Role of Invariant Natural Killer T (Inkt) Cells in Systemic Lupus Erythematosus

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Abstract: Natural killer T (NKT) cells represent a unique T cell lineage. The NKT cells bearing an invariant TCR (iNKT cells) recognize a small variety of glycolipid antigens in the context of CD1d (non-classical MHC-I) presentation. CD1d-restricted iNKT cells play a regulatory role during an immune response by producing cytokines (IFN-γ, and IL-4). The identification of α-galactosyl-ceramide (α-GalCer), a marine sponge derivative as a potent stimulator of iNKT cells has raised the potential of therapeutic iNKT cell activation.

Invariant NKT cells have been implicated in several different autoimmune diseases in mice and humans, including systemic lupus erythematosus (SLE). Abnormalities in the number and functions of NKT cells have been observed in SLE patients and mouse strains genetically predisposed to lupus (MRL/lpr, NZB/W F1). Moreover, inverse correlation between the frequency of NKT cells and IgG levels has been observed. Elevated IgG levels in relatives of patients with lupus as well as in patients with lupus were associated with low frequencies of NKT cells.

This review focuses on the potential roles of NKT cells in the pathogenesis of SLE. It summarizes recent advances in glycolipid therapy for murine lupus. First, it has been demonstrated, that repeated administration of α-GalCer to MRL/lpr mice alleviated inflammatory dermatitis but did not influence kidney disease. Treatment of NZB/W mice with α-GalCer resulted in amelioration of SLE symptoms in young mice, but treatment of older animals resulted in disease exacerbation.

The effects of NKT cell activation using α –GalCer, on disease progression, were influenced by a variety of parameters, including the genetic background of mice, the α –GalCer dose, number of injections and the stage of the disease process when treatment was performed.

Manipulation of NKT cells in the human system may be a promising treatment alternative for the future, however possible deleterious effects have to be carefully investigated first.

Keywords: Natural killer T (NKT) cells, CD1d, Systemic lupus erythematosus, glycolipids.

INTRODUCTION

Natural killer T (NKT) cells are a unique T cell subset. The name originates from the first description [1,2] in which a T-cell population was discovered to express the natural killer (NK) cell specific *NK1.1* marker and a T-cell receptor (TCR) [3,4]. The human equivalent of the mouse NK1.1 is CD161. NKT cells are a thymus-dependent [3,5] T-cell subset, but are developmentally and functionally distinct from mainstream T cells expressing $CD4^+$ and $CD8^+$ maturation markers [6].

Since this first description heterogeneous subpopulations of NKT cells have been described, with different specificities and functions. Antigens are presented to NKT cells by the MHC type one encoded CD1d molecule (Fig. **1**) is The two main groups are *CD1d*-dependent and CD1d-independent NKT-like cells (Table **1**) [7]. The predominant population of CD1d-dependent NKT cells expresses an invariant TCR (Fig. **2**) [8,9,10]. These TCRs recognize hydrophobic glycolipid antigens, presented by the MHC class I-like molecule hCD1d (human) or CD1d (mouse) [11,12]. Alpha-galactosyl-ceramide (*α-GalCer*) - isolated from a marine sponge (*Angelas mauritanius*) [13] - has been identified as an antigenic ligand, inducing a strong response in NKT cells [8,14,15]. A family of natural ligands for NKT cells are glycosyl-phosphatidyl-inositol (GPI)-anchors [16] and bacterial phosphoinositol mannosides [16]. These CD1d dependent NKT cells expressing the invariant TCR which can be identified by *α-GalCer* loaded CD1d tetramers are named *invariant* NKT (*i*NKT) cells. The α-GalCer-loaded CD1d tetramer – labeled NKT cells are detected by flow cytometry [18,19,20].

*i*NKT cells promptly produce both interferon-γ (IFN-γ) and interleukin-4 (IL-4) upon activation [8,21]. Classical *i*NKT cells are most abundant in the liver, where hepatocytes [22] express CD1d and thus participate in survival and/or expansion of classical *i*NKT cells [23,24]. Most antigen studies have used α-GalCer, to activate-human [25,26] or murine [20,27] *i*NKT cells *in vitro* and *in vivo*.

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In the present review we summarize the information available on CD1d dependent (classical) type I (*invariant*) *i*NKT cells. The information available on other subtypes is scarce at present due to the lack of known specific activating ligands.

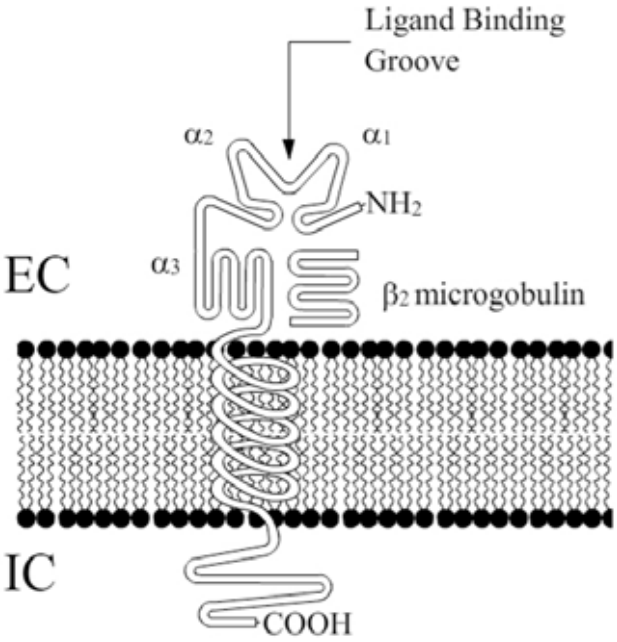

Fig. (1). Structure of the CD1d Antigen Presenting Complex.

The CD1d complex is composed of a polymorphic heavy chain (α) and a [β2]-microglobulin chain. The heavy chain is organized into three globular domains (α1, α2 and α3), from which the α1 and α2 domains form a cleft or a lipid binding groove. (EC: extracellular, IC: intracellular).

