unloaded CD1d tetramer (see Figure 5A-B).

As shown in figure 5C-D, CL-loaded CD1d-tetramers bound more cells than the unloaded tetramer. This observation was repeated and proved in several experiments. (Figure 6)

The number of measured events was stated as percentage of the total T cell population. 0,028 [\pm 0.01] percent of the T cells were able to recognize the CL-loaded CD1d-tetramers, whereas the unloaded tetramer bound significantly lower levels of T cells (0.012 % [\pm 0.004], $p = 0.001$).

Additionally, unloaded tetramers did not bind to CL NKT cells indicating that this newly discovered T cell subgroup specifically binds CL. (Figure 7)

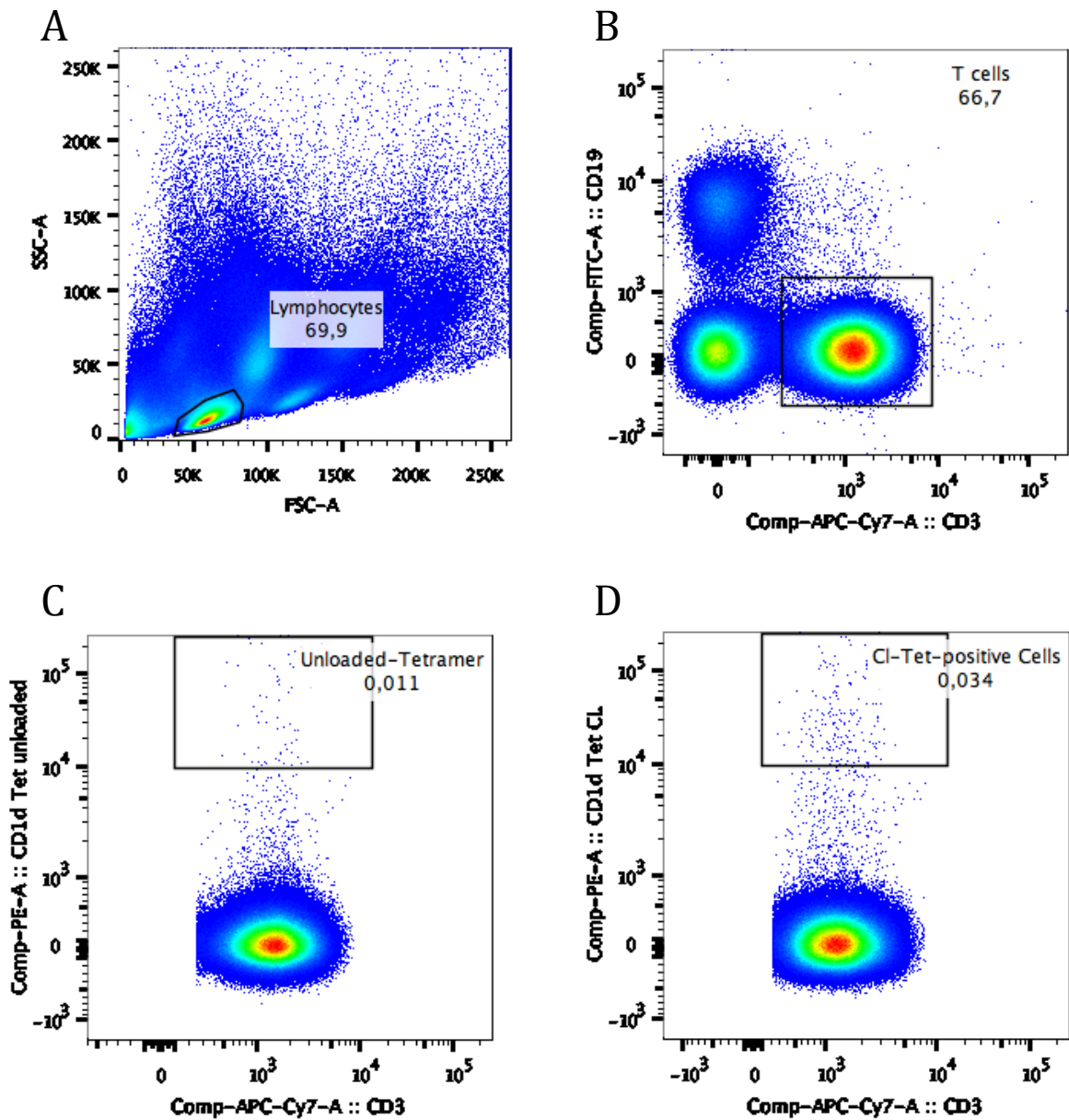


Figure 5 Gating strategy for flow cytometric analysis of CL-binding NKT cells

(A-B) Lymphocytes were first gated in the SSC vs FSC density plot, followed by anti-CD19 and anti-CD3 staining. Subsequently, B cells (CD19) were gated out, before the CD1d tetramer vs CD3 plotting.

(C-D) Comparison of binding between CL-loaded and unloaded tetramers.

