Identification of rat NKT cells and molecular analysis of their surface receptor mediated activation

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by:

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Preface:

The experimental work described in this thesis was carried out in the Institute for Virology and Immunobiology, University of Würzburg, Germany, from January 2000 to September 2003, under the supervision of Prof. Dr. Thomas Herrmann.

I herewith declare that the work contained in this dissertation is my own original work and that I have not previously submitted it at any university for a degree.

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ABBREVATIONS

Ab	- Antibody
Ag	- Antigen
α-GalCer	- α-Galactosylceramide (2S, 3S, 4R)-1-O-(α-$_D$-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol)
APC	- Antigen Presenting Cell
Bio	- Biotin
BSA	- Bovine Serum Albumin
BSS	- Balanced Salt Solution
cDNA	- Complementary DNA
CDR	- Complementary Determining Region
CIAP	- Calf Intestine Alkaline Phospatase
ConA	- Concanavalin A
DEPC	- Diethylpyrocarbonate
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
dNTP	- 100 mM solution of each dATP, dCTP, dGTP, dTTP
DP	- Double Positive
EDTA	- Ethylendiamintetracetic acid
FACS	- Fluorescence activated cell scan
F344	- Fischer rat
FCS	- Fetal Calf Serum
FITC	- Fluoresceinisothiocyanate
FL	- Fluorescence
FSC	- Forward scatter
G418	- Geneticin
h	- Hour
IFN	- Interferon
Ig	- Immunglobulin
IL	- Interleukin
J	- Joining segment of the T cell receptor variable domain

kDa	- Kilo Dalton
LEW	- Lewis rat
LN	- Lymph Node
M	- Molar
mAb	- Monoclonal Antibody
MHC	- Major Histocompatibility Complex
min	- Minutes
mRNA	- Messenger RNA
NK	- Natural Killer cell
NKT	- Natural Killer T cell
NMIg	- Normal mouse immunglobulin
OCH	- Analog of α-Galactosylceramide
ON	- Overnight incubation
PBS	- Phosphate Buffered Saline
PCR	- Polymerase chain reaction
PE	- Phycoerythrin
RNAase	- Ribonuclease A
RT	- Room Temperature
S	- Streptavidin
sec	- Seconds
SP	- Single Positive
SSC	- Side Scatter
TCR	- T-cell Receptor
TRIS	- N, N, N', N'-Tetramethylethylenediamine
V	- Variable segment of the T cell receptor variable domain
Zeo	- Zeozin

Chapter 1:
INTRODUCTION

1. MHC-restricted and MHC-unrestricted T lymphocytes

The immune sytem protects an individual organism against microorganisms, viruses and parasites. There are two types of immune responses: the innate and adaptive response. The innate system- "the first line of defence", mediated by cells including B-1 B, NK, NKT and γ/δT lymphocytes, immediately recognises and counters infection.

NK cells represent a major subpopulation of innate cells. They are preferentially expressed in the spleen and liver, but can be also found in the peripheral blood, thymus, BM and lymph nodes (Kiessling *et al.,* 1975). Activated NK cells possess anti-tumor activity (Brittenden *et al.,* 1996) and lyse cells with low expression level of syngeneic MHC or cells expressing allogeneic MHC class I molecules (Ljunggren *et al.,* 1990). NK cells produce large amounts of IFN-γ and play an important role in autoimmunity and in the defence to viral (Biron *et al.,* 1999), bacterial and parasite infections (Moretta *et al.,* 1994; Grunebaum *et al.,* 1989).

The second type of immunity- the adaptive immune system, which is regulated by B and T lymphocytes, recognises antigen and triggers the reaction to eliminate a source of Ag. B cells, through BCR, recognise Ag, proliferate and become plasma cells capable of producing and secreting Ag-specific antibodies.

T cells develop in thymus and migrate to peripheral lymphoid organs, where they mediate cellular immune responses. The majority of T lymphocytes express MHC-restricted αβTCR, which specifically recognises peptide Ags in an association with molecules encoded by class I or class II genes of the MHC (major histocompability complex).

Products of MHC class I genes (MHC class I), expressed on lymphocytes and most nucleated cells, present endogenous peptides to MHC class I restricted $CD8^+$ α/βT cells. By contrast, MHC class II is expressed in the thymus on epithelial cells as well as on APCs: B lymphocytes, macrophages and DC. MHC class II is able to present exogenous peptides, which arise from degradation of endocytosed Ags, to MHC II

restricted $CD4^+$ α/βT cells (Bevan *et al.,* 1975; Carbone *et al.,* 1990; Monaco *et al.,* 1995).

Since, $CD8^+$ T and $CD4^+$ T lymphocytes recognise different Ag, they differentially contribute to immune system. The majority of T lymphocytes are MHC class II-restricted $CD4^+$ helper cells, which activate cytotoxic T cells and regulate the functions of B cells. Whereas, $CD8^+$ T lymphocytes are cytotoxic and lyse host cells presenting "foreign" peptides on MHC class I molecules.

Almost all peripheral T helper and T cytotoxic cells express α/βTCR. Apart from classical T cells, some populations of unconventional T cells like T lymphocytes expressing γ/δTCR or CD1d-restricted NKT cells have been also described (Bendelac *et al.,* 2001). These unconventional T cells together with the B-1 subset of B lymphocytes belong to the group of innate cells, that are actively involved in innate responses and posses the ability to stimulate the cells of adaptive immunity (Benlagha and Bedelac 2000-a).

Apart from their specificity, innate T lymphocytes differ from conventional T cells. Firstly, they are autoreactive and express NK cell inhibitory receptors, which control their autoreactivity and that are absent on conventional T cells (Bendelac *et al.,* 2001). Secondly, innate lymphocytes possess germline-encoded seminvariant antigen receptor and have natural activated/memory or effector phenotype. Furthermore, unlike classical T cells, most cells of innate immunity have tissue specific distribution and are expressed at very low frequencies. Upon Ag stimulation they rapidly release cytokines and often possess cytolytic activity (Benlagha and Bendelac 2000-a; Bendelac *et al.,* 2001).

Therefore, it can be concluded that innate autoreactive γ/δT and CD1d-restricted T cells share phenotypic and functional features with NK and classical T cells. Table 1 and Table 2, presenting the classification and functional characterisation of innate T cells may be useful for understanding the correlation of these cells with conventional T lymphocytes.

Since unconventional T lymphocytes play important functions in the triggering of innate and adaptive responses, we took attempts to identify and characterise the population of rat NKT cells. Features and functions of human and mouse $V\alpha14^+$ T cells are described in the first part of the introduction. The second part of this chapter presents characteristics of the MHC class I like molecule CD1d.

Table 1: Classification of mouse T lymphocytes according to M. Emoto (Emoto and Kaufmann, 2003)

T cells															
Conventional T cells ($\alpha/\beta TCR^+NK1.1^-$)		**Unconventional T cells**													
		NK1.1$^+$ T cells						NK1.1$^-$ T cells							
		Classical NKT cells (TCRVα14$^+$, CD1d)		Nonclassical NKT cells (TCRVα14$^-$)				α/β T cells				γ/δ T cells			
				α/βTCR T cells		γ/δTCR T cells		Classical NKT cells? (TCRVα14$^+$, CD1d)		Others (TCRVα14$^-$)					
CD4$^+$T cells (MHC II)	CD8$^+$T cells (MHC I)	CD4$^+$ T cells	DN T cells	CD8$\alpha\alpha^+$ T cells	DN T cells	CD8$\alpha\alpha^+$ T cells	DN T cells	CD4$^+$ T cells	DN T cells	CD8$\alpha\alpha^+$ T cells	DN T cells	CD8$\alpha\alpha^+$ T cells	DN T cells		

Table 2: Characterisation of conventional and unconventional T lymphocytes (Emoto and Kaufmann, 2003; Bendelac 2001)

	Conventional T cells	**Unconventional T cells**	
		Classical NKT cells	**$\gamma\delta$ T cells (hVγ9/Vδ2)**
Receptor	α/βTCR	mVα14/Vβ8.2 hVα24/Vβ11 CDR3β diversity DN or CD4 NK cell markers	CDR3γ/δ DN or CD8 activating and inhibitory NK-receptors (2B4, NKG2D)
Antigen	endogenous peptides/MHCI exogenous peptides/MHCII	lipid Ags/CD1d	unknown phospoantigens/CD1c or MIC
Tissue distribution	lymphoid organs	liver, thymus, spleen, BM	epithelia, thymus, liver, peripherial blood, spleen
Effector properties	CD8$^+$T-cytotoxicity CD4$^+$T-helper functions	IL-4, IL-10 and IFN-γ	IFN-γ, TNF-α cytolysis
Functions	cellular immune responses	immunoregulation; anti-tumor, bacterial, viral and anti-parasite immunity	immunoregulation; anti-viral and anti-tumor defence; mycobacterial infections; wound healing

2. Phenotype, frequency and tissue distribution of NKT cells

Human and mouse natural killer T cells (NKT) constitute a population of lymphocytes that posses an invariant T cell receptor, expressed together with NK cell markers such as NK1.1 or NKRP1A^{int}, IL2Rβ^{int}, CD69^{int}, DX5, Ly49A and Ly49C (Bix *et al.*, 1995; Exley *et al.,* 1998).

The majority of mouse NKT cells have Vα14Jα18 TCR α-chain, preferentially paired either with Vβ8 (mainly Vβ8.2), Vβ7 or Vβ2 (Koseki *et al.,* 1991; Arase *et al.,* 1992; Lantz and Bendelac 1994; Makino *et al.,* 1995; Bendelac *et al.,* 1997).

Human CD1d-restricted NKRP1A^{+} NKT cells are characterised by the presence of an invariant Vα24JαQ TCR, which predominantly pairs with Vβ11 TCR (Porcelli *et al.,* 1993; Dellabona *et al.,* 1994; Lantz and Bendelac 1994; Exley *et al.,* 1997).

In terms of coreceptor expression, mouse NKT cells consist of CD4^{+} (60%) and DN cells (40%), with an activated T cell phenotype: CD62L^{low}, CD5^{high} CD44^{high} CD45Rb^{high} CD69^{+} (Hayakawa *et al.,* 1992; Bendelac *et al.*, 1994; MacDonald *et al.*, 1995).

The phenotype of human NKT cells may vary depending on the tissue distribution. It has been shown that CD1d-restricted Vα24^{+}JαQ TCR with no N region nucleotides is expressed by DN or CD4^{+} human PBMC, but it is absent on CD8^{+} cells (Dellabona *et al.*, 1994; Davodeau *et al.,* 1997; Porcelli *et al.,* 1993; Lee *et al.,* 2002). In the contrast to human blood NKT cells, the majority of hepatic Vα24Vβ11 NKT cells have been reported to be CD8^{+} or DN (Kenna *et al.*, 2003). However, it is not clear wether these cells are CD8αα^{+} or CD8αβ^{+} cells.

NKT cells have a tissue specific distribution. In mice, they represent 20% of α/β T cells in bone marrow, 2-5% of splenic, 0.3-0.5% of thymic and around 30% of hepatic T lymphocytes. NKT cells are rarer (0.1%-0.5%) in peripherial lymph nodes (Godfrey *et al.*, 2000; Bendelac *et al.,* 1997; Matsuda *et al.,* 2000; MacDonald 2000; Bendelac *et al.*, 1994).

Human Vα24JαQVβ11$^+$ cells are not as frequent as mouse Vα14Jα18$^+$ cells, however they can be detected among human PBLs (0.02%) or intrahepatic lymphocytes (0.5% of CD3$^+$ cells) by staining with mAbs or CD1d-tetramers (Kenna et al., 2003; Exley *et al.,* 2002).

3. Development of Vα14$^+$ T cells

The classical NKT cells originate in the thymus, while the extrathymic origin has been proposed for other NKT-like cells (Benlagha *et al.,* 2002; Eberl *et al.,* 1999-b; Bendelac *et al.,* 1995-a; Tilloy *et al.,* 1999; Pellicci *et al.,* 2002). Additionally, NKT cells may develop in the fetal liver or thymic organ cultures (Shimamura *et al.,* 1997; Makino *et al.,* 1994).

Thymic development

The canonical NKT cells originate in the thymus, where they undergo positive and negative selection. The positive selection of Vα14$^+$ cells, which requires β2m and the presence of CD1d$^+$ cortical thymocytes, results in CD4$^+$ or CD4$^-$CD8$^-$ NKT cells (Gapin *et al.,* 2001; Coles *et al.*, 2000; Bendelac *et al.,* 1995-a). CD8- a putative co-receptor for CD1d, contributes to the increased avidity of immature NKT cells for CD1d, and leads to negative selection of Vα14$^+$ autoreactive T cells (Bendelac *et al.,* 1994; Bendelac *et.al.*, 1997). However, the binding of CD8 to CD1d as well as negative selection of Vα14$^+$ cells have been tested only indirectly in CD8αβ transgenic mice, and have not been formally proven so far (Lantz and Bendelac 1994).

There are two models explaining the mechanisms of NKT cells development in thymus:

1. Mainstream model

The mainstream model, which seems to be more probable, suggests that NKT cells arise as by-product of conventional T cell development. The CD4$^+$CD8$^+$ precursors expressing invariant TCR (with randomly rearranged TCR-α genes) are able to interact with CD1d before lineage commitment, and can down regulate both CD4

and CD8, emerging as either $CD4^+$ or $CD4^-CD8^-$ (but not $CD8^+$) mature NKT cells (Pellicci *et al.*, 2002; Bendelac *et al.*, 1995-a; Lantz and Bendelac 1994).

After positive selection, immature DP $V\alpha14^+$ T cells are $CD44^{low}$ and do not express NK receptors (Gapin *et al.*, 2001). During further development they first become $CD44^{high}$ NK-receptor-negative and then they acquire the NK markers characteristic of the mature $V\alpha14^+$T cell population (Benlagha *et al.,* 2002).

2. The pre-commitment model

According to this model, NKT cells differentiate along an independent intrathymic NKT cell lineage, in which the NKT precursor cell would be rescued from cell death upon expression of invariant TCR and recognition of CD1d (Bendelac *et al.,* 1997).

Peripheral selection of $V\alpha14^+$ T cells

Mouse NKT cells are absent at birth, but gradually accumulate in the thymus, spleen and liver, reaching a plateau by 6-8 weeks of life (Bendelac *et al.*, 1994; Ohteki *et al.*, 1994).

Once NKT cells reach these organs, they might migrate to other organs or to the sites of inflammation. The circulation of $V\alpha14^+$ T cells is regulated by chemokines (MIP-2- macrophage inflammatory protein 2; MCP1- monocyte chemotactic protein) or by the presence of adherence molecules (LFA1- lymphocyte function-associated antigen) (Ohteki *et al.,* 1999; Miaymoto *et al.*, 2000).

Factors influencing the development of NKT cells

The presence of selection molecules as well as different cytokine and chemokines is necessary for development and function of NKT cells.

Mice deficient in genes encoding lymphotoxin (LT) (Elewaut *et al.*, 2000), IL-2 receptor β (IL-2Rβ) (Ohteki *et al.*, 1997), interferon (IFN)-regulatory factor 1 (IRF-1) (Ohteki *et al.*, 1998) and fyn (Eberl *et al.*, 1999-a) lack NKT cells, suggesting that these molecules are required for the generation of these cells.

The frequency and functions of NKT cells can be also regulated by cytokines. IL-18 might stimulate the cytotoxic activity of $V\alpha14^+$ T cells (Dao *et al.*, 1998), while IL-7 and IL-15 influence their frequency (Vicari *et al.,* 1994; Matsuda *et al.*, 2002; Ohteki *et al.*, 1997). Additionally, the survival of CD1d-restricted cells in the periphery can

be affected by other IL-15-responsive populations, such as NK cells or $CD8^+$ memory cells, which compete for IL-15 (Matsuda *et al.*, 2002).

4. Heterogeneity of NKT cells

Most of human and mouse TCR^{inv} positive NKT cells recognise the α-GalCer antigen, derived from a marine sponge, presented by CD1d. However, on the basis of differences in the phenotype, α-GalCer reactivity and CD1d restriction a few subpopulations of NKT cells can be distinguished.

1. Canonical NKT cells

$NK1.1^+CD1d\text{-}tetramer^+$ T cells expressing invariant TCR with $CD4^+$ or $CD4^-CD8^-$ phenotype. CD1d-dependent $CD4^+$, DN NKT cells have phenotype of activated T cells ($CD62L^-$, $CD69^+$) and express low levels of Ly49A and DX5 (Eberl *et al.*, 1999-c).They are mainly found in thymus and liver of normal mice but are absent in CD1d or Jα281 deficient mice (Chen *et al.*, 1997-a; Matsuda *et al.*, 2000; Hong *et al.*, 1999; Eberl *et al.*, 1999-c; Hammond *et al.*, 1999).

2. $NK1.1^+$, CD1d-α-GalCer $tetramer^-$ T cells

Abundant in spleen or BM, CD1d-independent $NK1.1^+$ $tetramer^-$ T cells are $CD8^+$ or DN, express NK markers (Ly49A, DX5) and have a phenotype corresponding to naive T cells ($CD62L^+$, $CD69^-$) (Eberl *et al.*, 1999-c). In contrast to classical CD1d-restricted NKT cells, such cells do not express TCR α-invariant, have different antigenic specificity and recognise self-antigens presented by CD1d, but do not respond to α-GalCer (Benlagha *et al.,* 2000-b).

3. The subset of $NK1.1^-$ but $tetramer^+$ cells

Despite the absence of NK1.1 expression, these cells are CD1d-dependent, express invariant TCR and bind CD1d-α-GalCer tetramer. The lack of NK1.1 marker might be caused by the loss of NK1.1 expression (Chen *et al.*, 1997-a) or can be dictated by the level of NKT cell maturation (Matsuda et al., 2000).

Similarly to canonical NKT cells, CD1d-restricted but $NK1.1^-$ T cells are $CD4^+$ or DN and are preferentially present in LN, in the small intestine and in gut epithelium (Hammond *et al.*, 2001; Benlagha *et al.*, 2000-b; Sykes *et al.*, 1990).

5. Antigen recognition by NKT cells

Site-directed mutagenesis of CDR3 residues of TCR, experiments with gene transfer as well as generation of Vα14Jα18 transgenic mice showed that the TCR of $V\alpha14^+$ T cells is responsible for recognition of lipid and glycolipid antigens (Grant *et al.*, 1999).

The specificity of the Ag recognition by NKT cell depends on type of carbohydrate rather than on the length of the lipid moieties. $V\alpha14^+$ TCR is able to discriminate a glucose from galactose, or an alpha from a beta anomeric form of galactose (Burdin *et al.,* 1998).
The α-anomeric form of the sugar in α-GalCer or α-GlcCer (glucosylceramide) is essential for contacting the invariant TCR of NKT cells. The β-GalCer and gangliosides can bind to CD1d but they are not antigenic because of their sugar β-linkage and failure to properly contact the TCR invariant (Kawano *et al.,* 1997; Nieda *et al.,* 1999; Sidobre *et al.,* 2002; Naidenko *et al.*, 1999). It is possible that self-antigens in β forms may be stress antigens, while exogenous α forms are not.

Self- or foreign-antigens can be recognised by CD1a, CD1b, and CD1c restricted T cells with various αβTCRs. By contrast, NKT cells, expressing invariant TCR recognise α-GalCer, self GPI-antigens (phosphatidylinositol or phosphatidylglycerol) and some unidentified lipid-antigens (Chiu *et al.*, 1999) in a CD1d-restricted manner.

6. Functions of NKT cells

NKT cells recognise glycolipid Ags presented by CD1d molecules (Bendelac *et al.,* 1995-b) in a similar manner as peptides are recognised by cytotoxic T lymphocytes (CTL). The engagement of $V\alpha14^+$ TCR with specific Ag induces a series of cellular activation events, which result in: cytokine release (Burdin *et al.,* 1999-a), the induction of co-stimulatory molecules (like B7.2, CD80, CD86) and the activation of other cell types of innate and adaptive immunity (Hermans *et al.,* 2003; Kitamura *et al.,* 1999; Carnaud *et al.,* 1999).

Activated NKT cells secrete Th1 (TNFβ, IFN-γ), and Th2 (IL-4, IL-10) cytokines, which might influence Th1-Th2 polarisation (Hayakawa *et al.,* 1992; Burdin *et al.,* 1999-a; Bendelac *et al.,* 1992; Joyce *et al.,* 2001; Singh *et al.,* 1999) and provide help for conventional T (Hermans *et al.,* 2003; Stober *et al.,* 2003; Nishimura *et al.,* 2000) or B cells (Gali *et al.*, 2003; Kitamura *et al.*, 2000; Hansen *et al.*, 2003), as well as NK cells (Carnaud *et al.*, 1999; Metelista *et al.,* 2001; Eberl *et al.*, 2000), DC cells (Gillessen *et al.*, 2003; Fujii *et al.,* 2003-b) and macrophages (Nakagawa *et al.*, 2000).