Invariant NKT (*i*NKT) cells bridge the innate and adaptive immune systems and hold significant promise for development of immunotherapies for a variety of diseases. *Invariant* NKT cells are multifunctional. *Invariant* NKT cells can enhance anti-microbial immunity and tumor rejection, suppress autoimmune disease and

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Table 1. Classification of Natural Killer T Cells into Three Groups has been Proposed [131]

	Type 1 NKT	Type 2 NKT	NKT-Like
Other names	classical NKT invariant NKT (iNKT) Vα24i NKT (human) Vα14i NKT (mouse)	non-classical NKT diverse NKT	NK1.1$^+$ T cells CD3$^+$ CD56$^+$ T cells
Restriction	CD1d	CD1d	MHC, other?
α-GalCer reactivity	+	+/-	-
TCR repertoire	Vα24-Jα18; Vβ11 (human) Vα14-Jα18; Vβ8.2, 7, 2 (mouse)	diverse	diverse
subtypes	NK1.1(CD161)$^+$ NK1.1$^-$		

promote tolerance [28]. Activation of *i*NKT cells result in the production of various immunoregulatory cytokines, including typical proinflammatory IL-2 (interleukin-2), IFN-γ (interferon-γ), TNF-α (tumor necrosis factor-α) and typical anti-inflammatory or humoral immunity promoting cytokines such as IL-4, IL-10, IL-13 [20]. *Invariant* NKT cells also trigger the activation and differentiation of a variety of other leukocytes including natural killer (NK) cells, dendritic (DC) cells, conventional CD4$^+$ and CD8$^+$ T cells, and B cells [6,29].

The prevalence of *i*NKT cells in humans is significantly lower than in mice. In humans, only approximately 0.1% of T-cells can be identified as *i*NKT cells through their reactivity with α-GalCer CD1d tetramers [30].

The tissue distribution of *i*NKT cells in mice and humans are similar, but mice tend to have higher frequencies at matched sites. This difference is most obvious in the liver, where 30-40 % of T cells are *i*NKT cells in mice but only 0.05 % in humans [31].

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease considered to result from disturbed tolerance to self antigens. SLE is characterized by antinuclear autoantibodies and affects multiple organ systems, including skin, joints, kidneys (immune complex glomerulonephritis) and the central nervous system in most sever cases [32]. The development of SLE is associated with the production of pathogenic autoantibodies, generation of autoreactive T helper (Th) cells, decline of regulatory T- and B-cell subsets and defective clearance of apoptotic products, auto antigens and immune complexes [33].

Several mouse strains, including the FAS deficient lupus prone (lpr) mouse on MRL background MRL/lpr and the New Zealand black*white F1 hybrid NZB/W F1 mice develop a spontaneous

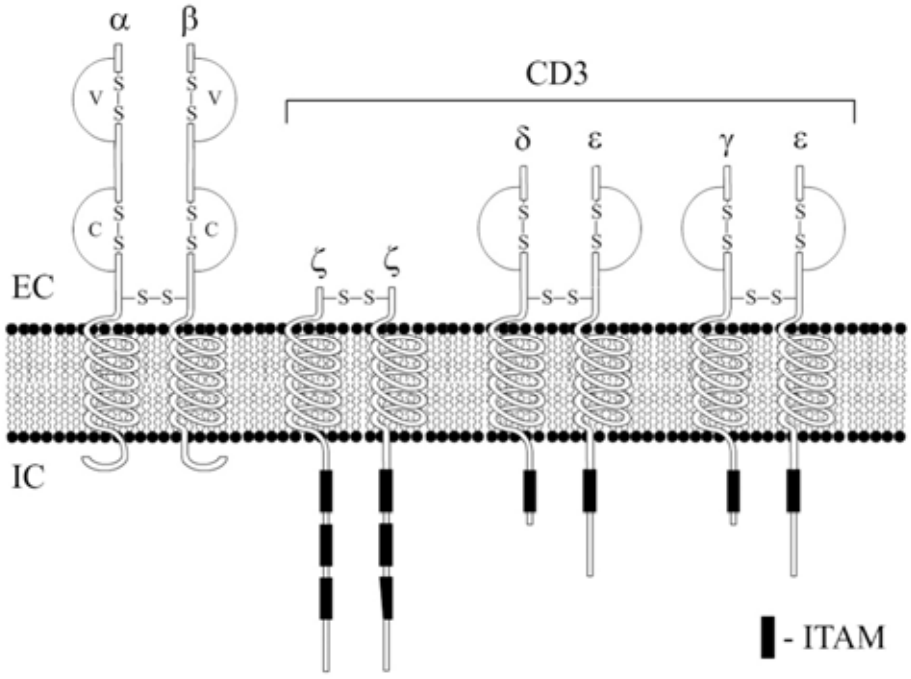

Fig. (2). The T-cell receptor (TCR).

The heterodimer T-cell receptor complex is composed of an α and β chain, each containing a Variable (V) a Joining (J) and a C onstant (C) region. Antigen recognition is achieved by the hypervariable region (HV). The α and β chains have no signaling regions. Signaling from the TCR is accomplished through the CD3 molecule. CD3 is composed of the γ, δ and ε chains, which have Immunoreceptor Tyrosine based Activation Motifs (ITAMs), required for the transduction of the α-β heterodimer signals into the cell. (EC: extracellular, IC: intracellular region).

lupus-like syndrome [34]. MRL/lpr mice develop autoantibodies, dermatitis, glomerulo- and interstitial- nephritis, and vasculitis due to immunecomplex deposition [35]. In NZB/W F1 mice, the production of IgG autoantibodies leads to the development of massive immune complex-mediated glomerulonephritis. Mice from both strains die prematurely before 12 months of age due to renal failure [34].

Besides the genetic mouse models of SLE, lupus-like autoimmunity can be induced chemically by pristane in different mouse strains [36]. In murine models of lupus, deficiency and reduced function of regulatory T cells, including natural killer T cells (NKT-cells) has been documented [12]. The present review focuses on the important role of iNKT cells in the pathogenesis of SLE, and on possible therapeutic benefits.

NKT CELLS ARE HETEROGENEOUS

NKT cells (expressing $NK1.1^+$ and a TCR) have two distinct groups: CD1d-dependent and CD1d-independent NKT-like cells [37] (Table **1**). CD1d-dependent cells need CD1d-linked antigen presentation for their maturation. Although many groups refer to CD1d-independent NK1.1+ T cells as "NKT cells", most investigators in the field prefer the name NKT-like cells or T cells with a phenotype of NKT cells. In the present review the nomenclature used is „CD1d-independent NKT-like cells".

CD1d-Dependent NKT Cells

CD1d-dependent NKT cells are defined by their reactivity to α-GalCer-loaded CD1d tetramer. CD1d-dependent NKT cells have two further subclasses: type I and type II NKT cells [37]. These subclasses mainly differ in their TCR which is invariant and thus recognize only a small group of ligands with similar structure in the type I NKT cells, whereas the TCR of type II NKT cells is more diverse. This review focuses on type I NKT cells, as information on type II NKT cells is limited at present.