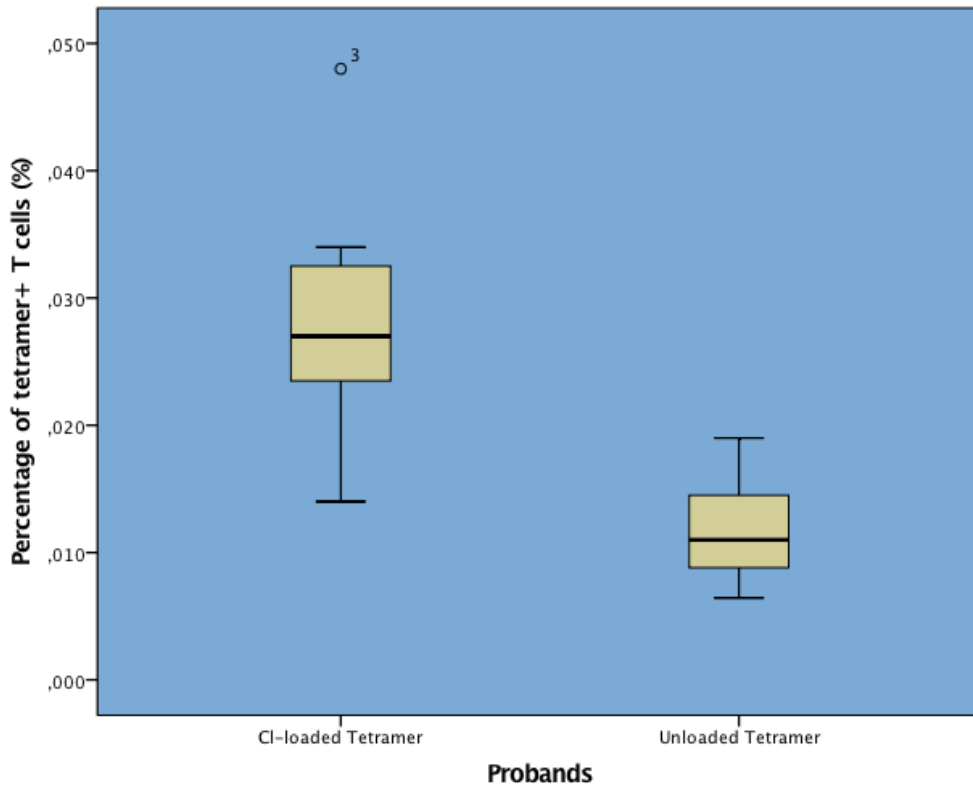


Figure 6 Comparison of CL-binding NKT cells and the negative control. Box plot show the percentage of CL-CD1d and unloaded tetramer-positive cells.

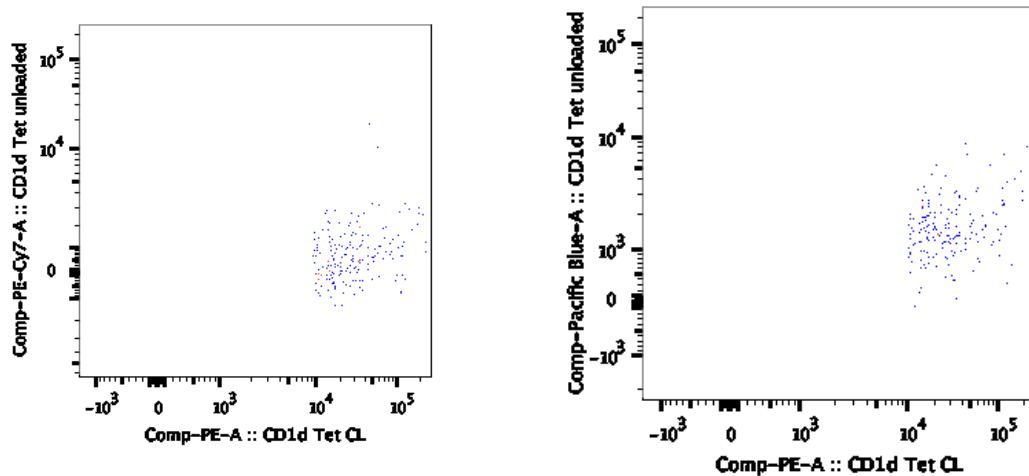


Figure 7 CD1d-unloaded tetramers do not bind to CL- NKT cells

Representative Plots show that CD1d unloaded tetramers (PE-Cy7, Pacific Blue) have no affinity to CL NKT cells.

Phenotypic properties of CL NKT Cells

iNKT cells do not recognize CL as an antigen

The next step was to characterize the properties of CL-positive T cells.

We examined if iNKT cells were the cells, which bound to CL-tetramers or a new T cell population that belongs to type II NKT cells. Anti-invariant TCR (anti-iNKT) and anti-CD161 antibody were used to detect iNKT cells. (Figures 8)

The results in Figure 8 demonstrate that iNKT cells do not bind to CL-tetramers, since anti-iNKT does not overlap with CL-tetramer-positive events.

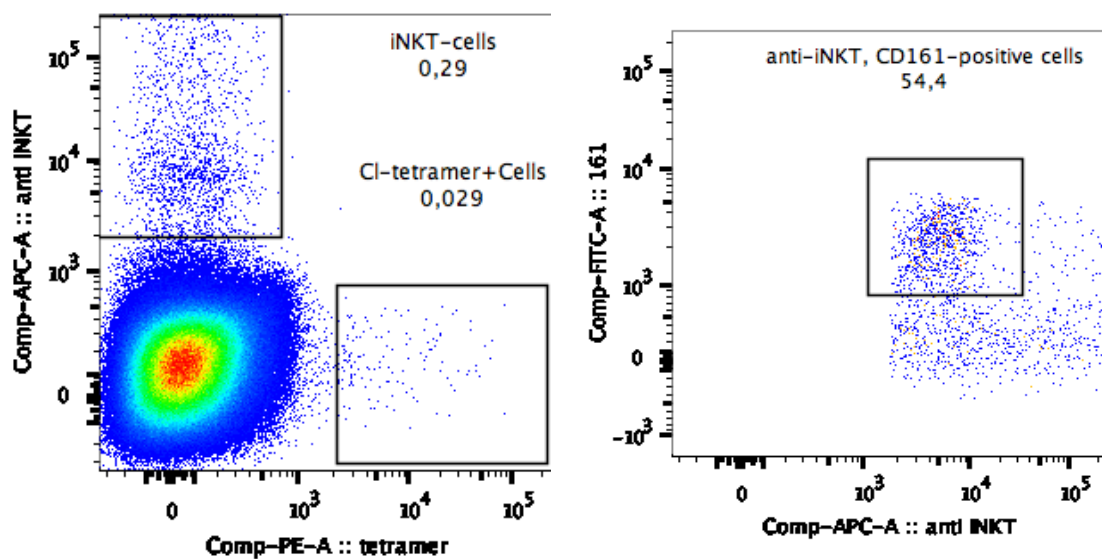


Figure 8 iNKT cells do not recognize CL loaded tetramer.

A-B: iNKT cells were defined as Valpha-24⁺ (anti iNKT) and CD161⁺ and did not show affinity to CL loaded CD1d tetramers.

$\gamma\delta$ T cells from human blood

Dieudé et al. identified $\gamma\delta$ T cells in mice, which recognized CL and additionally responded with secretion of INF- γ . (90)

Thus, we considered if $\gamma\delta$ T cells isolated from human peripheral blood could recognize CL. Anti- $\gamma\delta$ TCR antibodies were used to distinguish the $\gamma\delta$ T cells from conventional T cells. Figure 6 depicts that, similar to iNKT cells, nearly the entire range of $\gamma\delta$ T cells is negative for CL-loaded tetramers.