NKT cells influence other cells by producing cytokines or directly through surface receptor interaction (Nishimura *et al.,* 2000). They may also induce the maturation of other cells for example DC (Fuji *et al.,* 2003-a; Vincent *et al.,* 2002; Shreedhar *et al.*, 1999).

Additionally, the activated NKT cells, like CTLs and NK cells are able to kill target cells by Fas and perforin-dependent mechanism (Nicol *et al.,* 2000; Kawano *et al.,* 1998; Kaneko *et al.*, 2000).

The stimulation of CD1-restricted T cells may occur in lymphoid tissues as well as at the peripheral sites of inflammation. Because NKT cells express lymphoid tissue-homing chemokine receptors (CCR7, CXCR5) and homing receptors for inflamed tissue (CCR2, CCR5, CXCR3) (Kim *et al.*, 2002-b; Thomas *et al.*, 2003), they might migrate to sites of infection, where they directly or indirectly regulate immune responses (Gumperz *et al.*, 2002).

Therefore, NKT cells display regulatory functions at the onset of immune responses, long before classical B or T lymphocytes have been recruited. They influence the innate and the adaptive immune system and contribute to anti-tumor, and anti-microbial immunity, as well as autoimmunity (MacDonald *et al.,* 1995; Brutkiewicz *et al.,* 2002; Gumperz *et al.,* 2002; Schaible e*t al.*, 2000-b).

6.1. CD1-restricted cells in anti-microbial and anti-parasite immunity

CD1-restricted T cells improve host control of various bacterial *(Mycobacterium tuberculosis* (Behar *et al.,* 1999-a; Moody *et al.,* 2000; Schaible *et al.*, 2000-b)*, Mycobacterium bovis* (Emoto *et al.*, 1999; Dieli *et al.*, 2003)*, Borrelia burgdorferi*

(Kumar *et al.,* 2000*), Pseudomonas aeruginosa, Listeria* (Emoto *et al.*, 1995-b)*, Yersinia pseudotuberculosis* (Guinet *et al.*, 2002)) and protozoan infections *(Plasmodium yoelii, Toxoplasma gondii, Trypanosoma cruzi, Plasmodium berghei* (Denkers *et al.*, 1996; Hansen *et al.*, 2003; Miyahira *et al.*, 2003)).

Th1-biased human CD1a, b, and CD1c restricted T cells secrete IFN-γ and cytotoxic granules containing granulysin able to lyse immature DCs infected with *Mycobacterium tuberculosis, Mycobacterium bovis* or to directly kill the released mycobacteria (Sugita *et al.*, 2000-b; Emoto *et al.,* 1999; Schaible *et al.*, 2000-a).
In contrast, activation of CD1d-dependent NKT cells does not depend on foreign Ag. These cells usually migrate to sites of inflammation, where they influence the functions of macrophages, DCs, B cells and NK cells (Gillessen *et al.,* 2003; Carnaud *et al.,* 1999; Galli *et al.,* 2003).
Such helper function of NKT cells has been demonstrated in several infection models. During *Toxoplasma gondii* infection, $CD4^+$ NKT cells secrete IL-2 and provide help to CD8 cells, allowing them to clonally expand and respond to this parasite (Denkers *et al.*, 1996). However, by interfering with γδT cells, that have a protective role in *T.gondii* infections, NKT cells may suppress protective immunity against this parasite (Nakano *et al.,* 2001)
In experimental infection models of *Pseudomonas aeruginosa* or *Cryptococcus neoformans*, CD1d restricted T cells secrete IFN-γ, induce Th1 response and activate macrophages, which are important for resistance to these pathogens (Kawakami *et al.,* 1994; Kawakami *et al.,* 2001).
Furthermore, NKT cells, via IL-4 secretion, might promote B cell proliferation (Kitamura *et al.*, 2000) and enhance anti-parasite specific antibody (against GPI-anchored surface proteins from parasites such as *Plasmodium, Trypanosoma* and *Leischmania*) production (Schofield *et al.,* 1999; Gali *et al.*, 2003; Hansen *et al.*, 2003).

6.2. Antiviral functions of CD1-restricted T cells

NKT cells play an important role during viral infections of the liver. In mice, infection with hepatitis B virus directly or indirectly provokes activation of NKT cells and inhibition of virus replication (Kakimi *et al.*, 2000).

Furthermore, α-GalCer-activated NKT cells are known to protect wild type mice against the diabetogenic cytopathic virus, respiratory syncytial virus, genital Herpes Simplex Virus type 2 (HSV-2) or encephalomyocarditis virus (EMCV-D) (Exley *et al.,* 2001; Exley *et al.,* 2003; Ashkar *et al.*, 2003).
In the human system, NKT cells may play a role during HIV-1 infection, since the number of these cells was decreased in tested HIV-1 infected individuals (van der Vliet *et al.,* 2002; Motsinger *et al.,* 2002).

6.3. CD1-restricted T cells and cancer

NKT cells are usually required for rejection of tumors. To protect the host from certain tumors, NKT cells either directly recognise changes to self-glycolipid antigens presented on $CD1^+$ tumors, or produce cytokines and chemokines which activate anti-tumor cells, such as DC (Gillessen *et al.*, 2003), NK cells (Hayakawa *et al.,* 2002; Metelitsa *et al.*, 2001) and CTLs (Baxevanis *et al.*, 2003; Nishimura *et al.,* 2000). However, in some cases NKT cells may have opposite effect and may mediate the inhibition of anti-tumor immune responses via an IL-13- and IL-4R-STAT6-dependent suppression of CTLs (Terabe *et al.*, 2000).

6.4. CD1-restricted cells and autoimmunity

$V\alpha14^+$ T cells are regulatory and effector cells that may play a critical role in autoimmunity. The protective effect of NKT cells in variety of autoimmune diseases such as type I diabetes (Wilson *et al.,* 1998; Hong *et al.*, 2001), multiple sclerosis (MS) (Illes *et al.,* 2000; Araki *et al.*, 2003; Yamamura 2003), systemic sclerosis (Sumida *et al.,* 1995), EAE-experimental autoimmune encephalomyelitis (Jahng *et al.,* 2001; Singh *et al.*, 2001) or lupus (Zeng *et al.,* 1998) depends on their ability to secrete L-4 and/or IL-10 (Godfrey *et al.*, 2000; Miyamoto *et al.*, 2001).
Therefore, in the NOD mice, the reduced number of $NK1.1^+$ $V\alpha14J\alpha18^+$ T cells and inability to produce IL-4 usually correlates with enhanced disease (Gombert *et al.,* 1996; Hammond *et al.*, 1998).

6.5. NKT cells and tolerance

NKT cells may be crucial for the induction of tolerance. In ACAID (anterior chamber-associated immune deviation) model, systemic tolerance is induced to Ag introduced into the immunologically privileged anterior chamber of the eye. The induction of this tolerance depends on IL-10 produced by CD1-restricted NKT cells that regulates suppressor cells mediating tolerance. The tolerance in ACAID requires the presence of $V\alpha14^+$ T cells and it is abrogated in CD1-deficient mice (Sonoda *et al*., 1999; Sonoda *et al.,* 2002-a; Nakamura *et al.,* 2003).

NKT cells may also affect GVHD (graft-versus-host disease) by enhancing the effectiveness of BM transplantations. Bone marrow derived $NK1.1^+$ T cells, when added to the donor bone marrow cells infused into the recipient mice, could suppress GVHD. In this case, the protective effect strictly depended on IL-4 secreted by NKT cells (Zeng *et al.,* 1999).

Additionally, NKT cells are involved in the protection of embryos during pregnancy. Under physiological conditions, Vα14 NKT cells prevent the rejection of the fetus. In contrast, upon α-GalCer stimulation they secrete TNF-α and IFN-γ and provoke abortion by perforin-dependent killing (Ito *et al*., 2000; Mrakovcic-Sutic *et al*., 2003; Boyson *et al.,* 2002).

7. Properties of rat "NKT" cells

Mouse and human NKT cells are well characterised, while these cells remain uncharacterised in rats.

The presence of $NKRP1^+$ lymphocytes in rat spleen was first described by Kaufman and co-workers (Kaufman *et al.,* 1993). These splenic cells were of three different phenotypes: $NKRP1^{bright}\alpha\beta TCR^-$, $NKRP1^{dim}\alpha\beta TCR^+$, or $NKRP1^{dim}\alpha\beta TCR^-$ (Kaufman *et al.,* 1993) and were expressed at very low frequency.

The subset of $NKRP1^{dim}\alpha\beta TCR^+$ lymphocytes, which phenotypically would correspond to mouse NKT cells, expressed CD8 but did not contain cytoplasmic granules and did not exhibit NK lytic activity (Kaufman *et al.,* 1993; Brissette-

Storkus *et al.,* 1994). Since no information about functional features of these cells was given, it is not clear if rat NKRP1^{dim}αβTCR^{+} cells indeed represented the population of mouse Vα14^{+} T cells.

A second report on "rat NKT cells" describes PVG rat NKRP1^{dim}αβTCR^{+} clones, generated *in vitro* by culture of splenic cells with MIP-1α (macrophage inflammatory protein-1α) (Knudsen *et al.,* 1997). PVG rat Vβ8.2^{+} clones were either CD4^{+} or CD4^{-}CD8^{-} (DN) and could release various cytokines (Knudsen *et al.,* 1997). However, it was difficult to find a correlation between the phenotype of these clones and the cytokine secretion pattern.

The existence of the NKT cells in rats was questionable until 2000, when Matsuura and co-workers identified a population of F344 rat CD44^{high} T lymphocytes expressing the Vα14^{+} TCR, with Vα–Jα junctional region corresponding to mouse invariant TRAV14-J18 (Matsuura *et al.,* 2000). The nomenclature of TCRα given by Matsuura does not correspond to the generally accepted nomenclature of IMTG (http://imgt.cines.fr:8104). However, the Matsuura nomenclature was more appropriate for analysis of rat Vα14^{+} TCR cloned in this thesis, and was therefore used thoughout the thesis.

Rat invariant TCRα is coded by four TRAV14 genes, which on the basis of diversity accumulated in CDR2 region can be categorised into two groups (Matsuura *et al.,* 2000). Type 1 invariant TCRα, including products of TRAV14S1 and TRAV14S2 genes, is preferentially expressed by hepatic and thymic lymphocytes. Whereas, type II represented by the product of TRAV14S3 gene is expressed predominantly in the spleen (Matsuura *et al.,* 2000).

The presence of Vα14^{+} TCR cells in various F344 rat organs confirms the existence of NKT cells in species other than human or mouse. Unfortunately, data presented by Matsuura allow only the genomic and phenotypic characterisation of F344 NKT cells but functional capabilities of these cells remain unknown.

CD1d- Ag presenting molecule

8. The CD1 family

CD1 molecules are cell surface glycoproteins, able to present lipid and glycolipid antigens to T cells (Porcelli *et al.,* 1995-b; Bendelac *et al.*, 1997). The nonpolymorphic CD1 genes map outside the MHC region and encode: CD1a, CD1b, CD1c and CD1e in humans, and CD1d1 and CD1d2 proteins in mice (Calabi *et al.*, 1986; Martin *et al.,* 1986; Balk *et al.,* 1989). Based on sequence homology in leader α-1 and α-2 domains, CD1 molecules can be divided into two groups: group I comprises human CD1a, CD1b, and CD1c, and group II includs human and mice CD1d (Porcelli *et al.*, 1995-a; Sugita *et al.*, 2000-b; Calabi *et al.*, 1991). Human CD1e, for which the gene is known but the gene product has not been yet identified, is intermediate between group I and group II of CD1 molecules.

Except in humans, the CD1 family genes have been found in mouse (Bradbury *et al.,* 1990), rat (Ichimiya *et al.,* 1994), rabbit (Calabi *et al.,* 1989), cow (MacHugh *et al.,* 1988), pig (Chun *et al.*, 1999), cat (Woo *et al.,* 1997), guinea pig (Dascher *et al.*, 1999), rhesus macaques (Kashiwase *et al.,* 2003) and sheep (Ferguson *et al.,* 1996).
In mice, there are two CD1 homologous genes: CD1d1 and CD1d2 (carrying a point mutation, which prevents its surface expression), which are related to human CD1d (Porcelli *et al.,* 1995-a).
Rat has only one CD1 gene encoding CD1d protein with high structural similarity to human and mouse CD1ds (Ichimiya *et al.*, 1994). Two diverse allelic forms of CD1d, expressed in different rat strains, have been described (Katabami *et al.*, 1998).

9. Expression of CD1

Human group 1 CD1 molecules (CD1a, b, c) are expressed in thymus, on immature cortical thymocytes (Wang *et al.*, 2000). Extrathymically, CD1 is present on professional APCs, epidermal Langerhans cell, dendritic cells, activated

macrophages, and on subset of B cells (with high expression of CD1d by marginal zone B cells) (Porcelli *et al.*, 1995-a; Beckman *et al.,* 1995; Sonoda *et al.,* 2002-b; Amano *et al.,* 1998; Canchis *et al.,* 1993; Blumberg *et al.,* 1991).

Additionally, human group 2 CD1 (CD1d) appears to be highly expressed on liver hepatocytes (Bleicher *et al.,* 1990; Sugita *et al.*, 2000-b) and to some extent on the intestinal epithelial cells (Balk *et al.,* 1994; Blumerg *et al.,* 1991). By contrast, the presence of CD1 on mouse IEC is controversial (Brossay *et al.*, 1997).

In mouse and rat, CD1d can be detected in lymphoid organs including: lymph nodes, thymus and spleen as well as in nonlymphoid organs like liver, kidney and lung (Brandbury *et al.,* 1990; Ichimiya *et al.*, 1994; Brossay *et al.,* 1997).

10. Structure of CD1

Protein sequences of human and rodent CD1d show high structural homology. The heavy chain of CD1d protein contains leader sequence, three extracellular domains (α1, α2 and α3), a transmembrane region, and a short 6-10 amino acid cytoplasmic tail. A leader peptide signals co-translational insertion of the heavy chain into the ER membrane. The α1 and α2 domains of the extracellular region form the antigen binding pocket of CD1, while the α3 domain associates noncovalently with β2-microglobulin (Zeng et al., 1997; Calabi *et al.,* 1991; Ichimiya *et al.*, 1994).

Rat CD1d

Rat CD1d cDNA contains an open reading frame of 1008 bp, which encodes a polypeptide of 336 amino acids. A high level of sequence similarity between rat and mouse CD1d can be observed, especially in the extracellular domains. Rat CD1d shows more homology with mouse CD1d1 than CD1d2 (Ichimiya *et al.*, 1994; Kasai *et al.,* 1997).

Antigen-binding groove of CD1d

The antigen-binding groove of CD1d is narrower and deeper than that of MHC class I or II and forms a large hydrophobic pocket, well adapted to bind fatty-acid chains (Zeng *et al.*, 1997; Burdin *et al.,* 2000; Grant *et al.,* 1999). The polar head group of

presented lipid is important for the positioning and the presentation of antigenic residues to TCR (Cantu *et al*., 2003; Park *et al*., 1998-a).
Four amino acid residues of antigen-binding groove have been shown to be crucial for α-GalCer presentation. Asp80, Arg 79 and Glu 83 are important for binding to carbohydrate region, while Asp153 is necessary for interaction with amine group of fatty acyl chain (Kamada *et al.,* 2001).
The binding of ligands to antigen-binding groove, which occurs via hydrophobic interactions is stable but non-specific and of low affinity, allowing many types of lipids to be buried in different positions in the groove (Schaible *et al*., 2000-a; Park *et al*., 2000; Cantu *et al*., 2003).

11. Assembly of CD1d protein. Intracellular distribution of CD1 isoforms and pathways of CD1d antigen presentation.

During generation, the CD1 protein is translocated into the endoplasmatic reticulum (ER), where the heavy chain associates with calnexin and careticulin, (which have chaperone-like function) and assembles with β2m (Sugita *et al.*, 1997; Park *et al*., 1998-a; Jayawardena-Wolf *et al*., 2001; De Silva *et al*., 2002).
The binding of lipid Ags might occur in the secretory pathways, either directly at the cell surface or after internalisation in acidified intracellular compartments (Park *et al*., 2000). CD1 isoforms may have different cellular distribution. Human CD1b is mostly expressed intracellularly, accumulated in lysosomes. CD1c has been found on the plasma membrane and in intracellular vesicles (Sugita et al., 1996; Schaible *et al*., 2000-b). CD1a accumulates in endocytic compartments of Langerhans DCs, while CD1d is present in lysosomes. Therefore, human CD1a and CD1c can be detected mainly in early endosomes, CD1b and CD1d as well as mouse CD1d in late endosome and lysosome (Briken *et al*., 2000; Moody *et al*., 2003; Park *et al*., 1998-a).

Human CD1b and human or mouse CD1d proteins transit first to the cell surface, before they reach endosomes (Moody *et al*., 2003). The access to the endocytic pathway is regulated by tyrosine-based targeting motif YXXZ (where Y is tyrosine, X any amino acid and Z is a bulky hydrophobic aa), present in the cytoplasmic tail

of CD1 (Jackman *et al.*, 1998; Porcelli *et al.*, 1995-a; Cernadas *et al.*, 2003). The mutant CD1b and CD1d molecules, lacking this cytoplasmic tail motif fail to redistribute in the cell and lose the ability to present glycolipids to NKT cells (Chiu *et al.*, 2002; Sugita *et al.*, 2000-a; Park *et al.*, 1998-a; Park *et al.*, 2000; Briken *et al.*, 2000; Elewaut *et al.*, 2003).

12. Functions of CD1 molecules: presentation of lipid antigens to NKT cells

The CD1 molecules present lipid and glycolipid antigens to T cells (Briken *et al.*, 2000; Moody *et al.*, 2003; Gumperz et al., 2000). Distinct innate or acquired immunity derived lipid antigens, accumulated in endocytic compartments bind to group 1 of CD1 molecules. The endosomal pathways promote the presentation of foreign glycolipids that are internalized from exogenous sources by antigen-presenting cells. Self-lipids that comprise the membranes of APCs, can be presented to autoreactive T cells within non-endosomal compartments (Moody *et al.,* 2003).
Different classes of lipid Ags may be presented by CD1 to CD1-restricted T cells. Foreign mycobacterial lipids, which are structural components of the mycobacterial cell wall such as mycolic acids, glucose monomycolate and derivatives of lipoarabinomannans (LAM) (e.g.phospatidylinositolmannosides) are presented to human $CD4^-CD8^-$ $\alpha\beta TCR^+$ T cells by CD1b, whereas hexose-1- phosphoisoprenoids are presented in the context of CD1c (Sieling et al., 2000; Moody *et al.*, 1997; Porcelli *et al.*, 1992; Prigozy *et al.*, 1997; Sieling *et al.*, 1995; Moody *et al.,* 2000; Park *et al.*, 2000; Schaible *et al.*, 2000-b).

α-GalCer

Human and mouse CD1d have not yet been shown to present pathogen-derived lipid antigens. Nevertheless, various glycosylated ceramides, like α-GalCer and self glycosylphosphatidylinositol (GPI) anchors, that stimulate NK1.1 T cells have been described (Joyce *et al.,* 1998; Burdin *et al.,* 1998; Kawano *et al.,* 1997).
Obtained from the marine sponge *Agelas mauritanius*, α-GalCer-antigen has been first identified in the screen of reagents that could prevent tumor metastases in mouse liver (Morita *et al.,* 1995; Natori *et al.,* 1997; Kobayashi *et al.,* 1995). α-

GalCer contains α-anomeric sugar with sphingosine base and 26 carbon long acyl chain. Used in picomolar concentrations it specifically activates mouse Vα14/Vβ8.2 and human Vα24/Vβ11 NKT cells, making it a valuable reagent for the analysis of CD1d-restricted T cells (Morita *et al.*, 1995; Kawano *et al.*, 1997; Brossay *et al.*, 1998-c; Spada *et al.,* 1998; Matsuda *et al.*, 2000).

Other ceramide- and phosphatidylinositol-based glycolipids compete with α-GalCer for binding to CD1d. When loaded on CD1d, they inhibit presentation of α-GalCer to NKT cells, but themselves fail to stimulate α-GalCer-restricted cells (Benlagha *et al.*, 2000-b).