Type I CD1d-Dependent NKT (iNKT) Cells

The α chain of the *i*NKT TCR is the invariant: Vα24-Jα18 (humans) Vα14-Jα18 (mice) TCR chain. Bearing the invariant α chain these cells are also named *invariant* NKT (***i*NKT**) cells (Fig. **2**) [**Error! Bookmark not defined.**]. The variable region (V-domain) of a TCR is composed of 102-119 amino acids, and at least 3 subregions, which determine antigen specificity of the T-cell [15]. The rearrangement of the antigen recognizing hypervariable region (CDR3-third complementarity-determining region) of the TCR is encoded in iNKT cells by the CDRα3.

The β chain of the *i*NKT TCR is Vβ11 (human) or Vβ8.2, Vβ7 or Vβ2 (mice), [8,38].

The development of *i*NKT cells is controlled by CD1d: a β_2 – microglobulin (β_2m) associated nonpolymorphic molecule.

The CD161 NK cell receptor (human) (NK1.1 in mouse) is a type II membrane C-type lectin. NK1.1 expression depends on maturity, activation state, tissue location and expression of other receptors [39,40,41]. Mouse NK1.1 is also called NKR-P1C. The natural killer receptor-proteins (NKR-P) are "NK locus" –encoded surface molecules, expressed on rodent NK cells. In rodents there are three NKR-P1 molecules, NKR-P1A, -B, -C [42,43]. *i*NKT cells also constitutively express a variety of markers such as CD44, CD69 and CD122 that are typical of activated or memory T cells [44]. Murine NK1.1 $(NKR\text{-}P1C)^+$ T cells are either $CD4^+$ or double negative (DN) and subsets of NKT cells express additional markers, such as Ly-49C, a mostly inhibitory NK cell receptor not found on conventional T cells [8,23,45,46].

NK 1.1- iNKT Cells

Since the identification of α-galactosyl-ceramide (α GalCer) as a ligand which activates *i*NKT cells, it has been established that some type I *i*NKT cells do not express the NK1.1 (CD161) marker.

$NK1.1^-$ *i*NKT cells are CD4+. After *in vitro* stimulation, $NK1.1^-$ *i*NKT cells in the thymus produce higher levels of IL-4 and lower levels of IFN-γ than $NK1.1^+$ *i*NKT cells. At least in the thymus, $NK1.1^-$ *i*NKT cells are precursors of $NK1.1^+$ *i*NKT cells [41,47].

NK 1.1+ iNKT Cells

The NK 1.1+ *i*NKT cells have 2 further subclasses in mice. Most murine *i*NKT cells express CD4 ($CD4^+$). A small remaining portion does not express the typical T-cell maturation markers: CD4 or CD8, thus are double negative (DN) populations. Functional differences between these subpopulations however, are as yet unclear [48]. Mouse *i*NKT cells generally do not express CD8 [46], but 50% of human *i*NKT cells are $CD8\alpha^+$ and a small fraction is $CD8\beta^+$ [37,49]. The remaining group of *i*NKT cells in humans are DN [10,50,51,52]. The sequence homology of the invariant TCR chains is 75% at the nucleotide level and 90% in the CDR3 (complementarity determining regions) at the amino acid level, indicating that both mouse and human invariant TCRs recognize similar antigenic epitopes [53].

Type II NKT Cells

Type II NKT cells were first described in 1995 [54]. Type II NKT cells are also CD1d dependent, Vα14-Jα18-independent [55]. However, they express more diverse TCR Vα chains (Vα3-Jα9 or Vα8, in conjunction with Vβ8.2), which have also been identified in mice [56]. These cells have been less well characterized, mainly because they cannot be identified using, α-GalCer loaded CD1d tetramers, and therefore the exact prevalence of these cells and the extent to which they express NK1.1 is not precisely known. They nonetheless appear to constitute a considerable fraction of the CD4+ T cells in MHC class-II-deficient mice [54].

Some of the type II NKT cells recognize sulphatide molecules. Van Rhijn *et al*. demonstrated, that noninvariant CD1d-restricted T cells in humans are activated through their αβ TCR by nonlipidic sulfur-containing small molecules, including phenyl-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonate (PPBF) [57].

CD1d-Independent NKT-Like Cells

Analysis of CD1d-deficient mice revealed the existence of a residual population of Vα14-Jα18 and CD1d independent $NK1.1^+$ T cells. This subpopulation is predominantly made up of $CD8^+$, $NK1.1^+$ TCR^+ cells. Further subpopulations include $CD4^+$ or DN $NK1.1^+$ TCR^+ cells. CD1d independent NKT cells typically produce low levels of cytokines and are thymus independent but not much is known yet about their role and function [58,59,60]. Similar CD1d-independent NK-cell-marker-expressing T cells are present in humans. Co-expression of the invariable TCR chains Vα24 and Vβ11 in human lymphocytes does not completely coincide with α-GalCer binding CD1d-tetramer reactivity [61], suggesting the existence of CD1d independent iNKT cells in humans. This might also partly reflect a lack of specificity for α-GalCer rather than CD1d in general. Thus, type I *i*NKT cells represent only a subset of T cells that express NK-cell markers [61,62].

Invariant NKT Cell Ligands

There are many glycolipids that affect immune responses. Alpha-galactosyl-ceramide (α-GalCer) (Fig. **3**) is unique, as this compound is presented in association with CD1d. Less than 1nmole of α-GalCer is required to fully activate the *i*NKT subpopulation *in vivo*, and some studies indicate that a much lower dose may be also effective. Alpha-GalCer was discovered by the Kirin Pharmaceutical Research Corporation [13,63]. A synthetic analogue KRN7000 of this compound was developed for experimental studies and

clinical trials [64,65]. Alpha-GalCer is a glyco-sphingo-lipid, a chemical category that encompasses glycolipids in the body, including gangliosides. It is distinguished, by the stereochemistry of the bond that joins the asymmetric 1' carbon of the sugar to the lipid. In nearly all natural cases, this bond is in the β-anomeric form, but the sponge-derived glyco-sphingo-lipid has an α-linkage [66] (Fig. **3**).

After cleavage, the CD1d binds the aliphatic chains and presents it on the surface of the APC, and the iNKT TCR recognizes the carbohydrate group (Fig. **4**) [73].