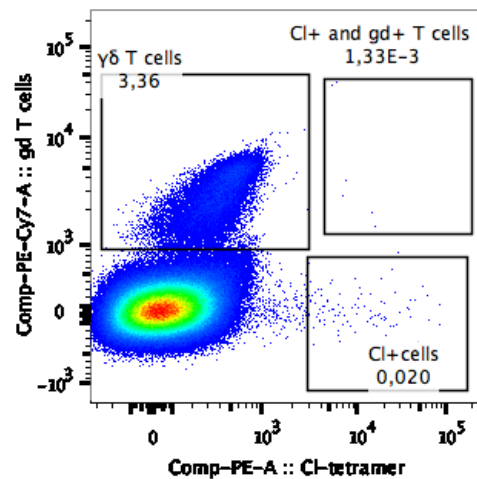


Figure 9 CL NKT cells do not express $\gamma\delta$ TCR

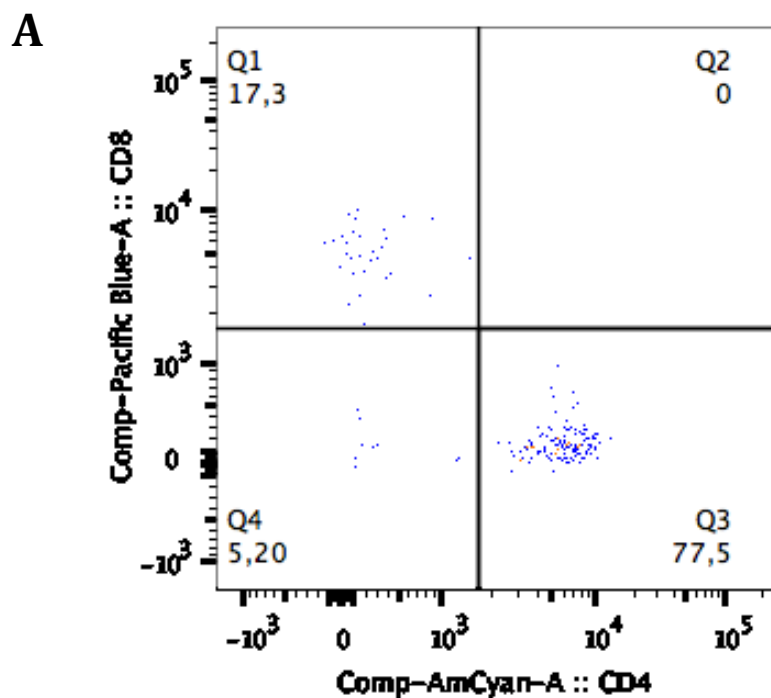
$\gamma\delta$ T cells were stained with anti- $\gamma\delta$ TCR and anti-CD3. Similar to iNKT cells, $\gamma\delta$ T cells were not able to bind to CL-loaded CD1d tetramer.

In conclusion, the circulating CL-binding cells neither belong to iNKT nor to $\gamma\delta$ T cell lineage. Further, our results indicate that the CL-binding T cells carry a $\alpha\beta$ -TCR, and, thus, belong to the diverse NKT cell population.

The distribution of CD4 and CD8 markers in CL and NKT Cells

The PBMCs were stained with anti-CD4- and anti-CD8 antibodies to further characterize the phenotypic properties of CL+ NKT cells.

Interestingly, the majority of CL+ NKT cells were CD4+ (n = 11; 70 % [\pm 15.1]) and only 17.9 % [\pm 7.7] were CD8+. Double positive events represent an infinitesimal number (0.68 % [\pm 1.02]) compared to single positive groups. Double negative cells also formed a small proportion of CL+ NKT cells (11.5 % [\pm 14.3])(Figure 10). The comparison between APS patients and the healthy group revealed no significant difference.



B

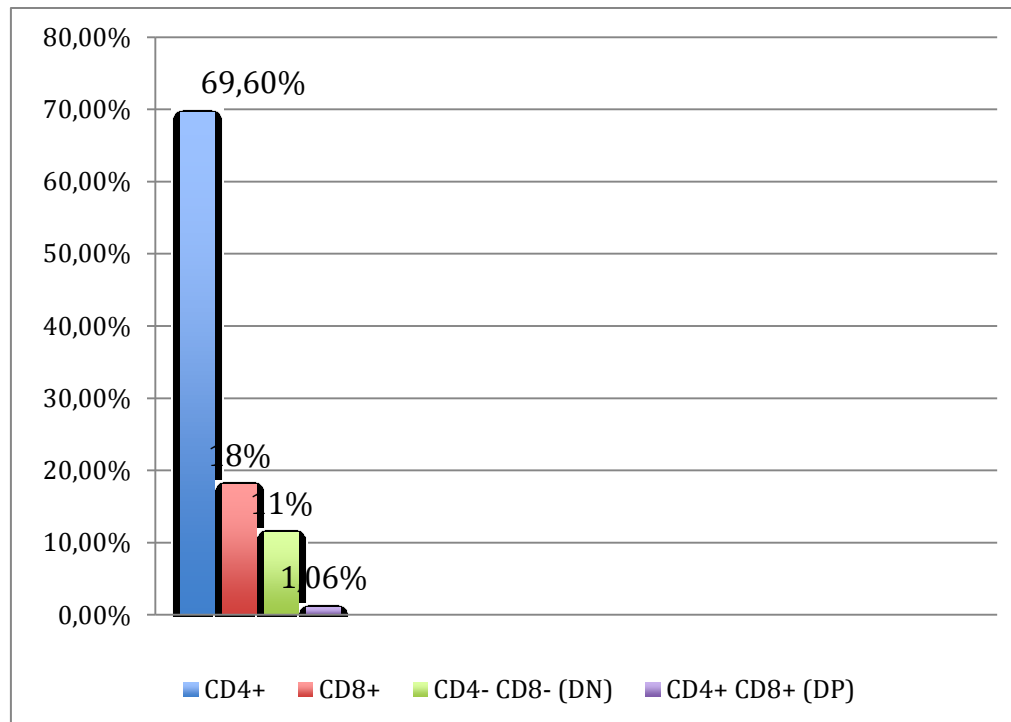


Figure 10 Relative distribution of CL NKT cells

A) Co-staining of CL NKT cells for CD4 and CD8 surface markers. Events in each quadrant represent the distribution of the CL+ NKT cells. (Q1:CD8+,Q2:DP; Q3:CD4+;Q4:DN)

The experiments were performed with 11 different test persons (APS & healthy group).

B) Bar diagram shows the average division of CL NKT cells.

APS patients have increased numbers of CL NKT cells

PBMCs of APS patients (n = 8) and the healthy control group (n = 11) were treated with CL-loaded tetramers to determine the number of CL-binding NKT cells. The percentage of CL⁺ NKT cells (0.024 % [\pm 0.006]) of the entire T cells was significantly (p = 0.017) higher than in the healthy group (0.016 % [\pm 0.007]).