Additionally, mouse CD1d was reported to present hydrophobic peptides (Castano *et al.*, 1995; Brossay *et al.*, 1998-b). As shown by binding-competition assays, the binding of peptide antigen to CD1d probably takes place outside the groove or to the groove of a subset of CD1d molecules. Thus, glycolipids and peptides do not compete for the same binding site (Naidenko *et al.,* 1999; Tangri *et al.,* 1998).

13.Tetramers

CD1d-α-GalCer loaded multimers have been recently created as a new, efficient tool to identify $V\alpha14^+$ T cells within complex mixtures of cells. These oligomers have long half-life and are usually cross-reactive. Mouse CD1d-α-GalCer complexes might be used to stain both mouse and human NKT cells (Benlagha *et al.,* 2000-b). Similarly, human CD1d oligomers positively stain mouse α-GalCer specific cells, confirming that CD1d/Vα14 T cell system is highly conserved across species (MacDonald 2000; Matsuda *et al.*, 2000; Brossay *et al.*, 1998-c).

The reactivity of CD1-α-GalCer complexes requires multimerization, and neutral pH for loading with Ag (Matsuda *et al.*, 2000*)*. Monomers composed of mouse CD1d molecule loaded with α-GalCer are able to significantly but weakly stain $V\alpha14^+J\alpha18^+$ cells. CD1d dimers are more effective than monomers, while CD1d-α-GalCer tetramers are the most efficient and bind to NKT cells with around 50-fold greater fluorescence intensity than monomers (Matsuda *et al.*, 2000; Benlagha *et al.,* 2000-b).

As previously reported, CD1d-tetramers are not restricted to a particular Vα14-Vβ combination (Matsuda *et al.*, 2000). They are also much more sensitive than classical monoclonal antibodies and are able to positively stain small populations of cells (e.g. intestine or lymph nodes $NK1.1^-$ but $tetramer^+$ lymphocytes), not detectable by conventional FACS staining (Benlagha *et al.,* 2000-b; Matsuda *et al.*, 2000).

Chapter 2.
MATERIALS AND METHODS

MATERIALS

Chemical reagents

Agar-agar	Roth (Karlsruhe, Germany)
α-GalCel	National Institute of Neuroscience, NCNP (Tokyo, Japan)
α-GalCel-mCD1d-PE tetramer	Olga V. Naidenko, Ph.D., La Jolla Institute for Allergy and Immunology San Diego, CA (reference: Hammond *et al.,* 2001)
Agarose	Roth (Karlsruhe, Germany)
Ammoniumchloride	Roth (Karlsruhe, Germany)
Ampicillin	Gibco BLR (Eggenstein, Germany)
APS	Roth (Karlsruhe, Germany)
β-mercaptoethanol	Gibco BLR (Eggenstein, Germany)
Boric acid	Roth (Karlsruhe, Germany)
Calcium Chloride	Roth (Karlsruhe, Germany)
Concavalin A	ICN (Meckenheim, Germany)
DEPC (Diethylpyrocarbonate)	Sigma (Deisenhofen, Germany)
Diatomaceous earth	Sigma (Deisenhofen, Germany)
DMSO (Dimethyl sulfoxide)	Sigma (Taufkirchen, Germany)
dNTP Set	Peqlab Biotechnologie (Erlangen, Germany)
Ethanol	Roth (Karlsruhe, Germany)
Ethidium bromide	Roth (Karlsruhe, Germany)
Ficoll-Paque	Pharmacia Biotech
Formaldehyde	Roth (Karlsruhe, Germany)
Formamide	Roth (Karlsruhe, Germany)
G418 (Geneticin solution)	Sigma (Schnelldorf, Germany)
Glycine	Roth (Karlsruhe, Germany)

IL-2 (recombinant human interleukin-2)	Hoechst (Frankfurt, Germany)
IL-6 (recombinant human interleukin-6)	Strathamann Biotec AG (Hamburg, Germany)
IPTG (Isopropyl-βD-Thiogalactopyrasoide)	Biomol Feinchemikalien (Ilvesheim, Germany)
Isopropanol	Roth (Karlsruhe, Germany)
LB (Broth Base medium)	Gibco BLR (Eggenstein, Germany)
Magnesium Chloride	Roth (Karlsruhe, Germany)
Mineral oil	Sigma (Schnelldorf, Germany)
Sodium Azide	E.Merck (Darmstadt, Germany)
Sodium Chloride	Roth (Karlsruhe, Germany)
Sodium Butyrate	Sigma (Deisenhofen, Germany)
Nylon wool	Fenwal Laboratories (Deerfield, USA)
OCH	National Institute of Neuroscience, NCNP (Tokyo, Japan) (reference: Miyamoto *et al.*, 2001)
PEG 1500 (Polyethylenglycol)	Boehringer (Mannheim, Germany)
Penicillin	Gibco BLR (Eggenstein, Germany)
Percoll	Amersham Pharmacia (Freiburg, Germany)
Polybrene (Hexadimthrinbromide)	Sigma (Deisenhofen, Germany)
SDS Sodium dodecyl sulfate	Sigma (Deisenhofen, Germany)
Trypan blue	Sigma (Deisenhofen, Germany)
Tween 20	Sigma (Deisenhofen, Germany)
X-Gal (5-Bromo-4-Chloro-3Indolyl-β-D-Galactopyranose)	Quantum Appligene
Zeozin	Cayla (Toulouse Cedex, France)

Media, Buffers, Solutions:

Media used for cell cultures were obtained from GibcoBRL (Eggenstein, Germany):

DMEM (Dulbecco's Mod Eagle Medium) with Pyruvate, without HEPES #41966-029

DMEM (Dulbecco's Mod Eagle Medium) without Pyruvate, with HEPES

#41966-027

$RPMI^-$	RPMI 1640 L-Glutamine	#21875-034
$RPMI^+$	$RPMI^-$ with 10% of SC (supplemet complete)	

- SC-supplement (50 ml of SC per 500 ml of $RPMI^-$):

500 ml	heat-deactivated FCS
100 ml	Na puryvate 100 mM
100 ml	non-essential amino acids
100 ml	Penicillin-Streptomycin (10000U/ml)
5 ml	β-Mercaptoethanol 50 mM
58.4 ml	L-Glutamine solution 5%

- ATV:

0.05%	Trypsin
0.02%	EDTA in PBS

- 10*Trypan Blue Buffer:

0.5%	Trypan Blue
0.5%	Xylenxyanol
50%	Glycine
10 mM	EDTA

- Trypan blue solution:

0.04%	Trypan blue in PBS/BSA/Azide

- BSS (Hanks balanced salt solution):

to prepare BSS 125 ml of BSS I was mixed with 125 ml of BSS II add filled with water to 1 L

BSS I:	50 g	Glucose
	3 g	KH_2PO_4
	11.9 g	NaH_2PO_4
	0.4 g	Phenylred
		water to 5 L

BSS II:

9.25 g	$CaCl_2$
20 g	KCl
320 g	NaCl
10 g	$MgCl_2$
10 g	$MgSO_4$
water to 5 L	

- BSS/BSA

  0.2 % BSA in BSS

- DEPC water

0.1 g	DEPC

  Water to 1 L, sterilised in autoclave

- 2*HBS

50 mM	HEPES pH 7.05
10 mM	KCl
12 mM	Glucose
280 mM	NaCl
1.5 mM	NaH_2PO_4

Three different pH (pH 7.0, 7.05, 7.1) were prepared and tested. Aliquots were stored at -20°C.

- LB- medium

  20 g LB

  Water to 1 L, autoclaved and stored at 4°C

- PBS (Phosphate buffered saline)

4 mM	KH_2PO_4
16 mM	Na_2HPO_4
115 mM	NaCl

  pH 7.3

- PBS/BSA/Azide

0.2%	BSA
0.02%	Na-Azide in PBS

- TAC (Tris-Ammoniumchlorid):

20 mM	Tris pH 7.2
0.82%	NH_4Cl

- 10*TBE

890 mM	Tris
890 mM	Boric acid
20 mM	EDTA

- <u>Hyper-Magic MiniPrep Reagents:</u>

Buffer 1:

50 mM	Tris-HCl pH 7.5
10 mM	EDTA pH 8.0
100 µg/ml	RNAse

Buffer2:

0,2 N	NaOH
1%	SDS

Buffer3:

2.55 M	K-Acetate pH 4.8
pH adjusted with glacial acid	

Buffer L6:

Guanidinium thiocyanyte	
0.1M	Tris-HCl pH 6.4
0.5M	EDTA pH8.0
destilled water	
Triton X100-	all dissolved at 37°C

Dia-Mix:

10 g	Diaetomaceous earth
50 ml	destilled water
50µl	concentrated HCl

Washing Buffer:

100 mM	NaCl
10 mM	Tris-HCl pH 7.5
2.5 mM	EDTA
destilled water	
ethanol	

- Reagents needed for cell fusion:

Following media and reagents were used during fusion procedure:

1 ml of PEG 1500 (Polyethylenglycol)- Boehringer (Mannheim, Germany)

15 ml of RPMI medium

30 ml of RPMI medium supplemented with 10% SC and 5% FCS (Gibco BRL # 10081; Karlsruhe, Germany)

100 ml of RPMI medium containing 10% of SC, 5% FCS and IL-6 (60 µl of stock 5 µg/ml per 100 ml medium)

To prepare 250 ml of selection HAT medium

202.5 ml of RPMI medium

10 ml of HAT

25 ml of SC

12.5 ml of FCS were taken.

For further incubation HT-medium was used. 500ml of HT medium consisted of:

415 ml of RPMI medium

10 ml of HAT

50 ml of SC

25 ml of FCS

300 µl of IL-6 (stock 5 µg/ml)

Cell lines:

293T	transformed primary human embryonal kidney cells ATCC#CRL 1573
BW5147	Do-11.10.7 hybridoma derived AKR/J mouse αβTCR-negative cell lymphoma; ATCC#TIB-233
M12.4.1.C.3	MHC II negative mouse B cell lymphoma (reference: Glimcher *et al.,* 1985)
P80	P815 mouse (DBA/2) cell mastocytoma (ATCC#TIB-64) transduced with rat CD80
RMA-S	RMA-derived TAP2-deficient mouse T lymphoma
A20J	mouse B cell lymphoma; ATCC#TIB-208
RAJI	human Burkin lymphoma; ATCC#CCL 86
H-4-II-E	rat hepatoma, Reuber H35 CRL-1548; Prof. Dr. O.Götze Zentrum für Hygiene und Humangenetik, Göttingen
FAO	rat hepatoma- a differential line derived from H4-11-E-C3ECACC 89042701; Prof. Dr. O.Götze Zentrum für Hygiene und Humangenetik, Göttingen
JM2	rat Fischer F344 liver hepatoma; ICLC ATL99009; B.Parodi, National Institute for Cancer Research, Genova
A20mCD1d	mouse A20 cell line infected with mouse CD1d
KT12	mouse KT12 hybridoma expressing mouse TCR^{inv}; last two cell lines were a gift of Susanna Cardell, Immunology section, Department of Cell and Molecular Biology, Lund University, Sweden; (reference: Makowska *et al*., 2000)
SP2/O	murine myeloma used as fusion partner, ATCC CRL 1581

Animals:

Mouse:

C57/Bl6J	were inbred in the Institute for Virology and Immunobiology animal facility
BALB/cJ $CD1d^{-/-}$	were provided by S. H. Kaufmann, Department of Immunology, Max-Planck-Institute, Berlin, Germany

Rats:

F344/Crl	were obtained from Charles River Wiga (Sulzfeld, Germany)
LEW/Crl	were inbred in the Institute for Virology and Immunobiology animal facility

Vectors:

Topo pCR®2.1-TOPO – TOPO Vector was obtained from Invitrogen Karlsruhe, Germany

pVSVG pCZVSV-G wt, containing *env* form Vesicular Stomatitis Virus (VSV) (Yee *et al.,* 1994)

pHIT-60 pHIT60-CMV-MVV-gag-pol-SV40ori; containing *gag* and *pol* from Moloney Murine Leukemia Virus (MoMLV) under the control of human Cytomegalovirus (CMV) promotor

pczCG5 IEGZ and pczCG5 IEGN- Retroviral vectors for the MuLV driven constitutive expression of the gene of interest inserted in the Polylinker (EcoRI/NcoI/BamHI) and through IRES mediated expression the EGFP-Zeo (EGZ) or EGFP-Neo (EGN) fusion protein; kindly provided by I.Berberich, Institut für Virologie und Immunbiologie, Universität Würzburg

Antibodies:

Mouse Abs:

F23.1	mouse anti-mouse Vβ8.1, 8.2 TCR (isotype: mouse IgG2a; reference: Behlke *et al.,* 1986)
mCD3	hamster anti-mouse CD3ε chain (isotype: hamster IgG1κ; reference: Leo *et al.,* 1987)
1B1	rat anti-mouse CD1d (isotype: rat IgG2b; reference: Brossay *et al.,* 1997)
CD4	anti-mouse CD4 (isotype: rat IgG2b; reference: Wineman *et al.,* 1992)

CD8	anti-mouse CD8β
	(isotype: rat IgG2b; reference: Takahashi *et al.,* 1992)
NK1.1	PK136 anti-mouse NK1.1
	(isotype: mouse IgG2a; reference: Koo *et al.,* 1984)

Rat Abs:

R73	anti-rat TCR-α/β
	(isotype: mouse IgG1κ; reference: Hünig *et al.,* 1989)
R78	rat TCR-Vβ8.2 in LEW rat TCR-Vβ8.4 in DA
	(isotype: mouse IgG1κ; reference: Torres-Nagel *et al.,* 1993)
W3/25	anti-rat CD4
	(isotype: mouse IgG1κ; reference: Mason *et al.,* 1983)
OX35	anti-rat CD4
	(isotype: mIgG2a; reference: Jefferies *et al.,* 1985)
OX38	anti-rat CD4
	(isotype: mIgG2a; reference: Jefferies *et al.,* 1985)
3.4.1	anti-rat CD8
	(isotype: mouse IgG1κ;reference: Torres-Nagel *et al.,* 1992)
NKRP1-A	anti-rat NKRP1-A
	(isotype: mouse IgG1κ; reference: Kraus *et al.,* 1996)

controls, secondary antibodies:

NMIg	- normal mouse Ig
L180	- mouse antibody specific to sheep red blood cells
	(reference: Hünig *et al.,* 1985)
2.4.G.2	- culture supernatant containing Abs against mouse Fcγ receptor
	anti rat CD16- Ab blocking binding to rat Fc receptor
	(reference: Latour *et al.,* 1992)
DαMIg	-PE or Cy5.5 conjugated $(Fab')_2$ fragment of donkey anti-mouse IgG

Streptavidin-Cy-Chrom

All monoclonal Abs were from PharMingen (Heidelberg Germany), while secondary detection Abs were from Serotec (Düsseldorf, Germany).

Kits:

Rneasy(r) MiniKit	# 74104 QIAGEN (Hilden, Germany)
Plasmid Maxi Kit	# 12163 QIAGEN (Hilden, Germany)
QIAquick PCR Purification Kit	# 28104 QIAGEN (Hilden, Germany)
First Strand cDNA Synthesis Kit	# K1612 MBI Fermentas (St.Leon-Rot, Germany)
BigDye Terminator Cycle Sequencing	# 4303152 Applied Biosystem (Warrington, United Kingdom)
Topo pCR®2.1-TOPO-TA Cloning®Kit;	# K450-01 Invittrogen (Karlsruhe, Germany)

ELISA kits were obtain from BD Bioscience Pharmigen (Heidelberg, Germany):

OPTEIA mouse IL-2 Set	# 555148
OPTEIA mouse IL-4 Set	# 555232
OPTEIA mouse IFN-γ Set	# 555138
OPTEIA rat IL-4 Set	# 2631KI
OPTEIA rat IFN-γ Set	# 2696KI

Oligonucleotides

Oligonucleotides were obtained from MWG- Biotech AG (Ebersberg, Germany)

- Control primers:

β-actin-Fow	5'-CTA TCG GCA ATG AGC GGT TC-3'
β-actin-Rev	5'-CTT AGG AGT TGG GGG TGG CT-3'

- Primers binding to the vector sequence, used for cloning:

M13F	5'-GTA AAA CGA CGG CCA-3'
M13R	5'-CAG GAA ACA GCT ATG AC-3'
PSI	5'-CAC GTG AAG GCT GCC GAC C-3'
IRES	5'-CTT CGG CCA GTA ACG TTA GG-3'

- Primers used for PCR of mouse and rat Vα14 TCR:

rVα14/1,2,3Fow	5'-TTT GGG GCT AGG CTT CTG-3'

Vα14Leader	5'-ATG AAA AAG CGC CTG AGT GCC TGC TGG-3'
RCαend-STOP-anti	5'-TCA ACT GGA CCA CAG CCT TAG CG-3'
RCαendRev	5'-TGA ATG GTC AGC AGC AGC AA-3'
mVα14kos	5'-TAA GCA CAG CAC GCT GCA CAT-3'
rVα14kos	5'-TAA GCA CAG CAC CCT GCA CAT-3'
Cαkos-anti	5'-AGT CGG TGA ACA GGC AGA GG-3'

- Primers used for cloning of mouse and rat Vα14 TCR as also for production of chimeric Vα14 TCRs:

rVα14-EcoRI-Fow	5'-GGG CTA GAA TTC TGC AGA AAA ACC ATG GGG AAG C-3'
rVα14-EcoRI-Rev	5'-CGC GAA TTC AAT CTC AAC TGG ACC ACA GCC-3'
mVα14-EcoRI-Fow	5'-GGG GAA TTC AAC CAT GAA AAA GCG CC-3'
mVα14-EcoRI-Rev	5'-CCC GAA TTC CTC AAC TGG ACC ACA GCC-3'
r/mVα14Rev	5'-CAC CAC ACA GAT GTA GGT GGC AG-3'
mVα14endSTOP	5'-TTA GCA TCT TTA TCC AGA GTT GC-3'
RVα14endSTOP	5'-TTA GCG CCT TCA TCC AGA GTT GCC G-3'

- Primers used for cloning of mouse Vβ8.2 TCR:

mVβ8.2-BamHI	5'-CGG GAT CCT GAG ATG GGC TCC AGG CTC TTC-3'
mCαend-BamHI	5'-GGG GGA TCC TCA GGA ATT TTT TTT CTT GAC C-3'

- Primers used for cloning and screening of rat CD1d:

rCD1dRev	5'-TTC TGA GCA GAC AAG GAC TGA-3'
N366	5'-TCG GAG CCC AGG GCT GTG TAG A-3'
E1R	5'-TTG ATA GGA GCG TCT CCT TT-3'
E2F	5'-TGT ACC TAC CGT GCC TGT TG-3'

E2R	5'-GAT GAT GTC CTG CCC CTC TA-3'
E3F	5'-TCC AGC AGA ATT ACA CCT TCC-3'

Enzymes, Inhibitors

Rnase A	Promega (Heidelberg, Germany)
T4-DNA-Ligase	Promega (Heidelberg, Germany)
Pfu-DNA-Polymerase	Stratagene (Heidelberg, Germany)
MBI Taq-DNA Polymerase	MBI Fermentas (St.Leon-Rot, Germany)
HotStar DNA Polymerase	Qiagen (Hilden, Germany)
CIAP	MBI Fermentas (St.Leon-Rot, Germany)
Restriction Enzymes: (EcoRI, BamHI, HindIII, PstI, EspI(Bpu1102I))	MBI Fermentas (St.Leon-Rot, Germany)

METHODS

Cell Culture Methods:

1. Cell culture

Cells were cultured in sterile conditions in CO_2 cell culture incubator at 37°C with 5% CO_2 and H_2O-saturated atmosphere. Almost all cell types were cultivated in $RPMI^+$ medium. DMEM medium was used for hepatomas and adherent cell lines. The cells were maintained in 24-well tissue culture plates and 50 ml culture flasks. For cell expansion 12, 6-well plates and 250 ml plastic flasks were used. Depending on cell type (cell line) cells were fed every three or four days. For adherent cells ATV or trypsin were used to detach cells from the plates.

2. Freezing

Frozen cells were stored at -140°C in freezing medium (10% DMSO, 50% FCS, 40% $RPMI^+$). Cells in a proliferative stage of growth were harvested, diluted in 0.5 ml of growth medium and stored on the ice for 30 min. Then 0.5 ml of freezing medium was added to each vial and cells were frozen at –70°C. On the next day cells were transferred to the –140°C freezer.