α-galactosyl-ceramide is the general designation for glyco-sphingo-lipids that contain a galactose carbohydrate (hexose) attached by an α-linkage to a ceramide lipid that has acyl and sphingosine chains of variable lengths. Thus, α-GalCer possesses a lipid as well as a carbohydrate moiety, both of which are ligands of *i*NKT cells. KRN7000 ((2S, 3S, 4R)-1-O-(α-D-galacto-pyranosyl)-*N*–hexacosanoyl-2-amino-1,3,4-octadecanetriol (Fig. **3**) is a synthetic α-GalCer that is most frequently used in experimental studies and is usually referred to as α-GalCer. The lipid portion of α-GalCer interacts with the hydrophobic antigen-binding groove of CD1d, and the carbohydrate portion is accessible for interaction with the *invariant* natural killer T (*i*NKT)-cell T-cell receptor. OCH (α-GCs9) (Fig. **3**) an α-GalCer analogue with short sphingosine base and truncation in the acyl chain (by 2 carbons) and the sphingosine chain (by 9 carbons) [67]. β-GalCer (Fig. **3**) is a C-glycoside (carbon glycoside) analogue of α-GalCer. β-GalCer differs from α-

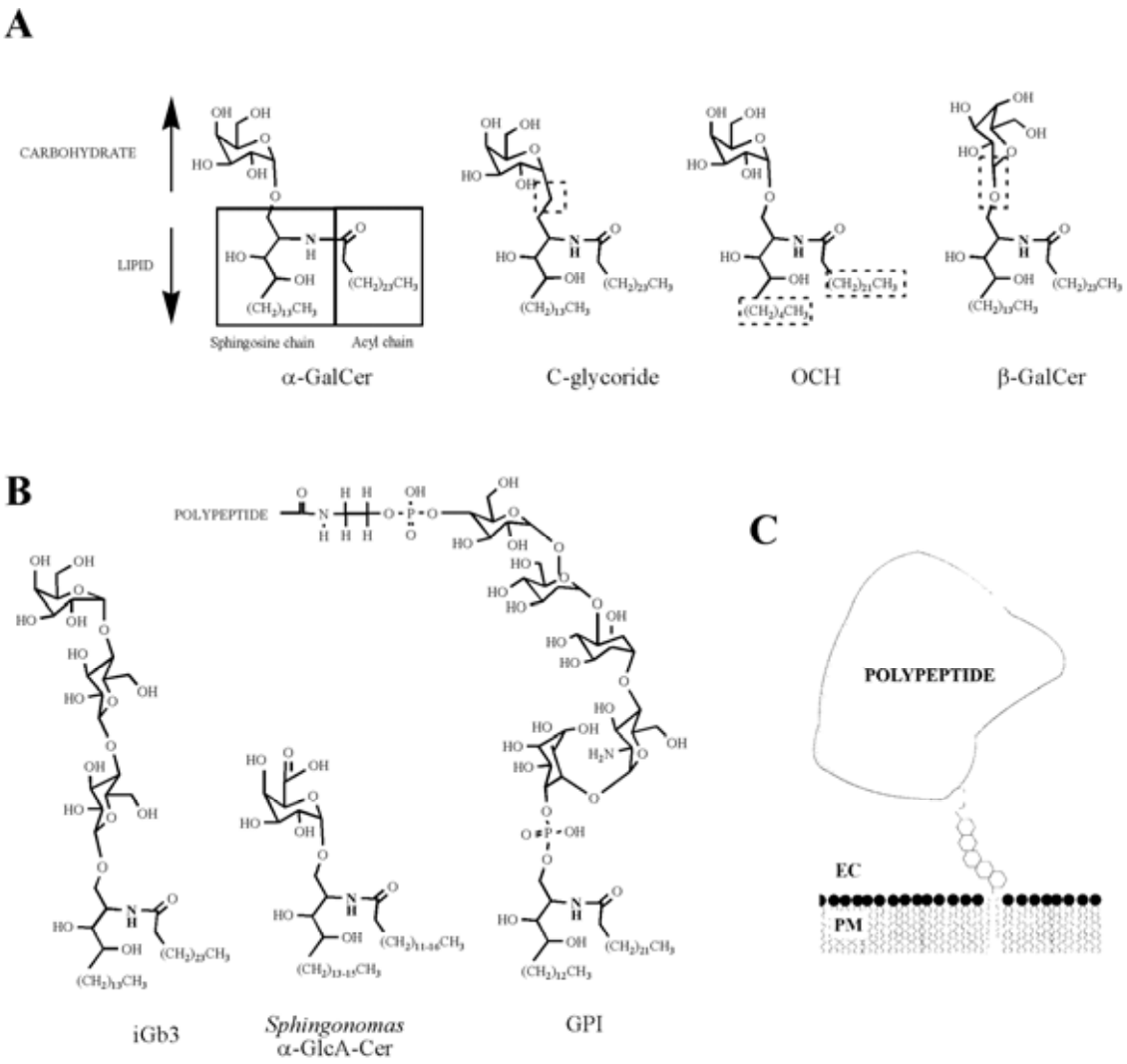

Fig. (3). Ligands of iNKT Cells

a) Structure of α-GalCer and Related Glycolipids: -Galactosylceramide (α-GalCer) or its synthetic analogoue KRN7000 (Kirin Corporation) are glycolipids. The lipid portions interact with the hydrophobic lipid binding groove of the CD1d, while the carbohydrate interacts with the TCR of an NKT cell (Fig. **4**). α-GalCer and its related glycolipids usually differ in the acyl-chains. β-Galcer differs from α-GalCer in that the lipid portion is attached to the carbohydrate through a β linkage.

b) Natural Ligands are glycosyl-phosphatidyl-inositol (GPI)-anchors [16] and bacterial phosphoinositol mannosides [17]. The glycosphingolipid: isoglobotri-hexosylceramide (iGb3) [138]. Furthermore, microbial cell wall components: α-glycuronosylceramide (α-GlcA-Cer) and the related glycolipid α-galacturonosylceramide (α-GalA-Cer) [139,140] derived from Shingomonas bacteria and α-galactosyl diacylglycerols (α-Gal-DAG) from B. burgdorferi (the causative agent of Lyme disease) directly stimulated iNKT cells through TCR engagement [141].

c) The Glycosyl-Phosphatidyl-inositol (GPI) Anchor: GPI is a glycolipid that can be attached to the C-terminus of a protein during posttranslational modification. It is composed of a hydrophobic phosphatidylinositol group linked through a carbohydrate containing linker (glucosamine and mannose bound to the inositol residue) to the C-terminal amino acid of the mature protein. The two fatty acids within the hydrophobic phosphatidyl-inositol group anchor the protein to the cell membrane.