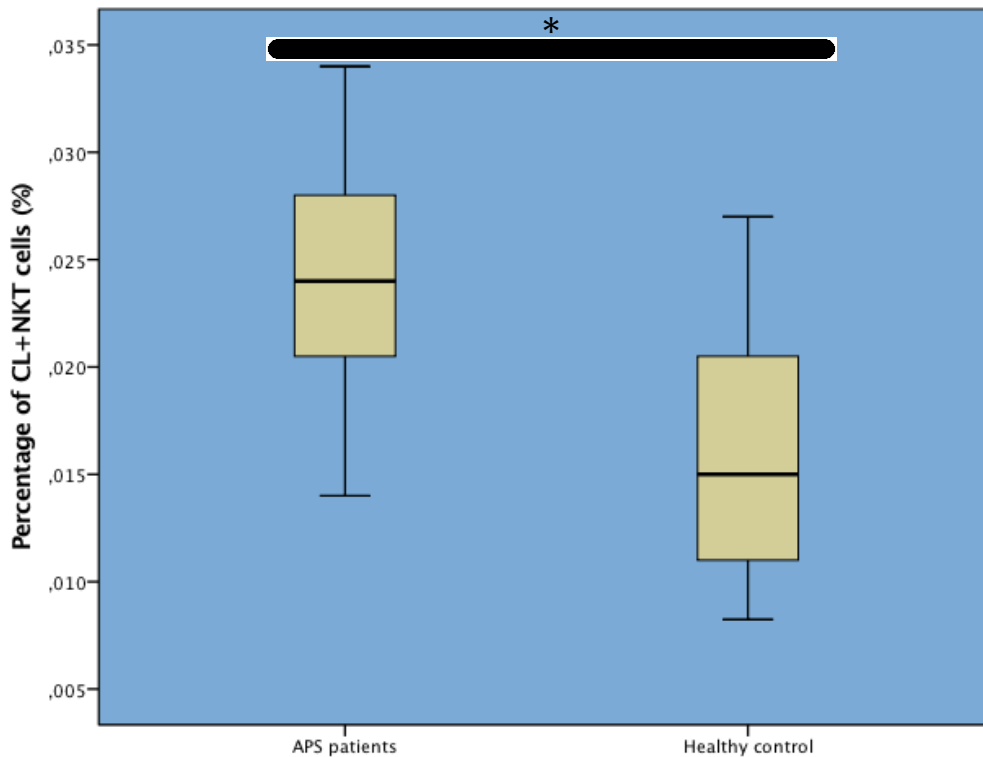


Figure 11 Increased prevalence of CL+ NKT cells in APS patients.

Boxplot shows the CL+ NKT cells percentage of T cells.

*Unpaired two-sample T test was performed to determine the statistical significance: P = 0,17 Mann-Whitney-Wilcoxon-Test (non-parametric test for unpaired samples) was also performed P=0,026

Association between the CL NKT Cells and the anti-CL antibody levels of APS patients

We next studied if there was any association between the anti-CL antibody levels the number of CL NKT cells. Interestingly, patients with lower levels of anti-CL antibody tended to have more CL NKT cells.