3. Thawing

To thaw cells stored in liquid nitrogen, cells were warmed at RT. The cell suspension was transferred by dropping into a 15 ml tube containing 5 ml medium and centrifuged at 1600 rpm (400 g) for 5 min. The medium was discarded, cell pellet was resuspended in culture medium and centrifuged. Washing step was repeated twice. At the end cells were diluted in culture medium and incubated at 37°C.

4. Cell viability

To determine the percentage of viable cells within population, the cells were diluted with a trypan blue solution and counted in a Neubauer Chamber under the microscope. Only unstained, living cells were counted.

5. Preparation of the cells:

5.1. Lymphoid organ cell suspensions

Animals used in experiments were sacrificed by anoxia with CO_2. All procedures to obtain single cell suspension were carried out in sterile conditions. Lymphoid organs: LN (a pool of mesenteric, cervical and submandibular lymph nodes), spleen and thymus were removed aseptically and placed into ice-cold centrifugation tubes

containing 5 ml of RPMI$^+$ medium. Organs were homogenised by passing through a metal sieve placed on tissue culture dish. At this step cells were released into medium. Cell suspension was transferred into the tube and left for 10 min to allow tissue debris to settle on the bottom of the tube. The supernatant containing cells was transferred to the new tube and centrifuged for 5 min at 1600 rpm (400 g). To remove erythrocytes from cell suspension, pellet was resuspended in 3 ml of TAC Buffer, incubated at RT for 10 min and centrifuged for 5 min at 1600 rpm (400 g). After erythrocyte lysis cells were washed twice and cell number was counted.

5.2. IHL cell suspensions

Mouse and rat intrahepatic lymphocytes were isolated according to two different protocols:

5.2.1. Protocol I

(by Olga Naidenko; personal communication)

- Liver was flushed of the blood with 20-50 ml syringe containing complete medium via portal vein
- Liver was mashed and passed through 70 μm nylon mesh. Cell suspension was centrifuged at 1200 rpm (225 g) for 10 min to pellet
- Pellet containing cells was resuspended in 28 ml of 40% Percoll (prepared in RPMI with 5% FCS) and layered over 12 ml 80% Percoll
- Percoll gradient was spun down for 20 min at 2500 rpm (970 g), RT, without brakes. After centrifugation the top face of the gradient contained liver tissue, the bottom face hepatocytes
- Lymphocytes from the interface were collected, resuspended in complete medium and washed twice to remove traces of the Percoll

5.2.2. Protocol II

(modified from Emoto *et al.*, 1995-a)

- Liver was perfused with 20-50 ml RPMI$^+$ medium to eliminate blood in the liver
- Liver was passed through a stainless steel mesh. Cells were washed for 10 min at 1200 rpm (225 g)

- Cell pellet was resuspended in 25 ml medium and centrifuged at 570 rpm (50 g) for 30-60 s. The supernatant was harvested and replaced with fresh medium. This washing-centrifugation procedure was repeated 2-4 times.
- Supernatants containing cells were centrifuged for 10 min at 1200 rpm (225 g)
- Pooled cells were purified from tissue debris and adherent cells by passing through a 20 ml syringe column packed loosely with nylon wool
- In order to obtain pure lymphocytes fraction Percoll density gradient was performed. 3 ml of 40% Percoll containing cells was layered onto 2 ml of 70% Percoll
- Percoll gradients were centrifuged at 2000 rpm (600 g), 20°C for 25 min
- Lymphocytes present in the interface were collected and washed for 5 min at 1600 rpm (400 g)
- After erythrocyte lysis lymphocytes were washed twice and counted

To prepare 100% Percoll, 90 ml Percoll was mixed with 10 ml of 10*PBS. This Percoll/PBS mixture was then considered 100% and used to make 40 and 80% by adding required amount of RPMI with 5% FCS.

6. Purification of the cells through nylon wool column

1.2 g of loosely connected nylon wool was packed in 20 ml syringe and autoclaved. Those columns were rinsed with around 60 ml of column medium (RPMI-5%FCS). In order to lyse erythrocytes, pellet containing splenic or hepatic cells was diluted in 5-20 ml of TAC buffer. After 10 min incubation at RT suspensions was spun down, washed twice and diluted in column medium. Cells isolated from one spleen were usually purified through one nylon wool column. Splenocytes were incubated on the columns for 45-60 min at 37°C to allow B cells and adherent cells to adhere to the wool. Then non-adherent cells were eluted at 1 drop per 3 sec by washing of the column with around 40 ml of column medium pre-warmed to 37°C. Cells were washed, counted and used in functional assays.

7. Immunofluorescence and flow cytometry

Fluorochrome labelled Abs are useful tools to detect surface or intracellular antigens. In order to determine the phenotype of rat hepatic and splenic NKT and also in order to detect surface expression of chimeric TCRs and rCD1d, different mouse and rat Abs were used. All antibodies were titrated and the optimal Ab concentration was used in staining. Isotype controls were included in labelling protocol as negative controls. $2*10^5$ cells diluted in 100 µl of FACS buffer (PBS/BSA/Azide) were usually used for immunofluorescent staining. To block unspecific binding or binding to Fc receptor, mouse cells were treated for 10 min at 4°C with NMIg or 2.4.G2 Ab. Then cells were stained with the first usually FITC conjugated Ab and incubated for 30 min at 4°C. After incubation cells were washed by centrifugation in 4 ml of FACS buffer for 5 min at 1600 rpm (400 g). Supernatants were discarded and cell pellet was labelled with second PE conjugated Ab for 30 min at 4°C. Washed cells were then incubated for 30 min in the presence of biotinylated Ab. The bio-Ab was visualised by incubation with Streptavidin-Cy-Chrome. Unconjugated antibodies, used in indirect immunofluorescent staining, were detected by using fluorochrome conjugated antisera (as secondary Ab). After binding of secondary Ab, cells were blocked by addition of 10 µg/ml mouse IgG before staining with other antibodies. After final washing the antigen expression was analysed by flow cytometry.

Staining with α-GalCer-mCD1d-PE tetramer:

α-GalCer-mCD1d-PE tetramer consisting of mouse CD1d loaded with α-GalCer, visualised with PE was used for specific identification of hepatic NKT cells within a mixture population of liver cells. This oligomer was also used for staining of $V\alpha14^+$ cell lines and hybridomas. Only cells containing TCR^{inv} recognising α-GalCer presented by CD1d molecule were positive in the staining and could bind to α-GalCer-mCD1d-PE tetramer.

The staining with α-GalCer-mCD1d-PE tetramer was performed as normal FACS staining with one difference: cells were incubated with oligomer not at 4°C but at RT in the dark for 30-60 min. Washed PE positive cells were analysed by flow cytometry.

8. Generation of anti-rat CD1d monoclonal antibody

The CD1d DNA obtained from F344 rat BM was cloned into the retroviral pczCG5 IEGZ vector and used for transfection of 293T and infection of M12 cells. GFP-positive cells were used for immunisation of Balb/c $CD1d^{-/-}$ mice. Animals received 5 weekly i.p. injections of GFP-rCD1d-M12 cells ($1*10^7$ cells diluted in 300-500 µl of PBS), and were rested for three weeks. After that time the last injection was done via i.v. route and 3 days later the fusion of mouse splenic cells with SP2/O fusion partner was done.

Splenic cells obtained according to standard preparation protocol, were washed twice with BSS and once with RPMI medium (without FCS). SP2/O fusion partner-myeloma cells were washed once with BSS and additionally once with RPMI medium. Both cell types were counted and mixed in 5:1 ratio (4 parts of splenic cells and one part of myeloma cells).

Cell fusion:

The cell mixture was resuspended in 50 ml warm $RPMI^-$ medium and centrifuged for 10 min at 1200 rpm (225 g). Then, all steps were performed at 37°C. The cell pellet was gently resuspended and 1ml of PEG was added dropwise over 1 min with shaking. Within next 10 min following solutions were added to fusion mixture:

5 ml of $RPMI^-$ (without FCS) at around 1ml/min

10 ml of $RPMI^-$ (2ml/min)

30 ml of $RPMI^+$ (RPMI medium containing 10% SC and 5% FCS).

Then the fusion mixture was incubated for 30 min at 37°C and centrifuged for 10 min at 1600 rpm (400 g). The pellet was carefully resuspended with 100 ml of $RPMI^+$ medium containing IL-6 and plated into 96-well flat bottom plates, by adding (using 1 ml plastic pipet) 100 µl of suspension per well. Fusion plates were incubated for 24h at 37°C and then 100 µl of 2 times concentrated HAT selection medium were added into each well. After 7-10 days incubation at 37°C, first positive clones were checked under microscope, supernatants were taken and tested. If necessary, clones were further expanded.

The CD1 specificity of clones was tested by FACS staining of rCD1d transduced cell lines and primary $CD1d^+$ rat cells. As a negative control wild type or mock-

infected cells were used. Hybridomas secreting antibodies, which stained $CD1d^+$ cells were cloned by single cell dilution.

9. Cell stimulation

Different cell types (primary cells, cell lines, hybridomas) were stimulated with several stimuli for either 24 or 72h. Then the levels of cytokines released by these cells into supernatants were analysed.

9.1. Ab stimulations:

Depending on the experiment Ab stimulation protocols were used:

9.1.1. R73/ R78 Stimulation

- 96 well flat bottom plate was coated with 50 μl/well ShαMIg (300 ng/ml in coating buffer) and incubated ON at 4°C
- next day the plate was washed 3 times with BSS and coated with 100 μl of R78 supernatant (previously diluted 1:3000 in BSS) or with 100 μl of R73 Ab (2 μg/ml). The plate was left for 2h at 37°C
- The plate was washed 3 times with BSS and appropriate cells in medium were plated.

9.1.2. CD3 Ab Stimulation

- 96 well flat bottom plate was coated with 100 μl/well of 10 μg/ml mCD3Ab diluted in PBS overnight at 4°C
- Next day the plate was washed 3 times with PBS and cells were plated. Different concentration of the cells were used for stimulation:

Cell lines	$5*10^4$ cell/well
APC: - splenocytes	$5*10^5$ cell/well
- thymocytes	$1*10^6$ cell/well
Cell lines used as APC	$5*10^4$ cell/well

Cells were cultured in the presence of Ab usually for 24h and 3 days and then supernatants were harvested and analysed by ELISA.

9.2. Ag (α-GalCer/OCH) stimulation

NKT cells are able to recognise α-GalCer presented by APC in the context of CD1d molecule. Once stimulated they become blasts, start to proliferate and produce cytokines like IL-4 and IFN-γ. In functional assays the response of mouse and rat splenic and liver cells to different doses of α-GalCer and OCH (the analog of α-GalCer) was tested *in vitro*.

10. Supernatant cytokine ELISA

In order to detect cytokines produced by activated cells the supernatants were harvested after 24h or 72h depending on the experiment setup. The samples were stored at –20°C until testing. IL-2, IL-4, IL-10 and IFN-γ in supernatants were measured using ELISA Kits (BD Pharmigen) according to the manufacturer's instructions.

Molecular biology methods

11. RNA isolation

RNA was isolated from cytoplasmic extracts of primary cells or cell lines according to the protocol of Rneasy®MiniKit(QIAGEN#74104). All steps of the procedure were carried with precautions to minimise RNAase activity:

- chemicals, plastic ware, and pipet tips were RNAase-free
- disposable gloves were worn during the preparation and analysis of RNA

To isolate RNA usually $1*10^7$ cells were used. At the end the RNA concentration was estimated spectrophotometrically.

12. Specrophotometric determination of the RNA (DNA) amount

To measure the amount of RNA or DNA an aliquot of the sample was prepared and read in spectrophotometer at wavelengths of 260 nm, 280 nm. The reading at 260 nm gives the concentration of nucleic acid in the sample. The ratio OD260/OD280 estimates the purity of the preparation.

13. RT-PCR

The synthesis of cDNA suitable for PCR amplification was usually performed according to manufacturer's RT-PCR protocol supplied with RT-PCR MBI Fermentas Kit (#K1612).

14. PCR

PCR reaction mixture (25 μl) containing:

2.5 μl 10*PCR buffer with $(NH4)_2SO_4$

1.5 μl MgCl2 (25 mM)

1 μl dNTP mix (each of 2,5 mM)

17.5 μl PCR water

0.04 μl MBI Taq Polymerase (5U/1μl)

1 μl cDNA (1pg-1ng)

1 μl primers (10 mM each)

was prepared and amplified as follows:

- denaturation: 4 min at 94°C
- amplification- 35 cycles:
  - 1 min at 94°C
  - 1 min at temp of anneling suitable for primers, counted according to: $T_m=((G/C)*4°C+(A/T)*2°C)-2°C$
  - 1 min at 72 °C
- 10 min at 72 °C.

Than samples were stored at 4°C until the electrophoresis was performed. As positive control PCR with β-actin specific primer was done. In some cases HotStar Taq Polymerase instead of MBI Taq Polymerase was used.

15. Transformation of competent cells

Transformation of competent *E.coli* was carried using TOPO-Cloning Kit (K450-01 Invitrogen), which allows the transformation of bacteria with PCR product, plasmid DNA or ligation product. Competent *E.coli* cells stored at -70°C were thawed on ice.1- 2 μl DNA of interest were gently added to vial containing 50 μl of competent *E.coli*. The tubes were incubated first on ice for 30 min and later in a preheated water bath at 42°C for 30 sec. The samples were rapidly cooled on the ice for 2 min. 250 μl of SOC medium was added to each tube and then probes were transfered to a shaking incubator and incubated for 30 min at 37°C, 200 rpm.
90 mm plate LB-ampicillin-agar were prepared in sterile conditions and then stored at 4°C until they were needed. Before use they were kept at RT for 30-60 min to eliminate problems of condensation. After 30 min incubation with rotation, DNA-transfected bacteria were ready to grow and divide. 50 μl or 150 μl of competent cells were spread on the agar plates. When a PCR product was cloned into TOPOCloning-Vector, plates contained both X-Gal and IPTG. The plates were inverted and incubated ON at 37°C. Only bacteria expressing an ampicillin resistance gene encoded by the plasmid could grow on the selection plates. On the next day positive white colonies were analysed either by PCR or by Miniprep and digestion with restriction enzymes.

16. Mini preparation of plasmid DNA

Positive colonies were picked from agar plates, transferred into 5 ml of LB medium containing 50 μl of ampicillin and incubated in shaking incubator ON at 37°C. Next day bacterial cells were harvested by centrifugation in Eppendorf centrifuge at 13000 rpm for 3 min. Bacterial pellet was resuspended in 150 μl of ice-cold Buffer 1 (components of buffers in “Media, Buffers, Solutions”). 150 μl of Buffer 2 was

added and eppendorf tubes were mixed 5 times. Then 150 µl of Buffer 3 was added and samples were spun down at 13000 rpm for 5 min. After centrifugation supernatants with plasmid DNA was transferred to the new tube containing 900 µl of Buffer L6 and 50 µl of Diatomacenous earth. Samples were left for 5 min at RT and then were loaded on the MiniWizard columns connected with vacuum. Columns were washed twice with 3 ml of washing buffer and spun down. DNA from minicolumn membrane was eluted with 30 µl water. Plasmid DNA was digested with restriction enzymes and DNA fragments were separated by electrophoresis

17. Maxi preparation of plasmid DNA

To obtain large amount of the plasmid DNA Maxi preparation was performed according to the orginal protocol from QIAGEN Plasmid Purification Handbook.

18. Cleavage of plasmid DNA by restriction enzymes

Restriction enzymes are able to cleave a specific sequence of nucleotides. They are useful at different steps of cloning: to analyse the restriction maps of plasmids or for preparation of the insert and vector before ligation. Digestion with appropriate restriction enzymes was done for plasmid DNA obtained after Mini or Maxi preparations following the producer instructions supplied with enzymes (MBI Ferments: St.Leon-Rot, Germany).

19. Sequencing

<u>Amplification of DNA</u>

PCR mix containing:

500 ng plasmid
1 µl primer (20 µM= 10pmol)
3 µl BD (big dye)
water to 10 µl

was amplified as follow:

25 cycles: 10 sec at 96°C
5 sec at 50°C
4 min at 60°C

DNA precipitation:

Each DNA sample was prepared for sequencing as follows: To 10 µl of PCR mix 90 µl of water and 10 µl of 3 M Sodium Acetate pH5.2 were added and mix was vortexed. Then 250 µl of 99.9% Ethanol was added and vortexed once again. Samples were centrifuged in Eppendorf centrifuge at 14000 rpm for 20-30 min, DNA pellet was washed twice with 250 µl of 70% Ethanol for 5 min or once for 20 min at 14000 rpm and then dried at RT. DNA was diluted in 25 µl of HiDi and samples were boiled at 94°C for 2 min, cooled on ice and transferred into new sequencing tube and sequenced using an ABI sequencer.

20. Cloning

20.1. Cloning of PCR product

Cloning of PCR product into TOPO-cloning vector was done according to manufactures instructions TOPO-cloning-Kit

20.2. Cloning into viral expression vectors

In order to express genes of interest two retroviral vectors were used

- pczCG5 IEGZ ("EGZ") containing genes for zeozin resistance,
- pczCG5 IEGN ("EGN") with genes for neomycin resistance.

Different restriction sites of those vectors were used for the cloning of different molecules:

Cloned molecule	Restrition sites	Vector
mouse Vα14 TCR mouse Vβ8.2 TCR rat CD1d molecule	EcoRI BamHI EcoRI	pczCG5 IEGZ
mouse Vα14-rat JC TCR rat Vα14-rat JC TCR	EcoRI/HindIII EcoRI/HindIII	pczCG5 IEGN

In all cases cloning method consisted following steps:

- Preparation of insert
- Vector preparation

- Ligation
- Screening of positive clones

20.2.1. Preparation of insert

- Digestion of the plasmid DNA with appropriate restriction enzyme was usually performed as follow:

  30 µl of plasmid with the gene of interest

  5 or 10 µl 10* appropriate buffer

  10 µl milipore water

  5 µl of restriction enzyme (10U/µl), sometimes two restriction enzymes were used.
- Digestion was carried out for 2h at 37°C and stopped by heating the samples for 20 min at 65°C.
- To isolate and to purify insert DNA from plasmid DNA, long agarose gel electrophoresis was done:

  0.99% agarose gel

  6 µl of size marker

  55 V

  3h
- The band of the right size was cut out from the gel warmed at 55°C in the presence of 600 µl buffer L6 and purified through MiniPrep column according to the standard MiniPrep protocol

20.2.2. Vector preparation

- Vector DNA was digested with the right restriction enzyme using a similar protocol as for insert preparation
- Additionally in order to protect against religation of vector DNA the dephosphorylation of 5' vector ends was done:

  to 50 µl of digestion mixtures

  5 µl of 10*CIAP reaction buffer and

  1 µl of CIAP (1U/µl)- Calf Intestine Alkaline Phosphatase were added and incubated for 15 min at 37°C. Then 0.5 µl of CIAP was added and mixture was incubated for another 15 min at 37°C. Dephosphorylation reaction was stopped by heating at 85°C for 15 min

- To purify vector DNA, long agarose gel electrophoresis was done, DNA band was cut out from the gel and was purified according to the procedure used for the purification of insert DNA

20.2.3. Ligation

In order to find the optimal conditions for ligation, agarose gel electrophoresis of purified insert and vector DNA was usually done before ligation. The insert DNA was cloned into retroviral vector DNA during 6h incubation at 12°C in the presence of T4 DNA Ligase. Ligation reaction was stopped by heating the reaction mix at 65°C for 10 min. To check efficiency of ligation a control electrophoresis was performed, *E.coli* bacteria were transformed and positive clones were screened by PCR and MiniPrep.

21. Transfection and infection

To express the gene of interest in mammalian cells, virus particles were produced by transfection of 293T cells. Then virus supernatants were used for infection of cell lines or primary cells. A transient three-plasmid expression system was used:

Day 1:

- 293T cells were plated into 6 cm dishes using $2*10^6$ cells in 5 ml DMEM, 10% IFS
- Plasmid DNA (EGN/EGZ-gene of interest + pHIT60 + pVSVG) were precipitated with 90% Ethanol and Sodium Acetate buffer, washed once with 70% Ethanol, dried at RT and resuspended in 100 µl of sterile H_2O. DNA samples were stored ON at 4°C. 15 µg of total DNA were used per 6 cm plate transfection.

Day 2:

- Medium from 6 cm dishes was aspirated and 4 ml of fresh media without Hepes were gently added. The plates were returned to the incubator.
- Combined DNA was brought to 438 µl with sterile H_2O then 62 µl of 2M $CaCl_2$ solution and 500 µl of 2*HBS were added. Eppendorfs were inverted 3-4 times and transfection solution was immediately added to the plates. Plates were gently

shaken to distribute the precipitate evenly and returned to the incubator for 7-10 hours.