GalCer in that its carbohydrate portion is attached to the lipid portion by β-linkage rather than by an α-linkage. β-GalCer is also a ligand of the *i*Vα14 TCR, albeit at reduced efficiency than α-anomeric GalCer [68].

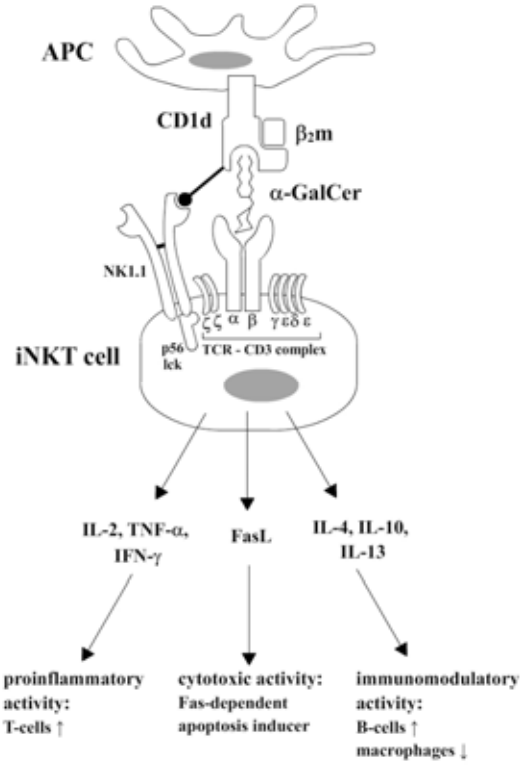

Fig. (4). Presentation of αGalCer by the CD1d molecule to iNKT cells and effector mechanisms.

The invariant TCR was first described by its recognition of α-GalCer. Similar antigens are widely used for iNKT cell identification and/or stimulation, and further ligands of the invariant TCR have been also described later. A recently discovered endogenous ligand of the invariant TCR is a glycosphingolipid: isoglobotrihexosylceramide (iGb3, a thymic selecting ceramide) [69]. Exogenous ligands are microbial cell wall components: α-glycuronosylceramide (α-GlcA-Cer) and the related glycolipid α-galacturonosylceramide (α-GalA-Cer) [70,71] derived from Shingomonas bacteria and α-galactosyl diacylglycerol (α-Gal-DAG) from B. burgdorferi, directly stimulated iNKT cells through TCR engagement [72]. A recent study by Zajonc *et al.* has investigated the crystal structure of the mouse CD1d-iGb3 complex and reviewed present knowledge about natural (endogenous and exogenous) and artificial (α-GalCer and analogue) ligands of iNKT cells [73].

The ligands are glyco- and phospholipids containing a sphingosine and an acyl chain. The two long (sphingosine and acyl) chains bind to the long apolar grooves of the CD1d. The iNKT TCR recognize the carbohydrate group of the ligand.

Function of INKT Cells

The range of actions attributed to *i*NKT cells is diverse (Fig. **4**). Administration of α-GalCer to mice results in robust activation of *i*NKT cells in a variety of organs, including spleen, liver, peripheral blood, and bone marrow. Activated iNKT cells rapidly produce a variety of cytokines and acquire a cytotoxic phenotype (Fas ligand expression). Activation of *i*NKT cells can also result in the secondary activation of a variety of other cell types, including NK cells, dendritic cells (DC), conventional CD4 and CD8 T cells, and B cells. Furthermore, α-GalCer may modulate lymphocyte differentiation, most notably the differentiation of naive $CD4^+$ T cells into proinflammatory or anti-inflammatory/humoral immunity promoting helper T cells.

The hallmark of the *i*NKT cell response is the rapid and copious production of cytokines (Fig. **4**). Secretion of the prototypical pro- and anti-inflammatory cytokines, IFN-γ and IL-4, has been most thoroughly documented, but TCR-activated *i*NKT cells produce many other cytokines, including IL-2, TNF, IL-5 and IL-13 [16].

Similarly to natural killer (NK) cells, activated *i*NKT cells have both perforin-dependent and FasL-dependent cytotoxic functions [55]. Activation of *i*NKT cells results in their apparent rapid disappearance *in vivo*, which also occurs when these cells are activated by stimuli such as anti-CD3 antibodies or IL-12 [74,75]. Thus it is generally held, that *i*NKT cells play a regulatory role in the immune response [reviewed in 76,77].

The immunoregulatory function of *i*NKT cells can be a consequence of the quality of the TCR signal. The quality of the TCR signal may influence the cytokine profile produced by the stimulated *i*NKT cell, by analogy to the effects of altered peptide ligands on conventional $CD4^+$ T cells. Perhaps the length of the ligand sphingosine or acyl chain influences the TCR signal [67,78]. IL-4 secretion was proportionally high compared to IFNγ secretion in cultured spleen cells stimulated with OCH, (an α-GalCer analogue with short sphingosine and truncated acyl chain – see above) [67,79]. On the other hand, stimulation with α-GalCer or C-glycoside analogue molecules with long chains induced a predominantly IFNγ response with low IL-4 secretion [80]. So far, it appears that iNKT cells can be polarized, similarly to conventional T-cells but contradictory data, suggesting, that iNKT cells can not be polarized have been also published. In one study, structure of the used ligand did not predict the cytokine production of *i*NKT. Furthermore, *i*NKT cells contain both IL-4 and IFNγ mRNA in similar amounts and intracellular distribution. However, one study demonstrated, that cytokine production of *i*NKT cells resulting from TCR stimulation was not influenced by IL-4 or IL-12 [81]. The type of cytokine secretion may be influenced by costimulatory signals in conventional T-cells, but activation of *i*NKT cells is not dependent on costimulatory molecules [81]. This further supports the hypothesis, that *i*NKT cells may produce pro- and anti-inflammatory cytokines simultaneously.

It has been suggested, that CD1d dependent iNKT cells primarily produce IL-4, whereas CD1d independent NKT cells produce IFN-γ in the New Zealand Black/White F1 hybrid (BWF1) mouse model (see later) of systemic lupus [82]. However, this pattern of cytokine production between the NKT cell subpopulations is probably not consistent, as the above listed publications generally report on both IL-4 and IFN-γ production after stimulating iNKT cells with α-GalCer analogues. Furthermore, α-GalCer treatment induced elevation of IFN-γ in old BWF1 mice [83]. In the same mouse model an age and/or disease related shift was observed in the predominant cytokine production of NKT cells. NKT cells of young animals (before the onset of lupus) produced IL-4 whereas NKT cells of old animals produced IFN-γ [83]. Thus it can be hypothesized, that lupus develops as a consequence of a shift in NKT cell cytokine production from IL-4 to IFN-γ.