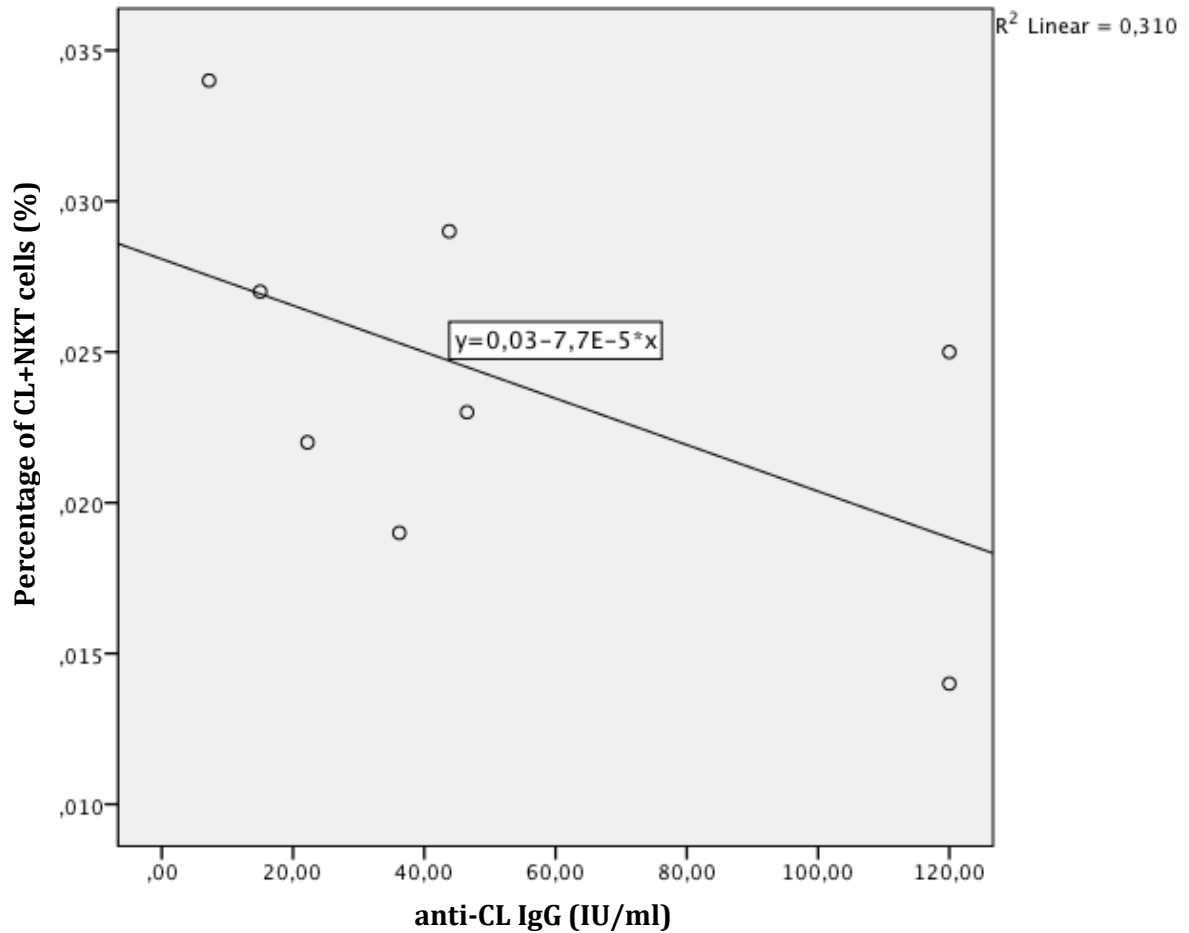


Figure12 CL NKT cells are increased in patients with lower anti-CL IgG levels

In vitro stimulation of CL NKT cells

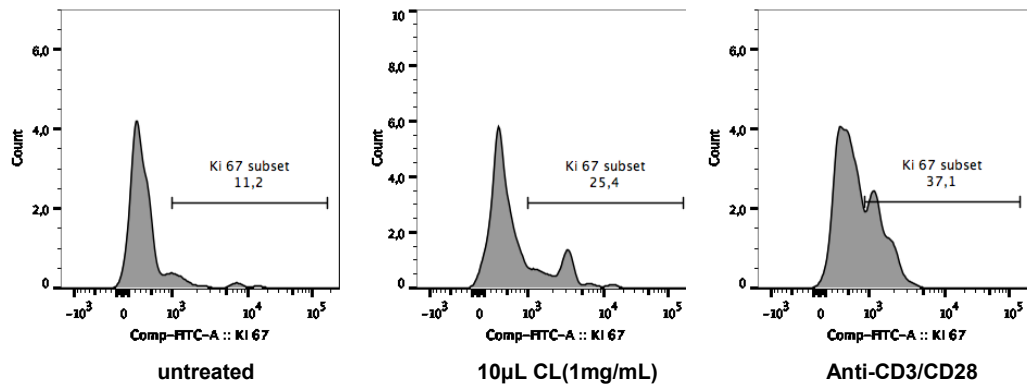
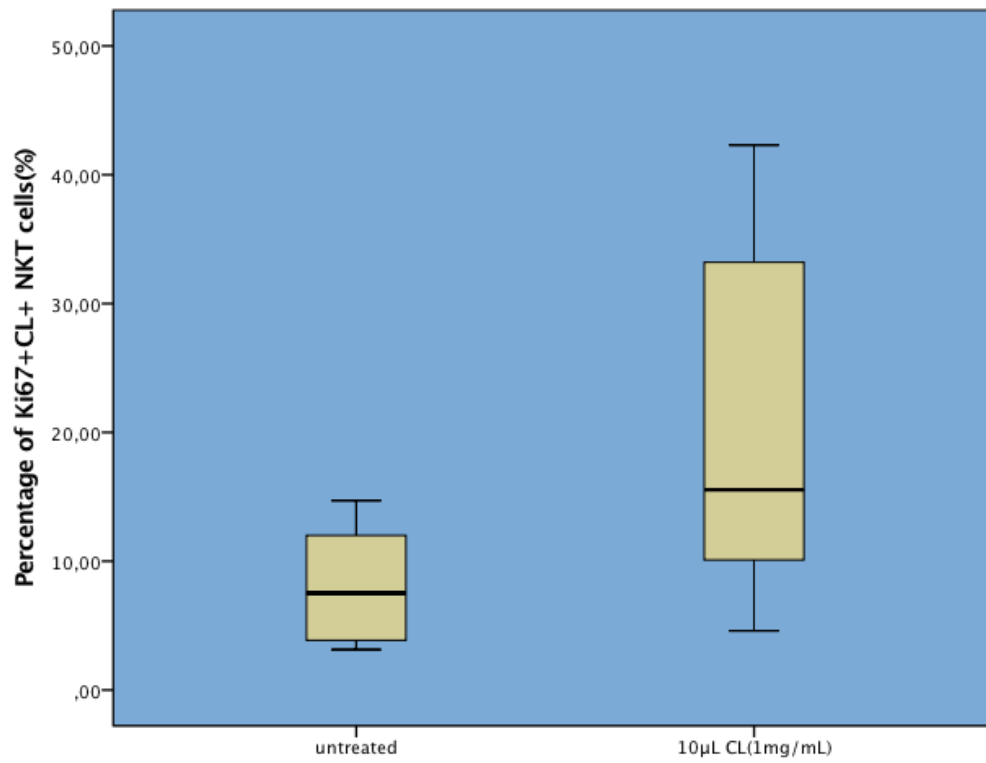
It is well known that α -GalCer can stimulate the proliferation of iNKT cells with a subsequent production of various cytokines. (66) Therefore, we examined whether or not it would be possible to stimulate the CL NKT cells by adding CL.

PBMCs of healthy test persons (ten) were either treated with CL or remained untreated (negative control). Additionally, IL-2 was added in both samples. Anti-CD3/CD28 antibody-coated beads were used as a positive control. After 24 hours, the cells were stained with anti-Ki-67 antibody to analyze the proliferation of CL NKT cells.

At first, we compared the upregulation of Ki-67 between CL-treated, untreated and positive control PBMC cultures (Figure 10a).

After the exposure to CL, the CL NKT cells showed a considerably higher expression of Ki67 proteins in comparison to untreated cells (20.37 % [\pm 13.40]) vs. 8.08 % [\pm 4.42] $p = 0.027$). (Figures 10A and 10B) In the backgating gate (FSC/SSC), we compared the distribution between untreated and treated Ki67⁺CL NKT cells. (Figure 10C)

Interestingly, besides the substantially increased Ki-67 expression, CL treated Ki-67⁺CL NKT cells had higher FSC and SSC, compared to untreated cells. High FSC and SSC correlate with the activation degree of lymphocytes. The results of positive control display an observation similar to that of the CL treated cells.