- After 7-10 hours incubation media were replaced carefully with 5 ml of fresh normal IFS media. Plates were incubated O/N at 37°C, 5% CO_2

Day 3:

- In order to activate the CMV Promoter media from the plates were replaced with 3 ml of fresh normal DMEM medium containing 10 mM Na-Butyrate.
- Plates were incubated at 37°C, 5% CO_2 for 8-12 hours and then Na-Butyrate containing media were aspirated, 293T cells were washed once with 3 ml normal medium and 3 ml of fresh medium were added for O/N incubation at 37°C, 5% CO_2.

Day 4:

- The 293T cell supernatant containing viral particles was harvested with a syringe and pushed through a 0.45 μm filter into a 15 ml Falcon tube containing 30 μl of Polybrene (800μg/ml, the final concentration of Polybrene was 8 μg/ml)
- Viral supernatants were plated onto 12 well plate and $1*10^5$ target cells per well were added. Plates were returned into incubator and incubated for 0.5-1.5h.
- To increase the efficiency of infection plates were centrifuged for 1.5h at 32-37°C at 2000 rpm
- After centrifugation plates were incubated at 37°C for 1h. Infection supernatants were then replaced by fresh growth medium
- The expression of gene of interest in target cells was analysed by FACS at the time point depending on the confluency of target cells.

Chapter 3:
RESULTS

I. Properties of rat NKRP1A$^+$TCR$^+$ cells

1.1. Phenotype

Human and mouse natural killer T cells express CD1d-restricted TCR invariant (Vα24JαQ/Vβ11 in human and Vα14Jα18/Vβ8.2 in mouse, respectively) together with NK cell specific markers. In terms of co-receptor expression, human and mouse NKT cells belong either to the single-positive CD4$^+$ or to the double-negative CD4$^-$ CD8α/β$^-$ subset of lymphocytes.
Mouse TCR^{int}NK1.1$^+$ cells show tissue-specific distribution- they are preferentially present in the liver and in a low proportion in: spleen, thymus, lymph nodes and bone marrow.

1.1.1. Phenotype of rat hepatic and splenic lymphocytes

Since mouse Vα14$^+$ T cells are most frequent in the liver and spleen, we determined if the population of cells with the same phenotype and CD1d reactivity also exists in rat. To this end, the phenotype of lymphocytes isolated from F344 rat liver and spleen was determined by FACS staining. Because no anti-TCR^{inv} monoclonal Ab is available, only cells co-expressing TCR (CD3ε) together with NK1.1 (NKRP1-A) were analysed as positive and were designated NKT cells.

Figure 1 shows a representative experiment, in which the expression of surface antigens on mouse and rat lymphocytes was analysed by FACS staining. Mouse cells, used as a control, were stained with culture supernatant containing anti-mouse NK1.1 antibodies, which then were detected with donkey anti-mouse IgG. Therefore, some unspecific binding of this secondary antibody to mouse cells could be seen, probably as a result of the population of B lymphocytes present in cell

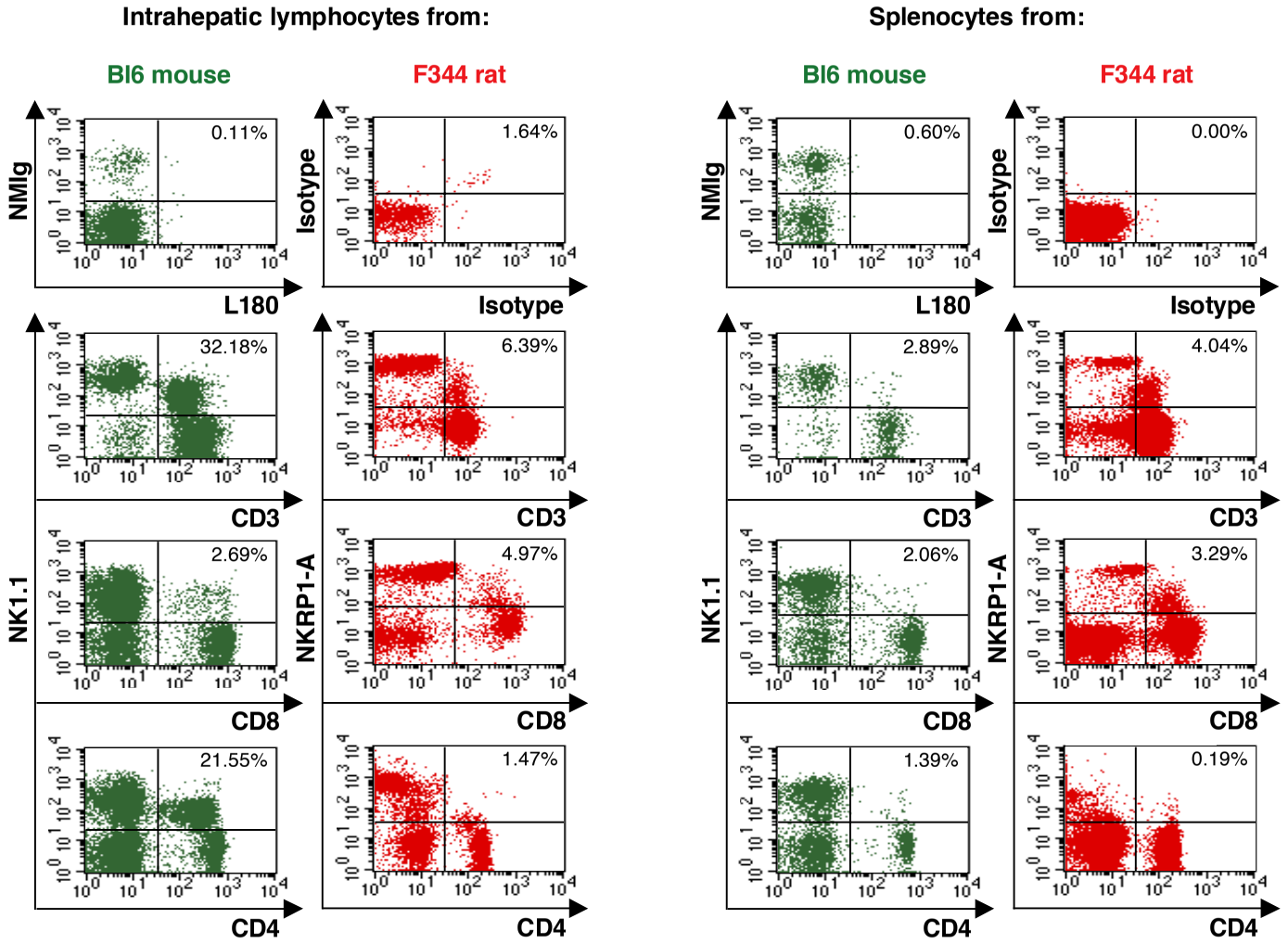


Figure 1:

The NKT cell population in F344 rats is smaller than in C57/Bl6 mice, and comprises both CD4⁺ and CD8⁺ cells.

$2*10^5$ of mouse liver and spleen lymphocytes were preincubated with anti-CD16 Ab for 15 min at 4°C and then were stained with NK1.1/DαMIgG-Cy5, and FITC labelled anti-CD3, anti-CD8, anti-CD4 mAbs. Rat cells were stained with purified anti-CD3, anti-CD8 and anti-CD4 mAbs, which were detected with DαMIgG-Cy5, and then anti-NKRP1A-FITC Ab was used.

preparations. Basing on the definition of NKT cells, we analysed only $CD3^+NK1.1^+$ cells present in the upper right quadrant, while "false positive" cells from the upper left quadrant were not taken into account.

As it is presented in Figure 1, the phenotype of rat liver lymphocytes was distinct from the phenotype of mouse NKT cells. The population of TCR^+NKRP1^+ cells in F344 rat liver (6.39%) was much smaller than the population of mouse IHLs (32.18%). In terms of CD4/CD8 co-expression, the majority of rat hepatic lymphocytes expressed CD8 (4.97%) rather than CD4 (1.47%), while mouse counterparts were $CD4^+$ (21.55%).

The frequency of TCR^+NKRP1^+ cells (figure 1, right panel) in rat spleen (4.04%) was higher than the frequency of mouse $NK1.1^+TCR^+$ splenocytes (2.89%). Rat spleen NKT cells were mostly $CD8^+$ (3.29%). This time, the CD8 marker was preferentially expressed also by the majority (2.06%) of mouse splenic NKT cells.

Therefore, rat TCR^+NKRP1^+ cells in liver were not as frequent as mouse NKT cells. With regard to CD8 expression rat NKT cells were more similar to mouse spleen rather than liver lymphocytes.

1.1.2. Phenotype of rat NKT cells in different rat organs

In order to test if $TCR^+NKRP1A^+$ cells were present in other rat tissues, lymphocytes isolated from F344 rat liver, spleen, lymph nodes, bone marrow and thymus were labelled with mAbs.

To stain CD8 positive cells, antibodies recognising the heterodimeric α/β form of CD8 were used, to exclude the possibility of detecting of α/αCD8 homodimer, which is often expressed by activated T cells.

Table 3:

Phenotype and frequency of rat NKT cells differ significantly from that of mouse NKT cells. FACS staining of F344 rat lymphocytes isolated from various organs- data from one representative experiment.

	% of lymphocytes				
	BM	**thymus**	**liver**	**spleen**	**LN**
$TCR^+NKRP1A^+$	1.09	0.32	6.21	3.85	1.51
$CD4^+NKRP1A^+$	2.92	0.41	3.97	4.18	2.05
$CD8^+NKRP1A^+$	2.57	0.10	3.12	2.53	1.08

The frequency of $TCR^+NKRP1A^+$ cells in rat organs varied between preparations but usually was much lower than frequency of mouse counterparts. F344 liver was an organ, where rat $NKRP1A^+TCR^+$ cells were the most frequent (6.21 % of total lymphocytes). BM contained 1.09 % and spleen around 3.85 % of $TCR^+NKRP1A^+$ cells. A very small proportion of cells with this phenotype could be found in thymus (0.32%) and LN (1.51%).
In contrast to mouse NKT cells, rat $TCR^+NKRP1A^+$ cells expressed not only CD4 but also CD8.

1.1.3. Staining of rat splenocytes and intrahepatic lymphocytes with α-GalCer-mCD1d-PE tetramer

Both mouse and human invariant NKT cells recognise lipid antigens presented by the MHC class I-like CD1d molecule. Basing on the mechanisms of natural antigen recognition by TCR^{inv}, α-GalCer loaded CD1d tetramers have been generated. Tetramers are able to bind to TCR^{inv} NKT cells within a mixed lymphocyte population.
To further characterise rat $TCR^+NKRP1A^+$ cells, we used α-GalCer-mCD1d-PE tetramer, which due to high homology between human and mouse CD1ds can positively stain NKT cells from both species.
Figure 2 shows that a significant proportion of mouse (26.61%) but not rat liver lymphocytes (0.25%) could bind to mouse α-GalCer-mCD1d-PE tetramer. Whether such weak binding of mCD1d tetramer to F344 rat lymphocytes was specific will be discussed in later chapters of the thesis.

It should be pointed out that the percentage of tetramer positive cells in mouse liver varied (in the range of 2-41%) between cell preparations and was strictly dependent on the frequency of $NK1.1^+TCR^+$ cells which could be obtained. Tetramer positive cells represented around 85-95% of mouse $NK1.1^+TCR^+$ hepatic lymphocytes.
Data presented in the Table 4 shows that tetramer-binding cells could also not be found in rat splenocytes. This might suggest that rat $NKRP1A^+TCR^+$ cells did not bind or bound very weakly to mouse CD1d tetramer.

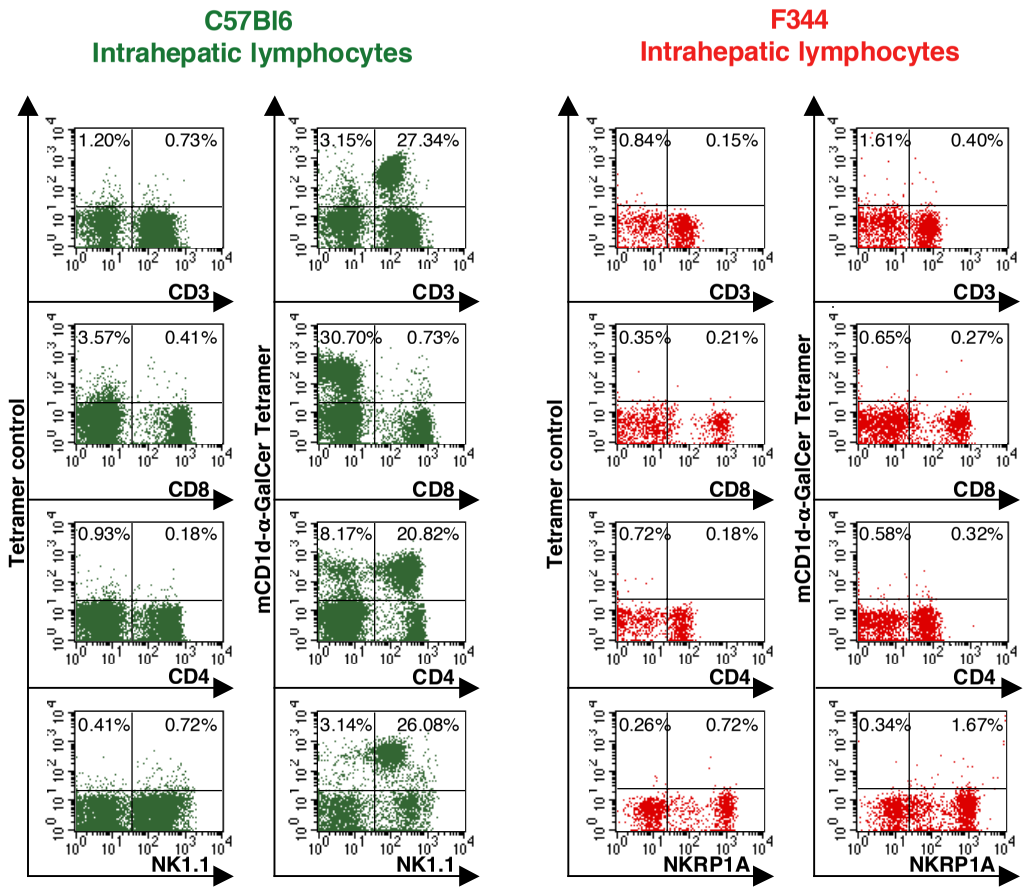


Figure 2:
F344 rat intrahepatic and splenic lymphocytes do not bind to mouse CD1d-α-GalCer tetramer.
Mouse splenocytes and IHLs were stained with NK1.1/DαMIgG-Cy5 and with FITC labelled anti-CD3, anti-CD8 and anti-CD4 antibodies. Rat cells were stained with purified anti-CD3, anti-CD8 and anti-CD4 and DαMIgG-Cy5, and then anti-NKRP1A FITC was used. After washing all cell types were stained for 45 min at RT with mouse CD1d-α-GalCer tetramer-PE or it's control. As many as possible of positive cells were acquired.

Table 4:
Comparison of the frequency of CD1d-α-GalCer tetramer positive cells in C57Bl6 mouse and F344 rat liver and spleen.
(The percentage of CD1d-α-GalCer tetramer positive cells was estimated as: % of tetramer positive cells -% of control positive cells)

	% of lymphocytes			
	C57Bl6 mouse		F344 rat	
	IHLs	splenocytes	IHLs	splenocytes
$CD3^+tetramer^+$	26.61	1.69	0.25	0.52
$CD8^+tetramer^+$	0.32	0.38	0.06	0.25
$CD4^+tetramer^+$	20.64	0.94	0.14	0.00
$NKRP1A^+tetramer^+$	25.36	2.94	0.95	0.06

When mouse spleen NKT cells (positive control) were stained with tetramer, 1.69% from 2.29% NKT cells in spleen could bind to this oligomer.
The lack of α-GalCer-mCD1d-PE tetramer-positive cells in rat liver and spleen raises the question if rat NKRP1A$^+$CD8$^+$ cells were CD1d-restricted and if they were able to bind to α-GalCer.

1.2. Functional characterisation of rat NKRP1A$^+$TCR$^+$ cells. Stimulation of rat IHL and splenocytes with α-GalCer *in vitro*

Upon α-GalCer stimulation mouse liver NKT cells become activated, they proliferate and produce cytokines like IL-4 and IFN-γ. Because rat splenocytes and IHLs showed no clear binding to mouse CD1d tetramer, there was a need to check if these cells, similarly to mouse cells, could respond to α-GalCer. To this end, lymphocytes prepared from F344 liver were cultured in the presence of α-GalCer or vehicle (DMSO) as negative control. The level of IL-4 and IFN-γ released into supernatants was analysed after 24h co-culture.
As shown in Figure 3, upon α-GalCer stimulation both mouse and rat IHLs were able to secrete cytokines in Ag-dependent manner. Rat liver lymphocytes, when compared with mouse counterparts released less IL-4 (around 15% of that produced by mouse IHLs) but produced higher levels of IFN-γ. Some background IFN-γ production (Figure 3B) was found for rat IHLs incubated in the presence of control medium or vehicle. However, when these cells were stimulated with 100 ng/ml of α-GalCer, the level of IFN-γ in supernatants increased suggesting Ag-dependent cytokine production.

The liver was an organ where rat NKRP1A$^+$TCR$^+$ cells were the most frequent but the numbers of IHLs obtained were always low. Therefore, in experiments in which high cell number was needed splenic rather than hepatic lymphocytes were used.
As it is shown in Figure 4, either total or nylon wool purified F344 spleen cells were co-cultured for 24 or 72 hours with different doses of α-GalCer, and then levels of IL-4 and IFN-γ in supernatants were measured by ELISA.

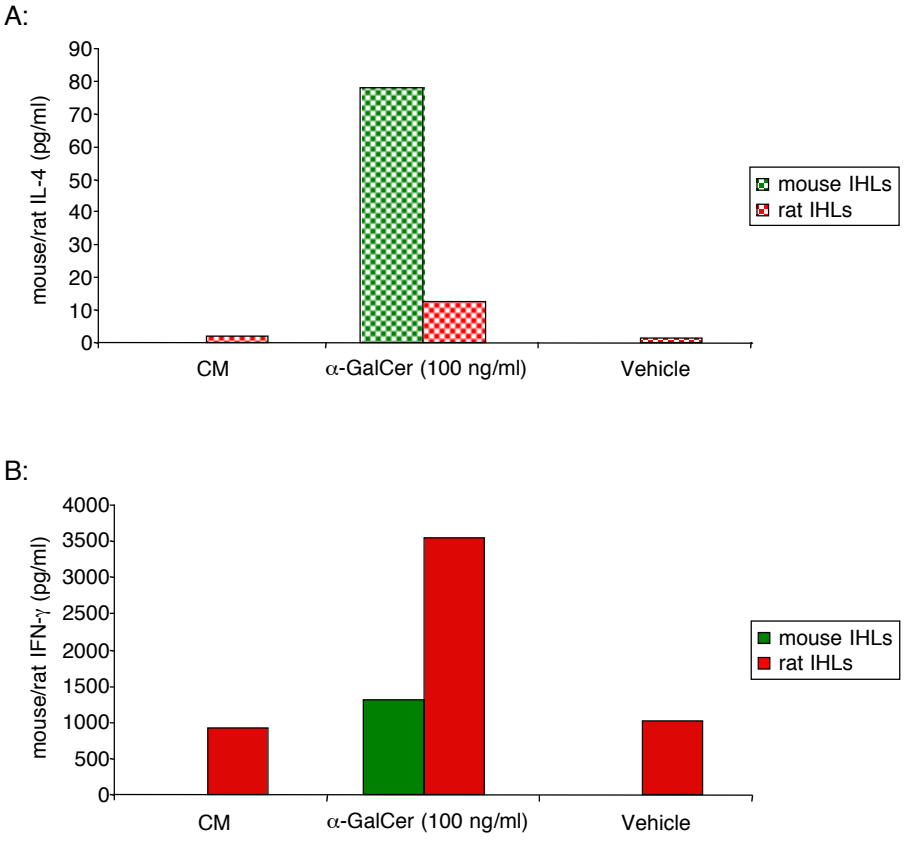


Figure 3: F344 rat intrahepatic lymphocytes (1*10^{5}cells) produce both IL-4 (3A) and IFN-γ (3B) after 24h stimulation with α-GalCer.
(CM- control medium)

Because we were not able to obtain enough purified lymphocytes from mouse spleen, the comparison of IFN-γ production by mouse and rat splenocytes only after 24h coculture is presented (Figure 4C).