NKT cells do not generally become polarized in their cytokine production capacity as is the case for conventional T cells. This is more obvious in mice than humans. However, cytokines can certainly impact the cytokine production profile of NKT cells. For example, IL-12 promotes the Th1 cytokine production capacity of NKT cells, but does not permanently polarize these cells. IL-7 promotes IL-4 production.

Activation of iNKT cells also plays an important role in the immediate activation of other cells (Fig. **4**) such as, NK cells. Within one hour of stimulating iNKT cells with α-GalCer, iNKT cells produced large amounts of IFNγ systemically, *in vivo* [81].

Invariant NKT- cell- derived cytokines can activate several other cell types besides NK cells [74,84], such as conventional T cells [84,85], and B cells [84,86], and recruit myeloid dendritic cells (DCs) [87,88].

In humans, *i*NKT cell numbers and CD1 expression have been associated with many autoimmune disorders, including SLE, psoriasis, rheumatoid arthritis and myasthenia gravis. *Invariant* NKT cells have a strong tendency to produce IL-4 and are especially important in its early production [8,89]. Thus, *i*NKT cells might be important in down-regulating IFNγ predominant autoimmunity [90] such as SLE [90,91]. Vα14 TCR transgenic mice, which have ten-fold increased *i*NKT-cell numbers, show elevated serum IgE and IL-4. *Invariant* NKT cell activation *in vivo* promotes IL-4-dominated immune response [84,86,93]. Furthermore, *i*NKT cells suppress cell-mediated immunity through the production of IL-4, IL-10 and/or TGF-$_\beta$ [94]. Bone-marrow DN *i*NKT cells were important in preventing graft-versus-host disease following allogenic bone-marrow transplantation, in an IL-4-dependent manner [94].

ROLE OF *I*NKT CELLS IN SLE

Human SLE

Systemic lupus erythematosus (lupus, SLE) is a chronic autoimmune inflammatory disease with complex clinical manifestations. In humans, SLE affects between 40 and 250 individuals, mostly females, in every 100 000 of the population [95]. It is generally held, that SLE is a prototype of type III hypersensitivity reaction driven autoimmunity [32] with a loss of tolerance to a variety of self-antigens [96]. Pathomechanic factors include autoantibody production against intranuclear antigens, such as double stranded (ds) and single stranded (ss) – DNA, histones, and other antigens such as the Smith antigen (Sm) and rheumatoid-factor (RF) etc. These antibodies form circulating immune-complexes with their corresponding antigens. The resulting immune complexes are deposited in capillaries of various organs, resulting in a systemic nature of the disease. Typical target organs include the skin (discoid lupus), kidney (lupus nephritis), joints and in most severe cases: the central nervous system.

The pathomechanism of SLE is unknown. The induction of human SLE is clearly dependent on an interplay between hereditary factors and exposure to environmental agents. Known risk factors include ultraviolet exposure, estorgens and smoking. A central effector mechanism is B-lymphocytic autoantibody production. Triggers may include cellular destruction, and reduced apoptotic clearance of dead cells, thus prolonged exposition of intranuclear antigens to the immune system. According to another theory, pathogen associated molecular patterns (PAMPs) recognized by toll-like-receptors (TLR) may trigger the onset of lupus [reviewed in 97]. Human SLE patients and murine models of lupus manifest a wide range of abnormalities in immune regulation. These include the production of pathogenic autoantibodies, defective clearance of apoptotic bodies, autoantibodies and immune complexes, lack of T- and B-lymphocyte regulation [63,98,99]. Both human and murine SLE is accompanied by decreased number and function of circulating *i*NKT cells [12,134,100].

As SLE develops, autoreactive helper T (Th) lymphocytes develop and regulatory T cell (Treg) numbers such as *i*NKT cells decrease, which recover with diminishing disease activity.

Murine Models

Genetic Models

MRL/lpr Mice

The lupus prone or lymphoproliferative (lpr) mice on MRL background (MRL/lpr mice), is a hereditary model of SLE, very similar to human lupus. These mice produce large amounts of IgG and IgM antibodies, including anti-DNA antibody and rheumatoid factor autoantibody [101]. The lpr phenotype is caused by a defective point mutation (isoleucine to asparagine (ILE -> ASP)) in the middle of the cytoplasmic region of the Fas apoptosis receptor leading to a truncated mRNA. This mutation abolishes the ability of Fas to transduce the apoptotic signal into cells [102]. FAS mediated apoptosis is critical in the thymic negative selection of autreactive lymphocyte clones during embryonic life. Consequently, proliferation of autoreactive T- and B- lymphocytes is initiated with the production of autoantibodies, and immunecomplex deposition. Notably, MRL/lpr mice are deficient in *i*NKT cell number and function [62].

Clinically, MRL/lpr mice experience hair loss and scab formation, typically on the dorsal neck region and the ears [103]. Light microscopy of this discoid lupus demonstrates hyperkeratosis, acanthosis, hypergranulosis, liquefaction, dermal vasodilation, and dermal T cell infiltration. Ig deposition also occurs along the dermal-epidermal junction, cellular infiltrates include $CD4^+$ and $CD8^+$ T cells as well as macrophages [104,105]. Cutaneous lesions usually occur after the onset of renal disease [2]. The mice die of nephritis or arthritis at approximately five months of age [6].

Autoimmune lymphoproliferative syndrome (ALPS) is a disorder of disrupted lymphocyte homeostasis. Patients with ALPS have mutations in the Fas apoptotic pathway, leading to abnormal lymphocyte survival, resulting in chronic lymphoproliferation and a breakdown in immunologic tolerance. ALPS was first characterized in 1992 in a group of patients who were found to have chronic lymphoproliferation, autoimmune manifestations, and an increased number of double-negative T cells (DN T cells phenotype $CD4^-$, $CD8^-$, $CD3^+$, $TCR\alpha\beta^+$) [106]. Similar DN T-cells dominate in MRL/lpr mice. These lymphocytes possess both T- (CD3) and B-lymphocytic (B220) markers without T-cell subtype/activation markers. These DN T cells are $CD4^-$, $CD8^-$, $CD3^+$ and TCR-α/β^+ and $B220^+$ and are inactive or anergic. DN $CD3^+$, $B220^+$ cells can represent more than 90% of all lymphocytes in older mice [107,108,109]. This functionally immature cell population leads to splenomegaly and lymphadenopathy.