A**B**

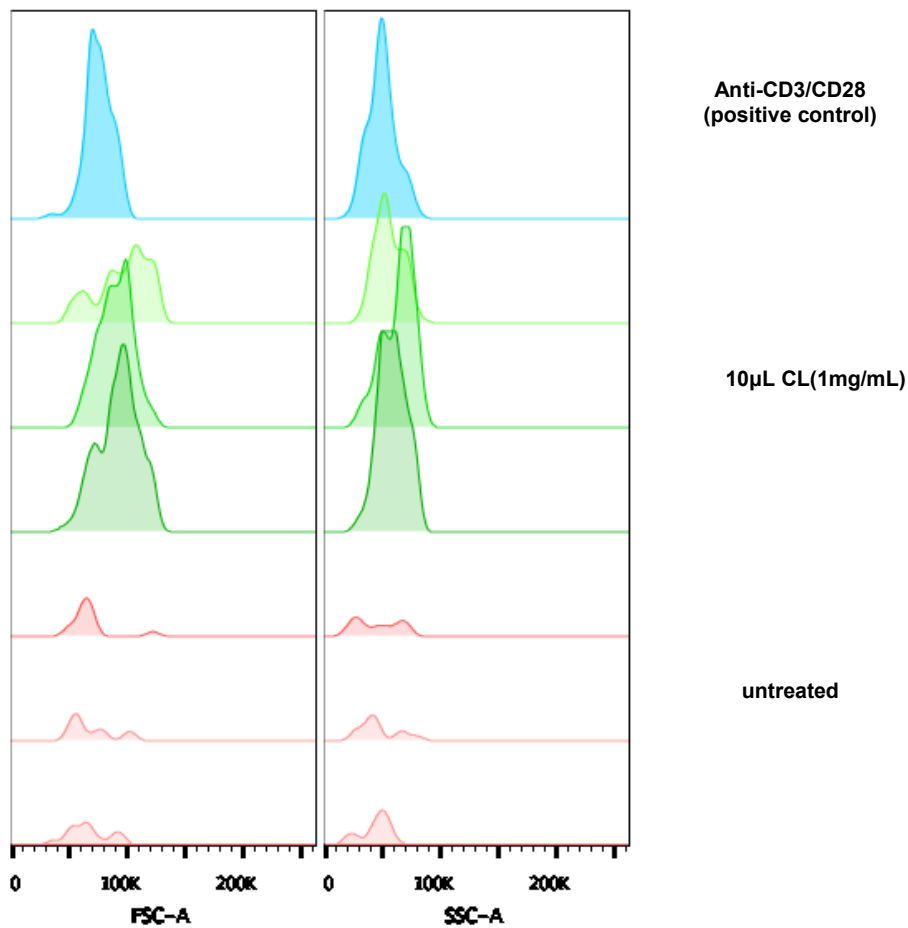


Figure 13 *In vitro* stimulation of CL+ NKT cells

A) Representative histogram shows Ki-67 expression of CL+ NKT cells in untreated, treated and positive control samples.

B) Box plots show the percentage of Ki67+ CL+ NKT cells in 10 healthy individuals. Paired two-sample T test was performed to determine the statistical significance: P=0,027

C) Backgating of Ki67+CL+NKT cells to FSC and SSC gate in untreated, treated and positive control experiments. Histograms display detected Ki67+CL+NKT cells in 3 representative individuals and a positive control.

Decreased cytokine secretion upon CL treatment

To investigate whether or not CL+ NKT cells secrete distinct cytokines after stimulation with CL, we determined the levels of the following cytokines: GM-CSF, Granzyme B, IFN- γ , IL-4, IL-6, IL-10, IL-17A, IL-21 and TNF- α .

Interestingly, compared to an untreated and a positive control, the majority of measured cytokines was significantly reduced in CL treated samples.

The positive control performed with anti-CD3/CD28 beads (two times) provided the unspecific activation of T cells and demonstrates that the cytokine measurements were carried out correctly.

CL treatment decreased the cytokine secretion under the basal levels of untreated PBMCs. The individual values and statistical analyzes are shown in table 5 and figure 14.

Table 6 Statistical results of cytokine measurements

Cytokine	Untreated range (pg/ml)	CL Treated range (pg/ml)	Untreated mean/SD (pg/ml)	Treated mean/SD (pg/ml)	Paired T Test	Wilcoxon Test
G-CFS	49,2- 375,9	3,8- 33,0	175,9 \pm 103,5	9,6 \pm 9,4	P=0,002	P=0,008
Granzyme B	69,30-3468,6	6,9-132,4	870,0 \pm 1049,7	37,8 \pm 41,4	P=0,046	P=0,008
IFN-γ	309,1-4893,3	1,0-12,5	1390,8 \pm 1568,9	5,7 \pm 3,6	P=0,029	P=0,008
IL-4	3,0-17,70	3,0-3,0	6,2 \pm 6,0	3,0 \pm 0	P=0,982	P=0,109
IL-6	10850,0-10850,0	74,4-2062,0	10850,0 \pm 0	900,8 \pm 613,4	P=0,000	P=0,008
IL-10	298,0-1386,2	1,7-15,5	637,7 \pm 340,4	5,7 \pm 4,0	P=0,001	P=0,008
IL-17A	3,8-32,7	0,6-4,0	11,2 \pm 8,6	2,0 \pm 1,3	P=0,017	P=0,008
IL-21	2,7-5,6	0,5-2,9	4,2 \pm 1,0	1,9 \pm 0,9	P=0,002	P=0,008
TNF-α	379,7-2751,1	11,1-235,4	1159,2 \pm 763,3	94,0 \pm 80,8	P=0,004	P=0,008