Upon α-GalCer stimulation F344 rat splenocytes, similar to mouse cells, were able to release IFN-γ. However, the level of rat IFN-γ was much lower than mouse cytokine, and could be detected only for cells incubated with the highest dose (20 ng/ml) of α-GalCer.

For IL-4, which is released usually shortly after α-GalCer stimulation, data after 24 and 72h co-culture are shown (Figure 4 A and 4 B). Both mouse and rat splenocytes showed the same pattern of activation. The amounts of IL-4 increased with the increments of α-GalCer in the culture.

After 24h culture total rat and mouse spleen cells released significant amounts of IL-4. After 3 days of stimulation, the cytokine secretion increased further in Ag-dose dependent manner.

When nylon wool purified splenocytes were used in these assays, the detection level of IL-4 in supernatants was much lower. Only in long term culture and at the highest α-GalCer (20 ng/ml) concentration could a low level of IL-4 be measured. The level of IL-4 released by rat splenic lymphocytes was lower than that produced by mouse counterparts- rat total splenocytes secreted 3.96% and purified 14.06% of that IL-4 released by mouse total and purified splenocytes, respectively. Given that APCs are removed during nylon wool purification, this could suggest that the activation of rat splenocytes was CD1d dependent and it could take place only in the presence of $CD1d^+$ APCs.

The presence of IL-4 and IFN-γ producing cells in rat liver and spleen confirmed that rat cells could respond to α-GalCer. The level of IL-4 produced by rat splenocytes or IHLs was lower than the level of IL-4 detected for mouse NKT, suggesting the presence much smaller population of “NKT” cells in rat organs. Alternatively, rat NKT cells may be biased toward producing more IFN-γ than IL-4.

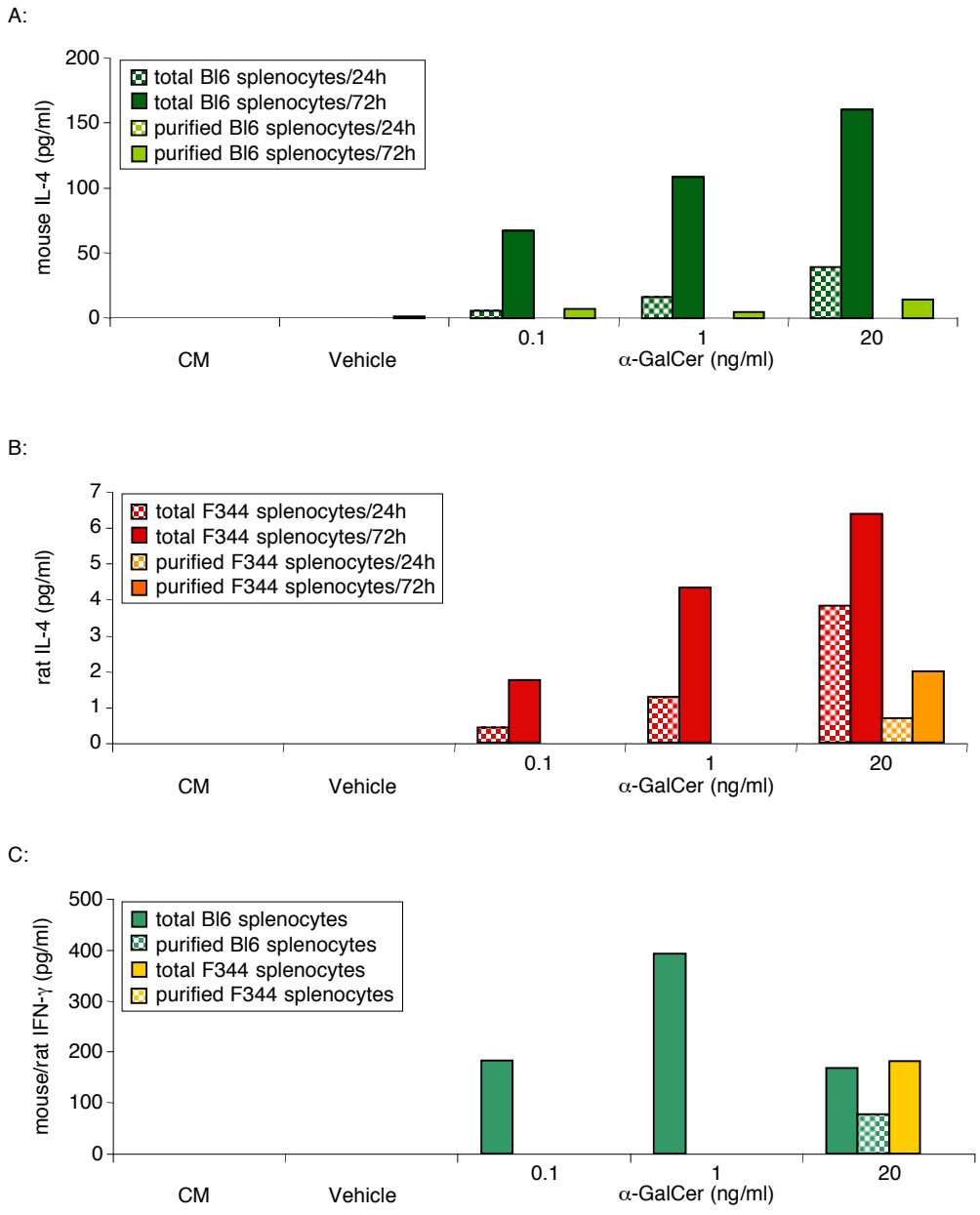


Figure 4: F344 rat splenocytes produce IL-4 and IFN-γ after stimulation with α-GalCer.
$5*10^5$ of total or nylon wool purified mouse and rat splenocytes were cultured for 24h with α-GalCer or Vehicle. The level of released IL-4 and IFN-γ was estimated by supernatant ELISA.

II. Characterisation of rat Vα14$^+$TCR

Mouse NKT cells express a particular invariant TCR consisting of Vα14Jα18 chain, paired together with Vβ8.2 chain. Thus, we wanted to check if the population of rat liver or spleen derived lymphocytes could express TCR^{inv}, which structurally and functionally would be similar to mouse receptor.

2.1. Frequency of Vα14$^+$Jα18$^+$ TCR in rat organs

In rats, four TRAV14 genes encoding TCR^{inv} have been described. According to the diversity accumulated in the CDR2 region, the products of TRAV14 genes may be categorised into two types:

Type I:

- TRAV14S1
- TRAV14S2
- TRAV14S4 (pseudo-gene)

Type II:

- TRAV14S3

The invariant TRAV14-positive rat NKT cells show tissue-specific distribution. Cells expressing type I invariant TCR are frequent in rat liver and thymus, while type II invariant TCR (TRAV14S3) positive cells are present in the spleen (Matsuura *et al.*, 2000).

To confirm the presence of Vα14 TCR positive cells in different F344 rat organs, TRAV specific RT-PCR was done. As a template for cDNA, the RNA isolated from lymphocytes obtained from different rat tissues was used.

Results presented in Table 5 (look: results/chapter 4/page 93) show that PCR positive signal was found for F344 rat thymus, liver and spleen. No or only weak band could be seen for cDNA from LN lymphocytes.

RT-PCR confirmed the presence of TCR^{inv} in different F344 rat organs. However, the Vα14$^+$ PCR signal for rat organs was much weaker than that from mouse tissues.

2.2. Cloning and expression of rat Vα14$^+$Jα18$^+$ chain from F344 liver cDNA functional characterisation of rat TCR^{inv}.

PCR products were cloned into TOPO-Cloning vector and sequenced. Analysis of Vα14$^+$ clones isolated from liver showed that rat Vα14 invariant TCR could be rearranged not only with Jα18 but also with other J segments. The low number of clones with in frame Vα14Jα18 rearrangement could suggest only a very small proportion of rat liver lymphocytes to be CD1d restricted NKT cells.

To facilitate the direct structural and functional characterisation of rat Vα14TCR, rat Vα14$^+$ TCR transgenic cells were generated and their reactivity to α-GalCer was tested.

The DNA of Vα14Jα218 TCR isolated from rat liver cDNA was sequenced. Two mutations in the V-region of cloned rat TCR^{inv} were found. The comparison of mutated rat receptor with mouse and two published in GeneBank rat Vα14$^+$ TCRs is presented in the Figure 10 (page 76) and will be discussed in chapter 2.2.4. Rat TCR^{inv} DNA was cloned into GFP$^+$- retroviral vector (EcoRI site) and used for transfection of 293T cells and infection of TCR$^{-/-}$ cell lines: BW58 or BW58 expressing a chimeric rat/mouse (r/m) CD28 molecule. Vα14$^+$-GFP$^+$ cells were then infected with rat Vβ8.2 chain. In parallel, as a control, mouse TCR^{inv} and mouse/rat chimeric TCRs were generated.

The surface expression of α/βTCRs was analysed by FACS staining with anti-mouse CD3ε and anti-mouse or anti-rat Vβ8.2 monoclonal antibodies. The representative staining of BWr/mCD28 cells expressing either mouse or rat TCR^{inv} with anti-mouse CD3 mAb is presented in Figure 5. Additionally, to check if cloned TCR^{inv} had the same specificity for CD1d-α-GalCer complex as primary liver or splenic lymphocytes, Vα14$^+$ transgenic cells were stained with mCD1d-α-GalCer tetramer.

Mock infected BWr/mCD28 cells lacked TCR on their surface therefore they were negative in anti-CD3 mAb or tetramer staining.

Both mouse (mVα14mVβ8.2) or rat (rVα14rVβ8.2) Vα14$^+$ TCRs were successfully expressed on the surface of BWr/mCD28 (Figure 5) or BW (data not shown) cells, since these cells were positive in the staining with anti-mouse CD3 mAb. It was also the case for positively labelled BWr/mCD28 cell lines expressing chimeric mouse/rat TCR (mVα14/rVβ8.2 and rVα14/mVβ8.2- data not shown).

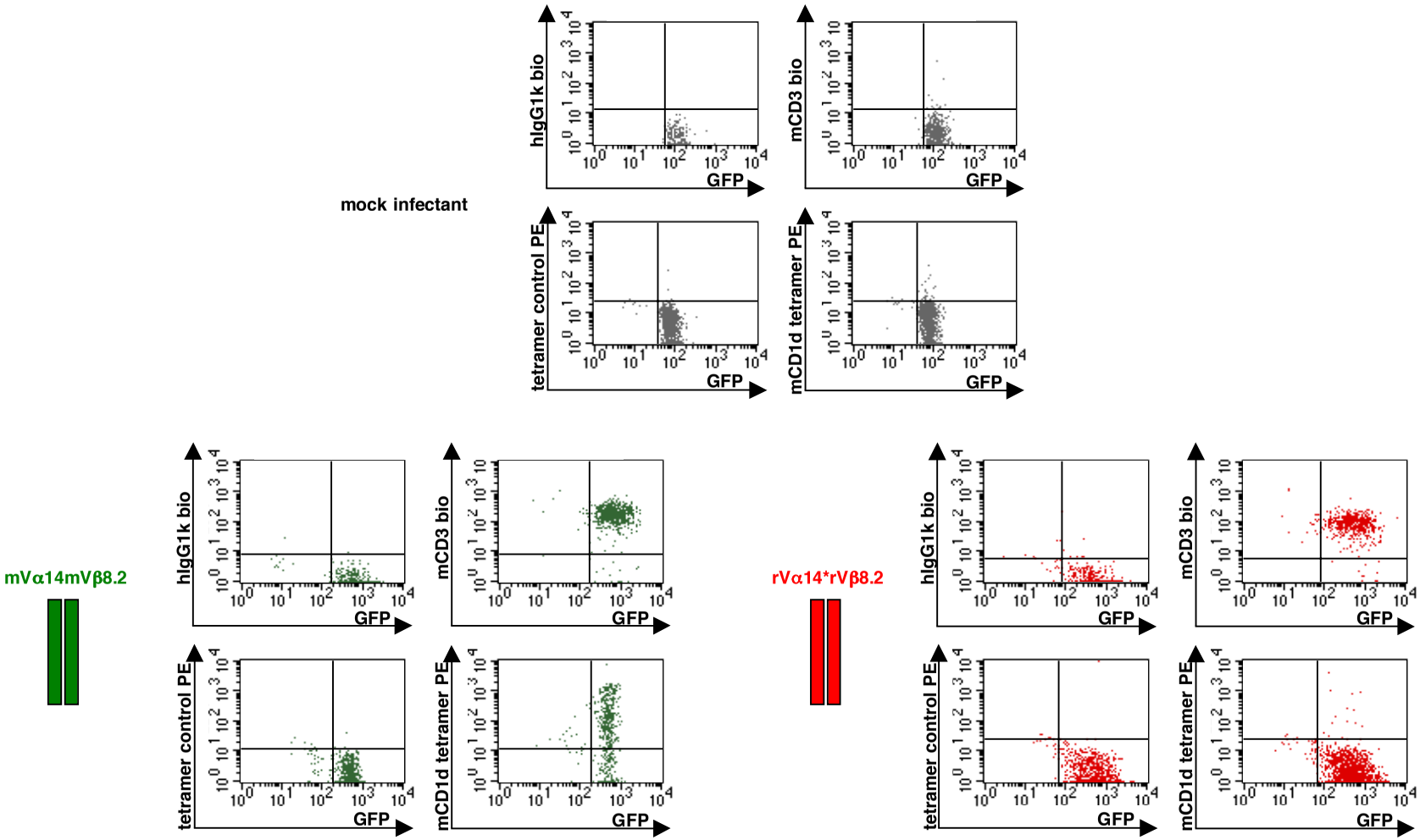


Figure5:
Expression of mouse or rat Vα14⁺TCR on the surface of BWr/mCD28 cell line and binding to mCD1d-α-GalCer tetramer.
$2*10^5$ cells were stained with anti-mouse CD3 bio mAb followed by Streptavidin-Cy-Chrom. For tetramer staining $1*10^6$ cells were incubated with mCD1d-α-GalCer tetramer PE or it's control. rVα14*- rat TCR cloned from F344 liver containing mutations in V-region as shown in Figure 10.

When BWr/mCD28 cell line expressing mouse or rat TCR^{inv} were stained with mCD1d-α-GalCer tetramer (Figure 5), only cells positive for mouse but not rat TCR could bind to tetramer. Cloned rat $V\alpha14^+$ TCR, similar to rat IHLs, did not have the specificity for mouse CD1d tetramer.

2.2.1. Cytokine production by rat $V\alpha14^+$ cells upon Ab and Ag stimulation

Purified by MACS or FACS sorting, TCR transgenic cells were used in functional assays, in which the capacity of these cells to produce IL-2 upon Ab (Figure 6A) or Ag (Figure 6B) stimulation was tested.

Upon anti-mouse CD3 or anti-Vβ8.2 Ab stimulation, BWr/mCD28 cells transduced with control GFP-vector did not secrete any IL-2, since they lacked the TCR on their surface. BWr/mCD28 cell line expressing rat TCR^{inv} was functional and able to release large amounts of IL-2 after 24h stimulation with anti-CD3 or anti-Vβ8.2 Abs. No IL-2 was found when cells were cultured with medium only.

To test the specificity of rat liver TCR^{inv} for α-GalCer, rat or mouse $V\alpha14^+$ TCR transgenic cells were stimulated with α-GalCer presented by $CD1d^+$ thymocytes. After 24h coculture, the IL-2 level in culture supernatants was measured by ELISA. The results are presented in Figure 6B.

Despite cytokine production upon Ab stimulation, BWr/mCD28 cell line expressing rat TCR^{inv} did not have the specificity for α-GalCer. No IL-2 could be detected in the supernatants of rat $V\alpha14^+$TCR-positive cells incubated with α-GalCer-loaded rat (both F344 and LEW) or mouse thymocytes. On the other hand, the positive control-BWr/mCD28mVα14mVβ8.2 cells secreted high amounts of IL-2 in the response to rat, and less IL-2 in the response to mouse APCs in an Ag-dose dependent manner.

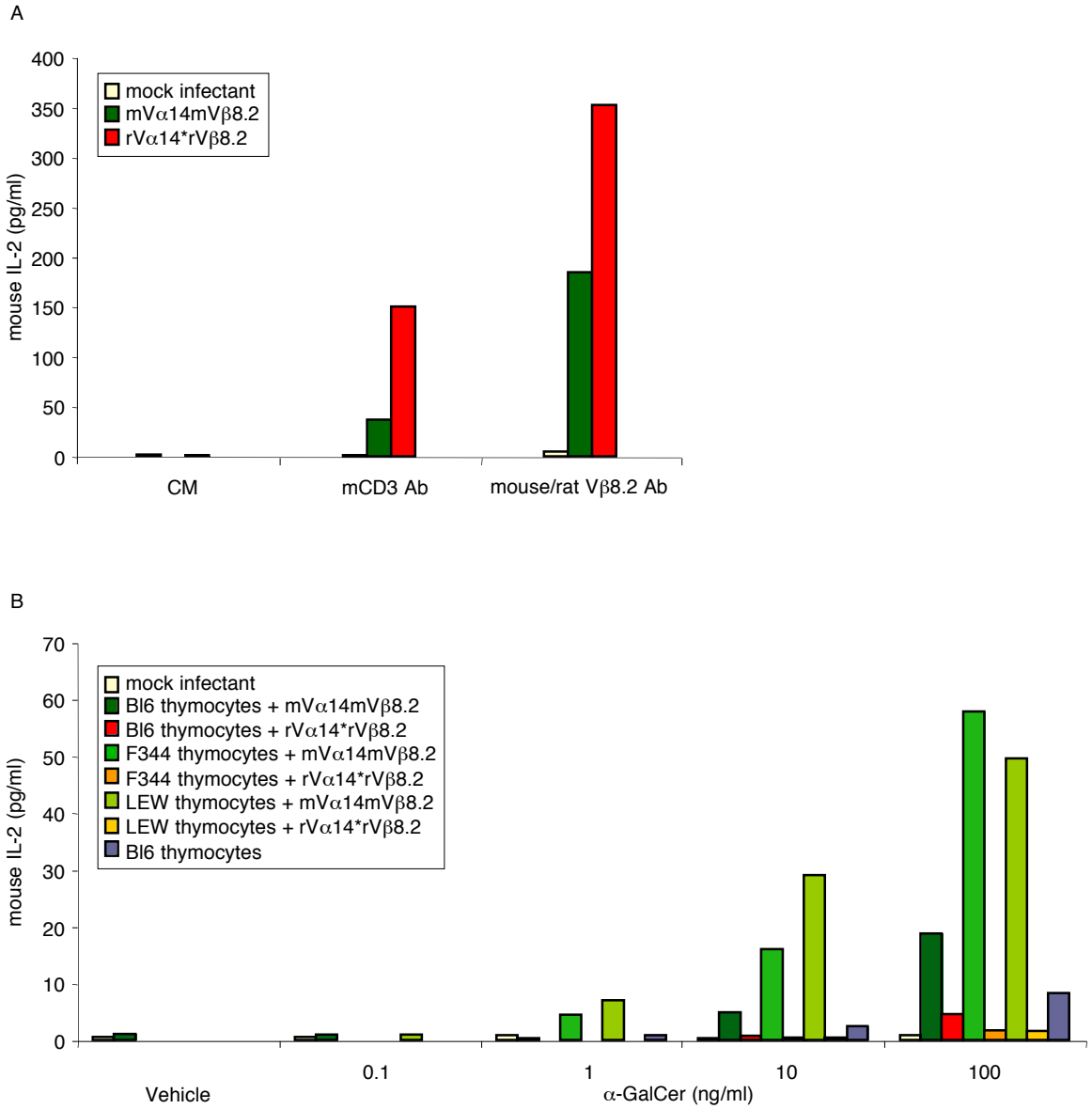


Figure 6:
Mouse but not rat Vα14⁺TCR produce IL-2 after stimulation with α-GalCer.
IL-2 production by BWr/mCD28 cell line expressing either mouse or rat Vα14⁺TCR ($5*10^4$ cells) after stimulation with anti-mCD3 (10 μg/ml) and anti-Vβ8.2 (F23.1 Ab-10 μg/ml, R78 Ab- 2 μg/ml) monoclonal antibodies (6A) or α-GalCer (6B).