NZB/W F_1 Mice

The New Zealand Black – New Zealand White F1 hybrid (NZB/W F_1) (BWF1) mice is another hereditary model of SLE, with striking similarities to human SLE. It is characterized by the production of IgG antinuclear antibodies, including elevated levels of IgM, anti-dsDNA antibodies, and the development of a severe immune complex-mediated glomerulonephritis in the kidney. Between 3 and 4 months of age, B cells undergo a class-switching from IgM to IgG of anti-dsDNA antibodies. Consequently, mice develop lupus nephritis and more than 95% of these mice die prematurely from renal failure before reaching 12 months of age [110,111]. The severe SLE like disease in BWF1mice is a consequence of multiple interacting genetic loci from both parental strains [112].

Chemically Induced Models

Pristane-Induced Lupus Model

In addition to the above hereditary models, lupus-like autoimmunity can be induced in otherwise non-autoimmune-prone mice by injection of hydrocarbon oils such as pristane [113,114]. Forms of chemically induced lupus, the isoprenoid alkane, pristane (2,6,10,14 tetramethylpentadecane), induces a wide spectrum of lupus-specific autoantibodies in mice. Pristane-induced lupus mirrors many characteristics of human SLE, such as autoantibody profiles, including anti-Sm and anti-dsDNA, as well as clinical features such as immune complex-mediated glomerulonephritis [113,115,116].

iNKT Cells in Murine Lupus Models

The number of *i*NKT cells and the CD1d expression is related to several autoimmune disorders such as SLE, psoriasis, primary biliary cirrhosis, rheumatoid arthritis, myasthenia gravis and ulcerative colitis [117,118,119,120,121]. Thus, CD1d deficiency and the number and function of *i*NKT cells have been investigated in most murine lupus models.

MRL/lpr Mice

MRL/lpr mice are deficient in *i*NKT cell number and function [11]. Furthermore, injection of MRL/lpr mice with anti-Vα14 antibody resulted in a reduced number of Vα14*i* NKT (*i*NKT) cells and earlier onset and exacerbation of lymposplenomegaly due to the accumulation of abnormal DN, $CD3^+$, $B220^+$ cells as well as higher titers of anti-dsDNA antibodies [101,106]. Thus, the specific reduction in Vα14*i* NKT cell number exacerbated both lupus-like disease and lymphoproliferation in MRL/lpr mice.

Beta-2-microglobulin (β_2m) deficiency inhibits the expression of classical and nonclassical (for eg. CD1d) MHC-I molecules. This prevents the normal development of CD1d-dependent *i*NKT cells, and other forms of MHC-I dependent antigen presentation. Deficiency of β_2-microglobulin (β_2m) and thus CD1d deficiency leads to a deficiency in *i*NKT cells in MRL/lpr mice. In β_2m deficient (thus also *i*NKT deficient) MRL/lpr mice an interesting dissociation of target organ disease was observed. Lupus skin lesions (inflammatory dermatitis) were accelerated, whereas nephritis was ameliorated. The authors propose that β_2m dependent iNKT regulation may operate in the skin, but not in the kidney. Similarly, exacerbation of inflammatory dermatitis have been observed by Yang *et al.* in CD1d (thus iNKT) deficient mice [122].

We have observed similar target organ dichotomy in pregnant and virgin MRL/lpr mice. Mice with repeated pregnancies had a more severe lupus nephritis progression, but skin disease was practically absent compared to virgin littermates [34].

Another study demonstrated, that activated NKT cells enhance CD8+ T cell response in the skin [123]. However, CD1-deficient MRL/lpr mice did not have similar accelerated skin or ameliorated kidney disease, suggesting that [124] regulation of autoreactive cells in this systemic disease is not solely governed by regulation of initial activation of autoreactive lymphocytes in secondary lymphoid tissues as seen in pregnant or β_2m–deficient mice [124,125]. Rather, it could be summarized, that autoimmune regulation was also affected at the target organ level, which regulation was not altered in CD1d-deficient mice [124,74].

NZB/W F_1 (BWF1) Mice

In contrast to MRL/lpr mice, the development of lupus in BWF1 mice is not linked to a deficiency in the number of *i*NKT cells. However, a recent study demonstrated that CD1d deficiency ($CD1d^0$) accelerated the onset and progression of nephritis in BWF1 mice. *Invariant* NKT cells and *i*NKT cell response is almost completely missing from the $CD1d^0$ BWF1 animals [82]. I*n vitro* stimulation of conventional T cells induced lower IL-4 production but normal IFNγ production in $CD1d^0$ compared to wild-type mice [60]. This report supports the regulatory role of CD1d and iNKT cells during the development of SLE in the BWF1 model [60]. Furthermore, a recent paper by Takahashi and Strober demonstrated, that T-helper cell dependent isotype switching of the autoantibody-production preceding glomerulonephritis require direct interactions of B-cells with iNKT cells. *Invariant* iNKT cells in this model stimulated anti-dsDNA antibody production of B-cells, while conventional T-cells did not [126]. This observation further supports a cardinal role of iNKT cells in lupus development.

Pristane-Induced Lupus Model

Similarly to the above SLE models, in the chemically induced lupus model, the regulatory role of iNKT cells have been demonstrated. In this paper by Yang *et al.* CD1d deficiency exacerbated lupus nephritis induced by the hydrocarbon oil pristane [127]. In this study CD1d -/- mice were backcrossed onto the Balb/cJ background for nine generations. Alpha-GalCer-induced expression of activation markers on spleen cells was reduced in pristane-inoculated mice. The proposed mechanism of SLE in this model is, that pristane itself reduces CD1d, leading to immunoregulatory iNKT cell deficiency and to activation of autoreactive marginal zone B-cells and lowering of IL-4 production.

CD1d Deficiency

The most convincing data on the essential role of iNKT cells in murine lupus is demonstrated by the study showing, that CD1d deficiency alone led to lupus-like syndrome with antinuclear antibody, glomerular IgG_3 and C3 deposition and proteinuria in CD1d knockout, old, non-autoimmune (Balb/c) mice [128]. The evolution of lesions was associated with age-dependent increase of anti-dsDNA and anti-cardiolipin autoantibody (preferentially IgG3) production, mainly secreted by marginal zone B cells.