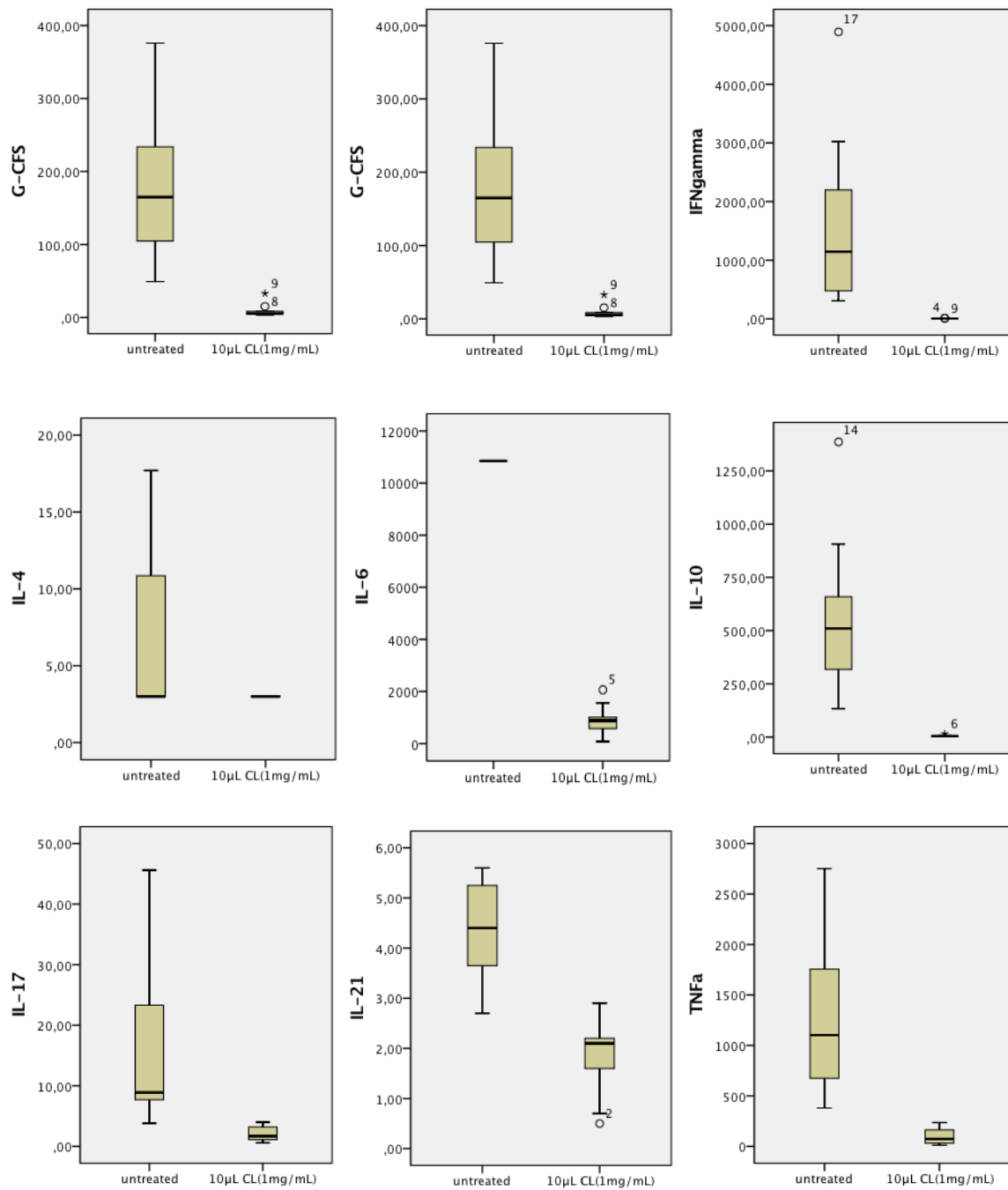


Figure 14 Decreased cytokine production in CL treated PBMCs

Box plots show the levels (pg/ml) of cytokines GM-CSF, Granzyme B, IFN- γ , IL-4, IL-6, IL-10, IL-17A, IL-21 and TNF- α in untreated and CL treated assays.

Determination of apoptosis in PBMCs upon CL stimulation

After the results of cytokine measurements, we determined whether CL could have toxic effects on lymphocytes. Therefore cells were stained with Annexin V upon 24-hour cultivation with 10 μ L and 20 μ L of CL (1 mg/mL). Compared to negative control (untreated cells), CL treated cells showed slightly increased apoptosis/necrosis in all experiments. In contrast, the apoptosis/necrosis in positive control samples (cells were exposed 65°C) was increased tremendously (see Table 7). The apoptosis/necrosis levels remained unchanged upon the treatment with 20 μ L CL, which indicates that CL does not induce dose-dependent apoptosis.

Table 7 Results of apoptosis determination

Trials	Apoptotic T cells in %	Necrotic T cells in %	Viable T cells in %
Positiv control			
1. sample	1,28	70,8	15,8
2. sample	0,83	66,6	17,1
3. sample	0,49	97,1	0,39
Negative control			
1. sample	8,25	3,23	88,5
2. sample	3,52	1,8	94,7
3. sample	4,84	2,75	92,4
CL 10 μL			
1. sample	13,6	8,89	75,9
2. sample	11,2	3,12	85,7
3. sample	14,5	3,57	81,9
CL 20 μL			
1. sample	10,6	11,3	77,9
2. sample	13	5,24	81,5
3. sample	12,3	5,15	82,4

Discussion

A growing body of evidence suggests that subsets of CD1d-restricted T cells recognize phospholipids, including CL. (108) (90)(132)

Upon the interaction with CD1d-restricted subpopulation, phospholipids were capable to trigger an immune response. The secretion of T_{h1} and T_{h2} cytokines by CD1d-restricted T cells was observed in several studies, which suggests that phospholipids can serve as potent antigens for the immune system. (90)(132) However, CL-binding T cells were previously only described in mouse studies.

In the current work, we identified a novel subpopulation of NKT cells from human peripheral blood, which can recognize CL in CD1d-dependent manner. Moreover, we showed in our experiments that these cells do not belong to iNKT cells or to $\gamma\delta$ T cells. Thus, these cells can be classified as a type II NKT cells.

Our results show a complete concordance with the study of Tatituri et al. (108) They demonstrated that dNKT cell hybridomas (type II NKT) bound to CL whereas iNKT cell hybridomas did not. These hybridomas were extracted from mice and not from a human being. (108) Therefore, our intention was to confirm the results of Tatituri et al. with NKT cells derived from human peripheral blood.

Concerning the recognition of CL by $\gamma\delta$ T cells, it has to be considered that Dieudé et al. enriched the $\gamma\delta$ T cells from murine livers. Therefore, it cannot be excluded that CL-binding $\gamma\delta$ T cells are also located in the human liver.

The majority of CL NKT cells is CD4 positive. T cells, which express CD4 protein on their surface, are referred to as T helper cells (T_h cells). T_h cells play a central role in immune response (activation of B cells, antibody class switching, activation of cytotoxic T cells, and modulation of immune response). (133)

$CD4^+$ iNKT cells are well known as potent activators of B cells. Several studies demonstrated that human $CD4^+$ iNKT cells are able to modulate the production of antibodies. (134)(135) Thus, it is conceivable that $CD4^+$ CL NKT cells have comparable effects in relation to antibody production. This suggests that $CD4^+$ CL NKT could be involved in the regulation of anti-CL antibody production.

As a next step, we raised the question whether or not there is a difference between APS patients and a healthy control group. Interestingly, the percentage of CL NKT cells was significantly increased in APS patients compared to the healthy control group, although at a low percentage.

As previously reported, Yang et al. demonstrated that CD1d-deficient mice showed decreased levels of anti-CL antibody, despite the presence of anti-phospholipid antibody producing autoreactive B cells. They suspect that CD1d-restricted T cells might regulate the production of aCL antibodies. Thus, it is reasonable to assume that the increased occurrence of CL NKT cells contributes to the regulation of anti-CL antibodies in APS patients. For this reason, we asked if there is a correlation between increased CL NKT cells and the antibody levels. Interestingly, we observed that patients with high anti-CL antibody levels appear to have fewer CL NKT cells than patients with lower antibody levels. Admittedly, the number of APS patients is not particularly representative. This could indicate that CL NKT cells seem to have a suppressive effect on the production of anti-CL antibody. A larger cohort of patients will be needed to investigate the observed association between CL NKT cells and antibody levels.