2.2.2. Generation of chimeric mouse/rat TCR$^+$ cell lines- functional characterisation of rat Vβ8.2 TCR

It was not clear why the rat TCR^{inv} was not stained with mCD1d tetramer and was unresponsive to α-GalCer stimulation. The explanation, that the cloned rat Vα14$^+$ TCR was not CD1d restricted is questionable, since IL-4 and IFN-γ secretion by rat splenic lymphocytes was strictly α-GalCer and CD1d-dependent.

However, it could be possible that the lack of the rat TCR^{inv} specificity for α-GalCer was caused by differences in α/β chain pairing of mouse and rat TCRs. To test if rat TCR β-chain was permissive, mouse Vα14 TCR was expressed together with rat Vβ8.2 or Vβ8.4 chain in BWr/mCD28 cells.

In rats, there are two BV segments: BV8S2 (Vβ8.2) and BV8S4 (Vβ8.4), which are highly homologous to mouse Vβ8.2. LEW rats of Tcrb^l haplotype possess only one functional allele-Tcrb-Vb8.2^l (BV8S2A1) (Zhang *et al.,* 1992). The Tcrb-Vb8.4^l (BV8S4A1) allele due to one nucleotide deletion is out of the frame and is not expressed (Asmuss *et al.,* 1996). By contrast, in rat strains of Tcrb^a haplotype (like F344 or DA) both Tcrb-Vb8.2^a (BV8S2A2) and Tcrb-Vb8.4^a (BV8S4A2) alleles are expressed (Herrmann *et al.,* 1994).

The analysis of genomic Tcrb sequences showed that Tcrb-V8.2 and Tcrb-V8.4 alleles of Tcrb^l and Tcrb^a hyplotypes are closely related (Asmuss *et al.,* 1996). The sequence of Tcrb-Vb8.2^a differs from the sequence of Tcrb-Vb8.2^l by seven nucleotides resulting in three aa substitutions (Gold *et al.,* 1994). Whereas, F344 Tcrb-V8.4^a allele differs from LEW Tcrb-V8.2^l in seven aa, which were predominantly located in the CDR2 and CDR4 regions (Asmuss *et al.,* 1996).

Due to differences in the aa sequence of Tcrb^a and Tcrb^l, some interstrain differences in terms of T cell activation by SAgs and recognition by R78 mAb, between F344 and LEW rats, can be observed. Only cells expressing Tcrb-V8.2^l or Tcrb-V8.4^a alleles carry the R78 epitope (R at position 9 and N at position 62) and can be stained with anti-TCR β-chain R78 mAb. F344 Tcrb-V8.2^a and non-functional LEW Tcrb-V8.4^l are negative in the R78 staining. In terms of stimulation by SAgs, only R78$^+$ cells of LEW haplotype (Tcrb^l) but not R78$^+$ cells of F344 rat Tcrb^a haplotype respond to the bacterial SAgs (e.g. SEB, staphylococcal enterotoxin B) (Asmuss *et al.,* 1996).

Since R78 mAb used in the thesis for detection of rat TCRβ positive cells was able to recognise LEW Tcrb-Vβ8.2[l] and F344 Tcrb-Vβ8.4[a], it might be concluded that Tcrb-Vβ8.4[a] of F344 is the functional analog of mouse Vβ8.2 and LEW Tcrb-Vβ8.2[l]. In order to check wether Vβ8 TCR of F344 and LEW rat could equally contribute to the TCR activation, both rat β-chains were expressed with mouse Vα14[+]TCR in BWr/mCD28 cells, and the response of chimeric mouse/rat TCRs to α-GalCer *in vitro* stimulation was tested.

Two rat Vβ8 TCRs were used: classical LEW Tcrb-Vβ8.2[l] (further called rVβ8.2) and it's mutated form-Vβ8.2TCR-CDR(2+4), which due to mutation in CDR2 and CDR4 region, showed high homology with F344 Tcrb-Vβ8.4[a]. Therefore, the mutated form of LEW Tcrb-V was used instead of F344 βTCR and was further referred to as rVβ8.4.

Table 6: Correlation between the classification and nomenclature of rat TCRβ:

Tcrb-V		specificity for anti-Vβ8.2 mAbs	name of TCRβ used in thesis	name of TCR, expressed in BWr/mCD28 cells
Mouse	Tcrb-V8.2	recognised by F23.1	mVβ8.2	mVβ14mVβ8.2
LEW	**Tcrb-V8.2[l]**	recognised by R78	**rVβ8.2**	mVβ14rVβ8.2
	Tcrb-V8.2[l] CDR(2+4)mutant	recognised by R78	**rVβ8.4**	mVβ14rVβ8.4
	Tcrb-V8.4[l]	not labelled by R78	-	-
F344	Tcrb-V8.2[a]	not labelled by R78	-	-
	Tcrb-V8.4[a]	recognised by R78	-	-

The response of either mouse or chimeric mouse/rat TCR[inv] positive cell lines to α-GalCer presented by mouse and rat thymocytes was tested (Figure 7 B-D). As positive control, stimulation of these cells with anti-mouse CD3 mAb was performed (Figure 7A).

In this experiment, the differences in Ag presentation by mouse and rat CD1d[+] APCs as well as the capability of TCR[+] cells to produce cytokine were tested.

Figures 7C and 7D show, that BWr/mCD28 cell line expressing mouse (mVα14mVβ8.2) or chimeric mouse/rat TCR[inv] (mouse Vα14 chain expressed together rat Vβ8.2) secreted comparable amounts of IL-2 in the response to α-GalCer-loaded rat (F344 or LEW) thymocytes.

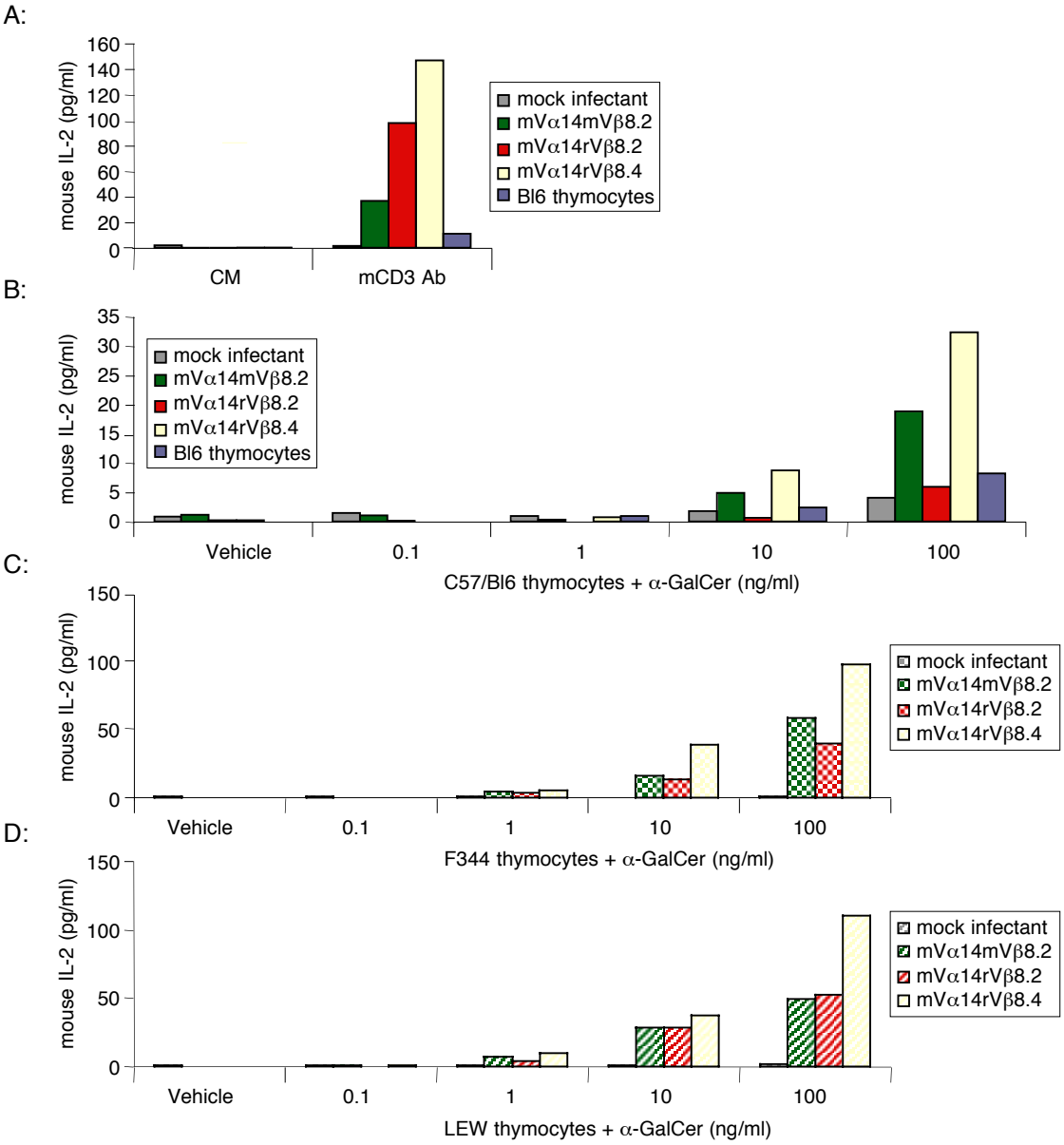


Figure 7:
Comparison of IL-2 production by BWr/mCD28 cells expressing mVα14 with rat Vβ8.2 or Vβ8.4 after 24h stimulation with mCD3 mAb or α-GalCer.
C57/Bl6, F344 or LEW thymocytes (1*10⁶) loaded with α-GalCer were used to 24h stimulation of BWr/mCD28 infectants.

When C57/Bl6 mouse CD1d$^+$ thymocytes were used as APCs (Figure 7B), some differences in the levels of produced IL-2 by TCR-transgenic cells, depending on expressed TCR, could be seen. Because mouse thymocytes cultured with α-GalCer secreted significant amounts of IL-2, it is not clear wether the cytokine production by activated BWr/mCD28 cells expressing mVα14rVβ8.2 was really specific. Nevertheless, data presented in Figure 7 suggest that BWr/mCD28 cells positive for mouse (mVα14mVβ8.2) but not mouse/rat (mVα14rVβ8.2) TCR^{inv} were responsive and could produce IL-2 in α-GalCer-dose dependent manner (Figure 7 B). In addition, the cytokine secretion by BWr/mCD28 cell line expressing mVα14rVβ8.4 TCR was the best regardless of the type of used APCs.

In conclusion, the α-GalCer recognition and the IL-2 production could be decreased by expression of mouse Vα14 chain together with LEW Vβ8.2, but on the other hand it could be enhanced by pairing of mouse α-chain with F344 rat Vβ8.4.

In this functional assay three TCR^{inv}-positive cell lines having the same α- but different β-chain had diverse reactivity to α-GalCer. Thus, it might be possible that β-chain of TCR^{inv} influences the Ag recognition depending on the CD1d used for Ag-presentation.

2.2.3. Staining of mouse TCR^{inv} transgenic cell lines with mCD1d α-GalCer tetramer

If the β-chain of TCR^{inv} indeed preferentially distinguishes between mouse and rat CD1d, it was important to check if the expression of different Vβ8.2 chains also influences the binding of CD1d tetramers. Therefore, cells used in functional assay were additionally stained with mCD1d-α-GalCer tetramer.

Figure 8 shows that the pattern of the tetramer staining of different mouse cell lines correlated with the results from the stimulation assay. The binding of mouse CD1d tetramer to mouse/rat (mVα14rVβ8.2) TCR^{inv} was much weaker than binding to the classical mouse (mVα14mVβ8.2) counterpart. BWr/mCD28 cell line expressing mVα14 chain paired together with rat Vβ8.4, had the comparable level of CD3 expression as mVα14rVβ8.2-positive cells, but it was the most positive in tetramer staining. Therefore cells, which produced less IL-2 upon α-GalCer stimulation, were

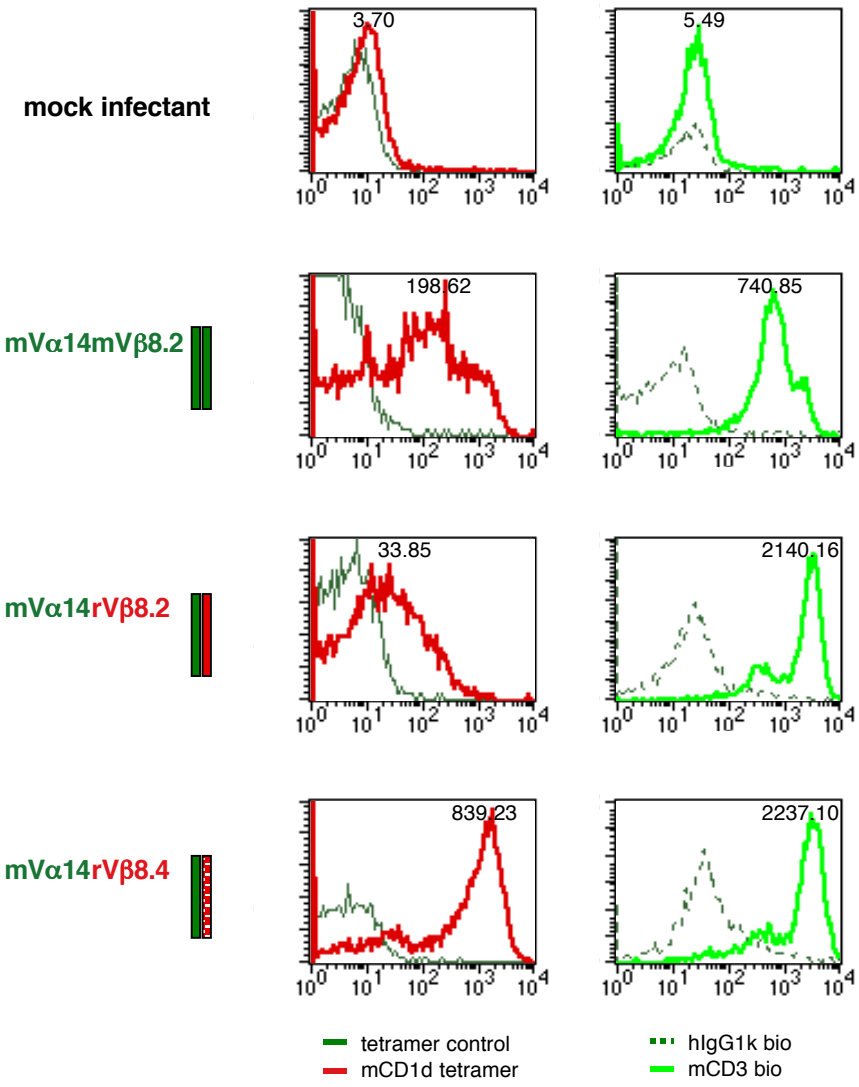


Figure 8:
The staining of BWr/mCD28 cells expressing mVα14⁺TCR and diverse Vβ8 chains with mCD1d-α-GalCer-tetramer PE and anti-mouse CD3 mAb.
The numbers in the histograms show mean fluorescence intensity of positively stained cells, corrected for background staining.

stained with tetramer only to some extent, while good IL-2 producers had high affinity for mCD1d-α-GalCer tetramer.
Thus, the coexpression of mouse Vα14-chain with diverse Vβ8.2 chain influenced both α-GalCer recognition and cytokine production as well as the binding to mouse CD1d-α-GalCer tetramer.

However, results from functional assay can be questionable. As it was presented in Figure 7A, upon anti-mouse CD3 mAb stimulation mouse $V\alpha14^+$ transgenic cells produced different amounts of IL-2. Furthermore, the level of IL-2 released by mouse thymocytes cocultured with α-GalCer was relatively high. To get more comparable results, the cytokine production upon α-GalCer was normalised to the cytokine production upon CD3 mAb stimulation. The results are presented in Figure 9. Similar to the results in Figure 7, F344 and LEW thymocytes used as APCs presented α-GalCer to $V\alpha14^+$ cells even better than mouse cells did. By contrast, the pattern of IL-2 production by BWr/mCD28 cells transduced with mouse Vα14 chain and mouse or rat Vβ8 chain was different from this before standardisation. This time, mouse TCR (Vα14/mVβ8.2) responded to α-GalCer stimulation the best, regardless of the type of APCs used.
After standardisation, the differences in the level of IL-2 released by BWr/mCD28 cells expressing two chimeric mouse/rat TCRs (Vα14/rVβ8.2 andVα14/rVβ8.4) were significant only for cells stimulated with α-GalCer presented by mouse thymocytes (Figure 9A). In agreement what was presented previously, mVα14/rVβ8.4-TCR was more responsive than mVα14/rVβ8.2-TCR. However, bearing in mind high background IL-2 production by mouse thymocytes, it is very likely that IL-2 detected in the supernatants of BWr/mCD28mVα14rVβ8.2 cells cocultured with α-Galcer loaded mouse thymocytes was produced not by TCR-transgenic cells but rather by APCs.
When BWr/mCD28mVα14/rVβ8.2 and BWr/mCD28mVα14/rVβ8.4 cells were stimulated with α-GalCer-loaded LEW and F344 thymocytes, both cell lines released comparable amounts of IL-2.
To conclude, one experiment, two different ways of presenting results, two different conclusions. From both Figure 7 and Figure 9, we can say that indeed there were differences in the response of TCR^{inv} transgenic cell lines to α-GalCer stimulation,

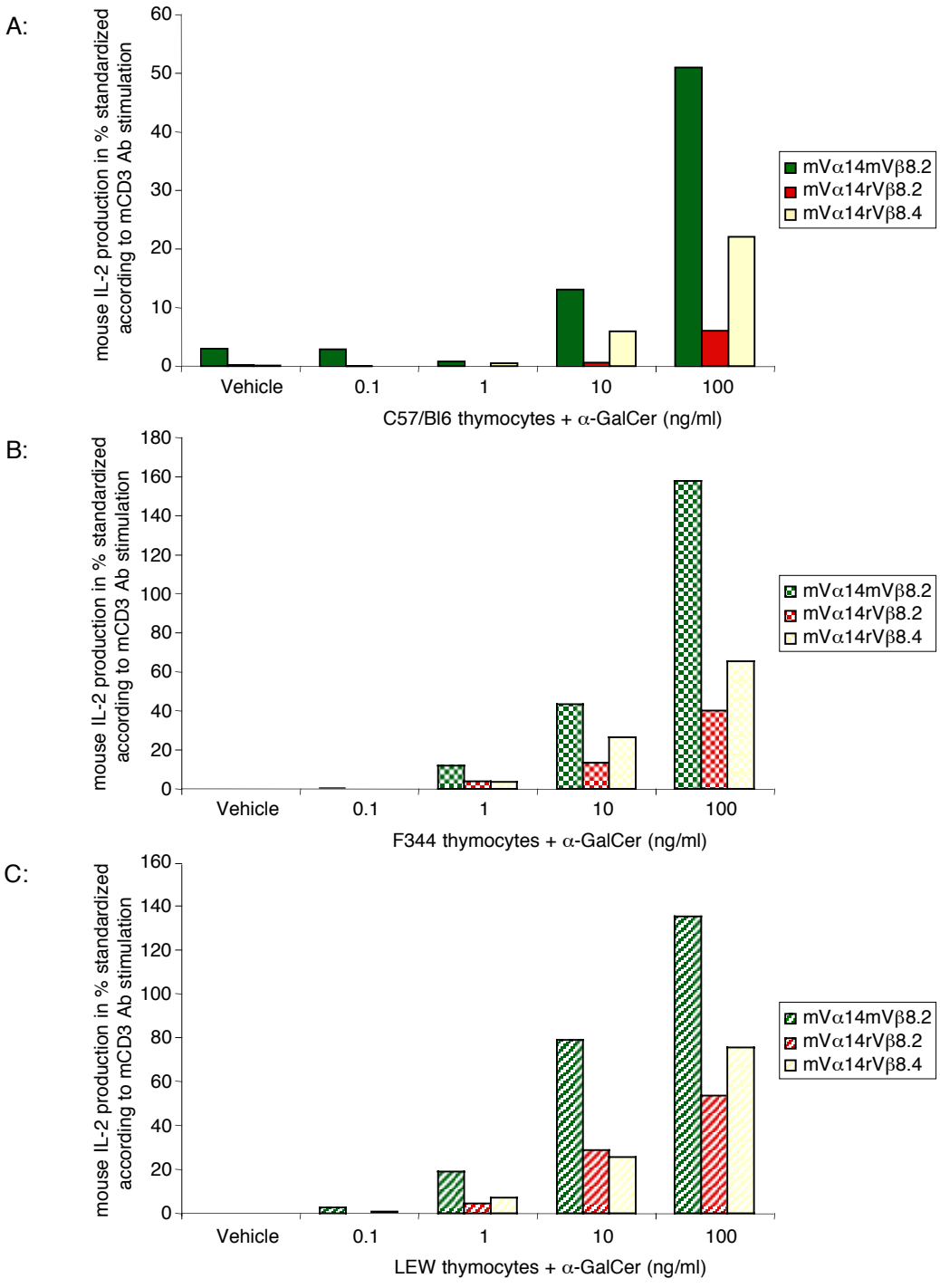


Figure 9:
Comparison of IL-2 production by BWr/mCD28 cells expressing mVα14 with rat Vβ8.2 or Vβ8.4 after 24h stimulation with α-GalCer.
The IL-2 production upon α-GalCer stimulation was standardized according to the IL-2 production upon mCD3 mAb stimulation.

depending on the combination of expressed Vα and Vβ8 of TCR. However, it was difficult to estimate if the Vβ8.2 TCR of LEW and the Vβ8.4 TCR of F344 rat had the same or different capacities to trigger the response of mVα14^{+}BWr/mCD28 cells to α-GalCer. Since the results from the tetramer staining of TCR^{inv} transgenic BWr/mCD28 cells (Figure 8) correlate more with the data from stimulation assay presented in Figure7, therefore we assume that the first interpretation of results was more likely.