Therapeutic Approaches

Alpha-galactosyl-ceramide (α-GalCer), an activating ligand of *i*NKT cells has been used extensively in various autoimmune and other immunological disease models, suggesting a possible role for its therapeutic application. The effects of α-GalCer on various manifestations of SLE have been investigated in both hereditary and induced models of SLE.

MRL/lpr Mice

Repeated administration of α-GalCer to MRL/lpr mice deficient in *i*NKT cell number and function could partially restore *i*NKT cell numbers, due to clonal expansion of *i*NKT cells. Marked clonal expansion of *i*NKT cells correlated with an increase in serum IgE antibodies, indicating a deviation to an IL-4 type cytokine response. This clonal expansion of *i*NKT cells due to α-GalCer treatment alleviated inflammatory dermatitis but did not influence nephritis [127].

NZB/W F_1 (BWF1) Mice

In contrast to MRL/lpr mice, the development of lupus in BWF1 mice is not linked to a deficiency in the number of *i*NKT cells. Forestier *et al.* investigated *i*NKT cell expansion and activation in BWF1 mice. In young, 4 weeks old BWF1 mice *i*NKT cell numbers were normal. However, with increasing age and lupus progression activated *i*NKT cells accumulated in the thymus and liver. Alpha-GalCer stimulation hyperactivated these *i*NKT cells which then produced predominantly IFNγ which is unusual. This unusual phenotype of *i*NKT cells may thus be a contributor to disease progression [129]. These results are contradictory to the beneficial role of *i*NKT cells in MRL/lpr or human lupus, discussed intensely in the paper. The authors suggest, that during disease progression lipid antigens liberated due to cell deterioration are presented to *i*NKT cells *via* CD1d inducing this unusual IFNγ producing phenotype in liver and spleen. However, the paper is not conclusive regarding the pathogenecity of these IFNγ producing *i*NKT cells. They may be a consequence and not causative in the lupus-like disease. Further dichotomous results of α-GalCer treatment have been observed in BWF1 mice. Treatment of adult mice (age 8-12 weeks) resulted in disease exacerbation, but treatment of young NZB/W F_1 mice (age 4 weeks) ameliorated SLE symptoms [83]. The authors reported that α-GalCer treatment induced IFNγ predominant cytokine immune response in old mice, but an IL-4 type cytokine response in young animals [63]. These findings suggest that the impact of α-GalCer treatment on disease in BWF1 mice depends on the stage of the disease at which treatment is initiated. In conclusion, *i*NKT cells seem to be protective before lupus initiation, but the *i*NKT cell population expands during lupus progression. IFNγ producing *i*NKT cells may contribute to disease progression in BWF1 mice.

Pristane-Induced Lupus Model

SJL (Swiss Jim Lambert) mice are susceptible [130] to autoimmune disease models including the hydrocarbon oil: pristane induced lupus nephritis whereas BALB/c and several other strains are less sensitive [116]. SJL mice have significantly less *i*NKT cells compared to BALB/c and B6 mice [131].

Pristane injection resulted in the decline of *i*NKT cell numbers and functions and cytokine production shifted from IL-4 to IFNγ production in BALB/c mice. Also, CD1d (*i*NKT) deficiency exacerbated pristane-induced SLE-like disease in BALB/c animals [130]. These data support a central role of *i*NKT cells in murine lupus [40].

Treatment with α-GalCer protected BALB/c mice against pristane induced SLE but exacerbated pristane-induced disease in SJL mice. Disease protection in BALB/c mice correlated with mixed IFNγ and IL-4 cytokine-production profile of iNKT cells and the tendency of α-GalCer to promote IL-4 type cytokine responses in this mouse strain. Conversely, α-GalCer induced disease in SJL mice correlated with an IFNγ bias in the cytokine profile of iNKT cells. So, consistent with the findings obtained using the disease models discussed here, the capacity of α-GalCer to prevent or promote SLE-like autoimmunity might be associated with its effects on the IFNγ/IL-4 balance of auto antigen-specific immune responses [132].

i*NKT Cells in Human SLE*

Humans that are genetically susceptible to autoimmune diseases, including systemic lupus erythematosus (SLE) have reduced numbers and functional defects in *i*NKT cells. These, selectively recover during remission [133].

A few human studies have investigated the presence and function of *i*NKT cells in peripheral blood in lupus patients and healthy control subjects. Two studies have demonstrated significantly lower numbers of circulating *i*NKT cells ($V\alpha24^+$, $V\beta11^+$, DN NKT cells) in SLE patients [134,135]. Another group observed reduced *i*NKT number during active SLE flare. However, successful corticosteroid therapy normalized the *i*NKT cell number [133]. The role of α-GalCer has also been investigated by Kojo S. *et al*. in different autoimmune diseases. Freshly isolated peripheral blood lymphocytes were treated with α-GalCer *in vitro* in cell culture. *i*NKT cell number was detected by flow cytometry before and after treatment. α-GalCer upregulated *i*NKT cell number in all 7 healthy controls but no such response was detectable in 5 out of 10 SLE patients [134]. The authors concluded that approximately 50% of autoimmune diseases react to α-GalCer treatment. In non-responders a functional defect of the *i*NKT cells can be suspected.

Another group investigated different rheumatic disease patients including SLE. In SLE patients $CD8^+$ *i*NKT cell number was significantly reduced in the peripheral blood as compared to healthy controls [136]. The authors suggest, that a reason for this reduced iNKT cell number could be that the *i*NKT cells migrate into inflamed tissues.

In a recent study, IgG production and NKT-like cell ($CD3^+$, $CD56^+$, see Table **1**) number inversely correlated in SLE patients. NKT-like cell number was reduced in SLE patients as compared to the control group which had high IgG and low IgM levels. Low NKT-like cell number was accompanied by high anti-dsDNA IgG levels [137].

CONCLUSIONS

The immunoregulatory function of *i*NKT cells seems to be quite well supported by data.

Based on the negative results in some animal experiments *i*NKT cells may have a complex and variable role in SLE, with a potential protective role before disease onset, followed by a potential pathogenic role once the disease is established. Such possible deleterious effects have to be carefully evaluated in the human system before introducing modulators of this T cell subset as a therapeutic approach.

The possible therapeutic application of NKT cell induction with α-GalCer or other ligands has been suggested by many papers dealing with this subject. However, to our best knowledge, no clinical trial has been initiated to date in SLE or other patients with autoimmune disease. On the other hand, in a phase I clinical study on malignant head-neck cancer patients, α-GalCer pulsed submucosal APC therapy induced the elevation of NKT cell number and function in peripheral blood. This induction was accompanied by tumor regression in some patients [65].

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