The entire healthy group was negative for anti-CL antibodies. This raises the question why CL NKT cells occur in healthy persons and what functions they have. Research of the last two decades showed that CD1d-restricted T cells are a thymus-dependent population and require self-lipid antigens presented by CD1d for the positive selection, even though the identification of self-lipids remains difficult until today. (136)(137) It is quite conceivable that CL serves as a self-antigen during the development of CD1d-restricted T cells. The thymus is a tissue with a high rate of cell apoptosis. Cell death by apoptosis makes CL accessible for APCs. (138)(139) Thus, CL may be present for positive selection of NKT cells. Another source of CL is the membrane of bacteria, which can possibly occur during an infection. (140) Upon engulfment of bacteria by macrophages, CL could be loaded on CD1d molecules, followed by the presentation to CL NKT cells. In summary, due to ubiquitous availability of CL, the occurrence of CL NKT in APS patients as well as in healthy individuals appears to be comprehensible.

It does, however, remain unclear what kind of process triggers an autoimmune reaction, followed by auto-antibody production. Apoptotic cells have a central role in the pathogenesis of SLE. (141) The impaired clearance of apoptotic cells by macrophages increases the spread of cell fragments in extracellular space. Antigen-presenting cells can take up these fragments to present these self-antigens to autoreactive T and B cells. This condition is supposed to contribute to

the pathogenesis of SLE. In a similar way, CL could serve as an auto-antigen to interact with CD1d-restricted T cells. (141) Diverse bacteria and viruses are well known to be a potential trigger of autoimmune diseases through the effects of molecular mimicry. Thus, it is also possible that bacterial infections could increase the CL presentation in a CD1d-dependent manner and provoke an immune reaction. The amount of the auto-antigens, as well as exposure time to the immune system could also play a crucial role in the development of an autoimmune response. Ongoing apoptosis or a chronic infection might ensure CL exposure over a longer period of time. Several studies seem to support our hypothesis. (142)(139) Sorice and co-workers figured out that CL becomes exposed on the cell surface after CD95/Fas-induced apoptosis. (142)(139) Further, they revealed that anti-CL antibodies purified from APS patients bind to CL on the surface membrane of apoptotic cells.

Besides the increased number of CL NKT cells in APS patients, it is certainly possible that CL NKT cells could be abnormally altered in their phenotype. This could result from altered receptor and signaling pathways or gene expression. Such pathological modifications of T cells are well described in Lupus disease. (143)

We examined whether or not it would be possible to stimulate the CL NKT cells by adding CL. It is well known that α -GalCer can stimulate the proliferation of iNKT cells with the subsequent production of various cytokines. (66) To analyze the activation of NKT cells, we measured the Ki67 expression (an intracellular marker of proliferation) and the cytokine secretion after the CL treatment. Our results suggest that CL can activate CL NKT cells in healthy persons. The CL treatment of PBMCs showed significantly more Ki-67+ CL NKT cells in comparison to untreated cells. Another difference between treated and untreated cells was the different localization of Ki-67+CL+ NKT cells in the FSC/SSC dot plot. Activated cells are characterized by an increase of FSC (cell size) and SSC (cell granularity). (144) The increase of both properties was observed in CL-treated PBMCs.

This would imply that CL-treated Ki-67+ CL NKT cells seem to be activated in comparison to untreated cells. The positive control with anti-CD3/CD28 antibody-coated beads supports this consideration, since the anti-CD3/CD28-treated Ki-67+ CL+ NKT cells also have higher FSC and SSC.

However, the majority of CL NKT cells remained inactivated after the CL treatment. The possibility exists that the majority of the CL⁺ NKT cells require more than 24 hours to express Ki67. A further factor could be the limited availability of CD1d presenting APCs. The expression of CD1d depends on the type of APC and their exposure to cytokines and other signals. APCs with higher CD1d expression more efficiently stimulate NKT cells.(145) Cell types such as marginal zone B cells (MZ B cells), which have highest expression of CD1d are located in the spleen and belong to non-circulating B cells. (68)

The determination of cytokine levels after CL stimulation provided some unexpected results. Instead of the expected increase in cytokines, we observed a suppressive effect of CL. All cytokines levels were significantly reduced compared to untreated cells. In search of a further comprehensible explanation, we raised the question whether CL has a toxicological effect, since we also observed a reduction of the cell number on a high-dose CL treatment. In our apoptosis experiments, we observed a slight increase of apoptotic cells upon CL treatment. Therefore, it appears unlikely that apoptosis with such negligible difference could induce the observed cytokine suppression. Another possible explanation for the observed suppression could be a recently described property of CL. According to the study of Balasubramanian et al., CL can promote an attenuation of the innate immune response through the suppression of a TLR4-mediated cytokine secretion. (146) The simultaneous addition of CL and LPS prevented the activation of macrophages since CL competitively inhibited the LPS-binding to myeloid differentiation factor 2 (MD-2). Together with TLR-4 and LPS, MD-2 forms a complex, which initiates an immune response to LPS by cytokine secretion. Balasubramanian et al. suggest that LPS-expressing bacteria have evolved to use CL as a protection against TLR4-induced immune response. Whether these characteristics of CL can also explain our observation needs to be confirmed by further investigations. It should be mentioned that CL does not only have effects on CD1d-restricted T cells, but also on many other cells and receptor-mediated pathways. Therefore, CD1d-restricted T cells should be analyzed separately from PBMCs to minimize the side effects of CL. (147) We supposed that this generalized suppression was rather due to systemic effects of CL than suppression through CL NKT cells. It appears highly unlikely that a small

subpopulation, such as CL NKT cells have regulative properties to such an extent that inflammatory as well as anti-inflammatory cytokines were simultaneously reduced.

This study has limiting factors: First, the value of the study was limited by the low number of APS patient cases. Patients with diagnosed APS who have no anti-CL antibodies, were excluded from the study. Therefore, the search for a suitable patient proved difficult.

Furthermore, the stimulation experiment was only performed with ten healthy test persons, since it was not possible to acquire sufficient PBMCs from APS patients for in vitro stimulation assays. Of particular interest would be a stimulation test with APS patients, which will allow comparing them to healthy individuals. Further stimulation assays are needed to confirm the initial findings.

In conclusion, we identified a novel subpopulation of CD1d-restricted T cells with the ability to recognize CL in a CD1d-dependent manner. Moreover, these NKT cell subsets occur more frequently in patients with antiphospholipid syndrome than in healthy persons. CL induces an activation of CL NKT cells and it seems possible that CL has suppressive effects with regard to cytokine secretion. This pioneering work serves as a pilot project for further investigations aimed at clarifying the role of CL NKT cells in the pathogenesis of APS.

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