2.2.4. Sequencing and amino acid analysis of rat liver Vα14^{+}TCR

The pairing of mouse Vα14 chain with Vβ8 chains representing two rat strains resulted in different response to α-GalCer. When Ag was presented by mouse CD1d^{+} thymocytes, BWr/mCD28 cell line expressing rat Vβ8.2 was less functional than cells positive for mouse Vβ8.2 or rat Vβ8.4 chain.
Because the same Vβ8.2 chain was a part of rat TCR^{inv}, therefore, it could be possible that the lack of the rat TCR^{inv} reactivity to α-GalCer was partially due to the presence of "not functional" Vβ-chain.

Another reason for the lack of α-GalCer specificity could be due to the Vα-chain of rat TCR^{inv}. To check this possibility, the amino acid sequence of rat liver Vα14 chain (marked in Figure 10 in black) was compared with the Vα14.1 (AB036694) and Vα14.1 (AB036695) TCR sequences, representing type I of rat TCR^{inv}, that is predominantly expressed in rat liver (Matsuura *et al.,* 2000).
The alignment (presented as Figure 10) of the sequence of cloned rat Vα14-chain with the published in GeneBank rat Vα14 TCR sequences (here in blue) revealed the presence of two alterations in the V-region.
In this thesis, the amino acid numbering of TCR V-regions was according to Matsuura (Matasuura *et* al., 2000). Since this nomenclature differs from that of Sim (Sim *et al.*, 2003) and the commonly used nomenclature of IMTG (http://imgt.cines.fr:8140), the amino acids depicted in Figure 10 and according to two nomenclatures are presented in Table 7.

Table 7:

	Matsuura *et al.*, 2000	**IMTG** (Sim *et al.*, 2003)
Start codon	1	1 (responds to 22 aa by Matsuura)
First aa alteration	22	1
Second aa alteration	72	51
CDR1-region	47-53	25-32
CDR2-region	70-77	49-55

The arginine (R) at position 22 (according to Matsuura's numeration, responds to aa 1 according to IMTG nomenclature), as well as threonine (T) at position 72 (51 according to IMTG) in published rat TCR sequence were exchanged for lysine (K). The first K at position 22 of cloned rat TCR sequence was also present in the functional mouse Vα14-chain, therefore we suggest that this amino acid was not responsible for lack of rat TCR reactivity to α-GalCer.

Matsuura and coworkers have reported that the CDR1 (amino acids from 47 to 53) region of rat TCR invariant is conserved between four rat TRAV14 proteins. By contrast, the CDR2 region (amino acids from 70 to 77) is variable (Matsuura *et al.,* 2000). Additionally, six amino acids at positions: 4H (histidine), 72T (threonine), 73N (asparagine), 75E (glutamic acid), 77K (lysine) and 80R (argine) are common for TRAV14S1, TRAV14S2 and TRAV14S4 (Matsuura *et al.,* 2000). Therefore, we suggest that the amino acid alteration at position 72 of cloned rat TCR might have been critical for Ag-recognition. The exchange of threonine having uncharged side group into basic lysine, which has a charged side chain, in the CDR2 region (complementarity determining region) of rat TCRα could have destroyed the interaction of TCR with CD1d molecule and abolished α-GalCer recognition.
Furthermore, when the sequence of cloned rat TCR^{inv} was compared with responding mouse sequence (M14506), one amino acid difference within the CDR3 regions was found. Instead of alanine (A) present in mouse sequence, the rat CDR3 contained valine (V).
The V-region and J18 region of rat TCR are known but the region where V and J regions are joined was predicted according to IMGT database and might contain either alanine (A) or valine (V), while in the mouse sequence alanine (A) or glycine (G) can be found. Therefore, it is not clear if valine, which was the last aa of the V-region, could negatively influence the α-GalCer recognition by rat TCR^{inv}.

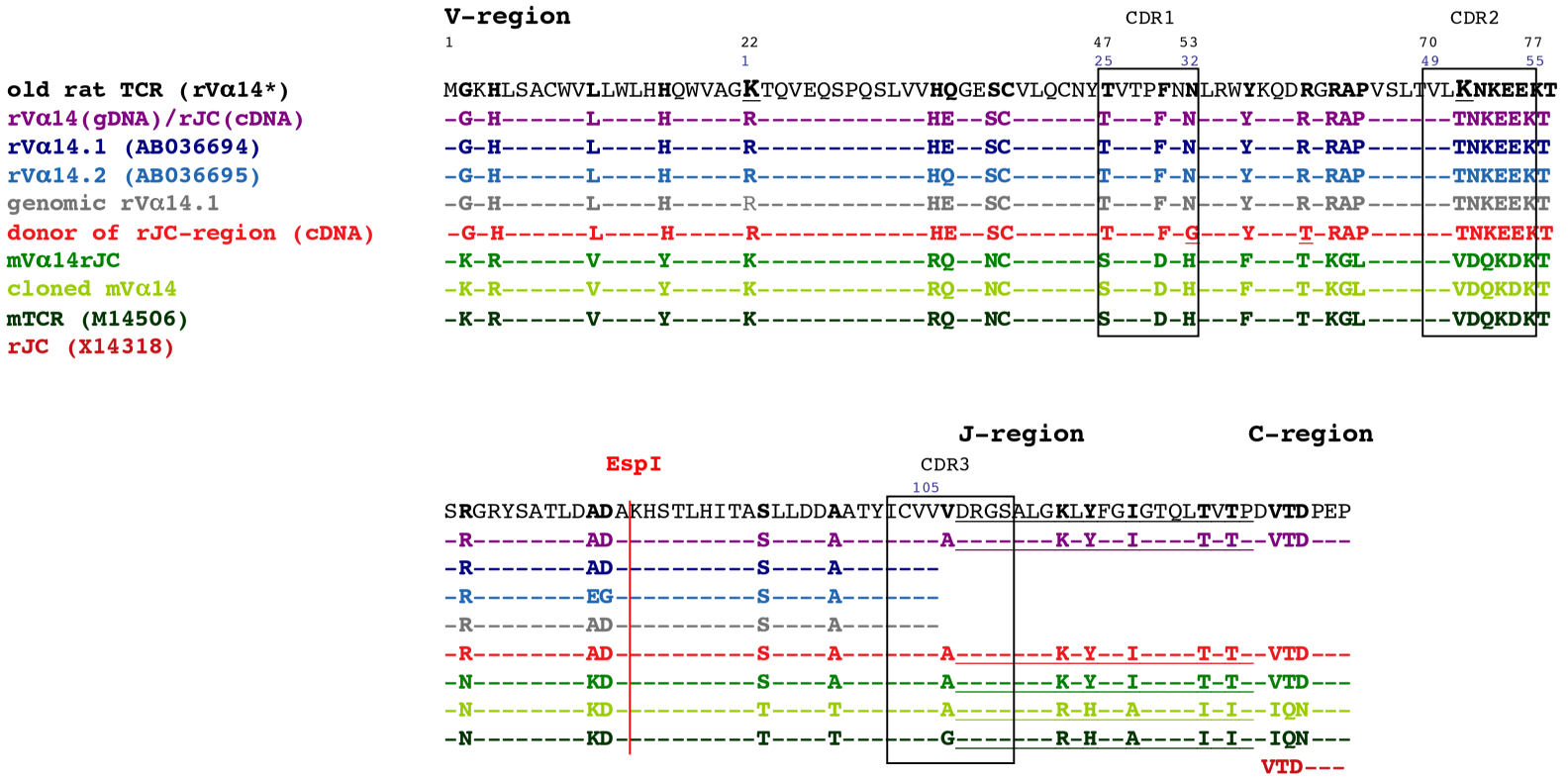


Figure 10: Comparison of cloned rat, genomic rat and mouse Vα14 TCR sequences.

Identical aa are presented as (-), while different aa are marked as bold letters. K22 and K72 in the rVα14* sequence, which differ from those in published rVα14 sequences, are underlined as is the joining region starting with DRGSAL…The CDR1, CDR2, and CDR3 regions are boxed, and numbering according to Matsuura *et al*., (2000) is in black. Numbers in blue correspond to the nomenclature by Sim *et al., (*2003) and IMGT (http://imgt.cines.fr:8104), in which CDR1 comprises aa 25-32, CDR2 comprises aa 49-55 and CDR3 starts at aa 105. The EspI site used for the generation of chimeric TCR is depicted by a red line.

2.3. Generation and expression of chimeric rat Vα14^{+} TCR

To generate the rat TCR^{inv} free of mutations, the cloning procedure was repeated. Several cDNA-derived F344 liver and splenic Vα14^{+} TCRs were analysed but each time the cloned sequences were different with published ones.

To determine whether aa differences in cDNA sequence appeared as PCR errors, the expression of four TRAV14 gene products was analysed on the genomic level, using high fidelity DNA Polymerase. The germline sequence was identical with published one- the PCR product was free of mutations.

In order to generate rat TCR invariant containing a canonical J-region, the sequence of rat TCR obtained from cDNA ("F344 liver rVα14*TCR" marked in Figure 10 in black) containing mutations was replaced for the right Vα14 sequence from gDNA (in grey), creating in this way chimeric rVα14(gDNA)/rJC(cDNA) (depicted in violet) rat TCR. As a source of rat JC-region another rat liver TCR (sequence in red) was used. Similarly to the previously cloned rat Vα14^{+} TCR, the new TCR had mutations in V-region but in contrast with old rat TCR, it contained at the VJ-joining region not valine but alanine, typical for mouse TCR.

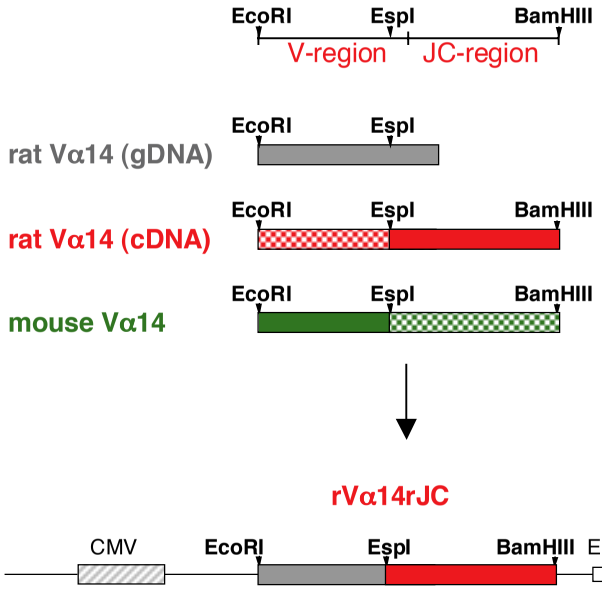
The cloning strategy is presented in Figure 11. The digestion with EspI restriction enzyme allowed excision of the part of V-region containing mutations off leaving the CDR3 region and V-J juncional region untouched.

In parallel, the mouse (Figure 10: bright green sequence- "cloned mVa14") and mouse/rat chimeric ("mVα14rJC" marked in dark green) TCRs were generated as positive control.

Cloned into retroviral GFP vector (EcoRI/BamHIII site) rVα14^{+}rJC or mVα14^{+}rJC TCRs were used for transfection of 293T cells and infection of BW58 or BWr/mCD28 cell lines. Similar to previously, Vα14^{+}-GFP^{+} cells were infected with either mouse or rat Vβ8.2 chain generating the panel of chimeric TCRs.

The surface expression of new TCRs was analysed by FACS staining. Only cell lines positive for Vβ8.2 and mCD3 Abs had TCR receptor on the surface.

Data from representative staining, presented as Figure 12, showed that only BWr/mCD28 expressing mouse canonical mVα14TCR rearranged with rat Vβ8.2 was positively stained with anti-Vβ8.2 and anti-CD3 mAbs. BWr/mCD28 cells positive for mVα14rJC or rVα14rJC TCR, paired with rat Vβ8.2 chain, were



rVα14(gDNA)/rJC (cDNA) MGKHLSACWVLLWLHHQWVAGRTQVEQSPQSLVVHEGESCVLQCNYTVTPFNNLRWYKQDRGRAPVSLTVLTNKEEKT
SRGRYSATLDADAKHSTLHITASLLDDAATYICVVADRGSALGKLYFGIGTQLTVTPDVTDPEPAVYQLKDPQSDNITLCLFTDFDSQTNVSQSASSEAFVSGNTV
MDMKAMDSKSNGAIAWSNQTSFSCKDAFKKNASHPTSDVPCDAKLVEKSFETDMNLNFQNLSVMGLRILLLKVAGFNLLMTLRLWSS

mVα14/rJC MKKRLSACWVLLWLHYQWVAGKTQVEQSPQSLVVRQGENCVLQCNYSVTPDNNLRWFKQDTGKGLVSLTVLVDQKDKT
SNGRYSATLDKDAKHSTLHITASLLDDAATYICVVADRGSALGKLYFGIGTQLTVTPDVTDPEPAVYQLKDPQSDNITLCLFTDFDSQTNVSQSASSEAFVSGNTV
MDMKAMDSKSNGAIAWSNQTSFSCKDAFKKNASHPTSDVPCDAKLVEKSFETDMNLNFQNLSVMGLRILLLKVAGFNLLMTLRLWSS

Figure 11: Generation of chimeric Vα14 TCRs.

The rat Vα14 TCR obtained from gDNA was digested with EcoRI/EspI, while rat Vα14rJC TCR obtained from cDNA as well as mouse Vα14mJC TCR were digested with EcoRI/EspI/BamHIII enzymes. Appropriate DNA fragments were purified after agarose gel electrophoresis and were ligated with T4 Ligase into EcoRI/BamHIII site of retroviral pczCFG5-IEGN vector.

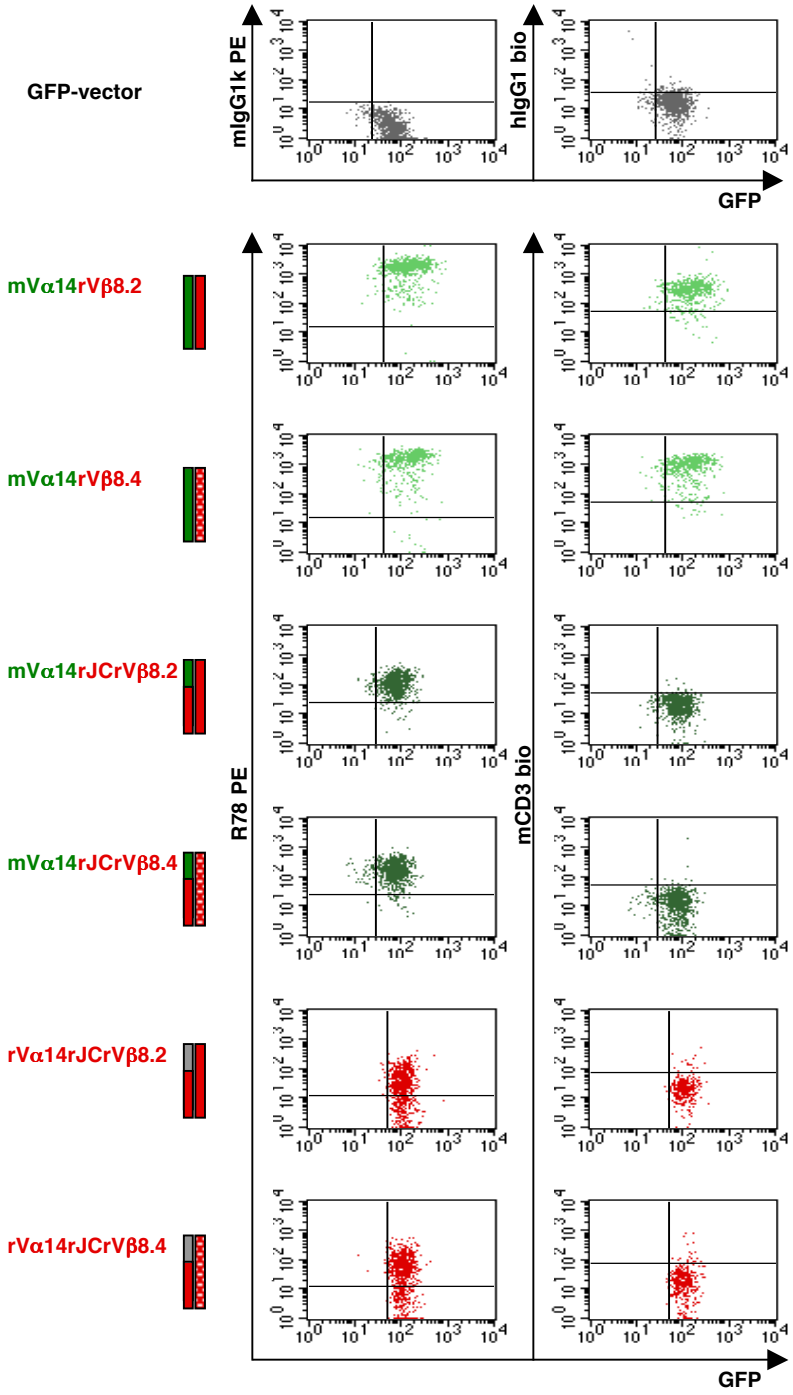


Figure 12:
Expression of rat Vβ8.2 and Vβ8.4 but not mouse CD3 on the surface of BWr/mCD28 cells transduced with chimeric mouse and rat Vα14⁺TCR.

positively stained with anti-rat Vβ8.2 mAb but were negative for anti-mouse CD3 Ab, suggesting that the rat Vβ8.2 chain could appear on the surface of BWr/mCD28 cells as a homodimer. These observations are consistent with a previous study showing rat but not mouse Vβ8.2 expression on the surface of BW cells without CD3 (Anne Asmuss's PhD thesis). Neither rat/rat (rVα14rJC) nor mouse/rat (mVα14rJC) chimeric TCR could be successfully expressed with mouse Vβ8.2 chain. The absence of CD3-α/βTCR complex on the surface made these transgenic cells unresponsive to Ab or Ag stimulation.

The rat Vα14$^+$ TCR was cloned twice. First TCR could be successfully expressed on the surface of BWr/mCD28 cells but probably due to mutation present in V-region it could respond to Ab but not to α-GalCer stimulation. The second Vα14$^+$TCR, generated using molecular biology methods had the canonical amino acid sequence but it could not be paired with any of Vβ-chains and therefore it could not be expressed on the surface of BWr/mCD28 cells.
The comparison of the sequences of both cloned rat TCR^{inv} (Figure 10) as well as the generation of chimeric TCR comprising rat Vα14-region rearranged with mouse JC region will allow the definition of the positions in rat TCR responsible for the lack of α-GalCer reactivity.

III. RAT CD1d

3.1. Expression of rat CD1d in different rat organs

To closer identify rat NKRP1A$^+$TCR$^+$ cells and the mechanisms of their α-GalCer recognition, the capacity of rat CD1d molecule to present this Ag was tested.
Rat CD1d has been described as nonpolymorphic surface glycoprotein consisting of a heterodimer of α-chain (45kDa) associated with β2m. CD1d can be expressed in lymphoid (thymus, LN, spleen) and nonlymphoid (liver, kidney, heart, lung) organs.

No anti-rat CD1d monoclonal antibody is available for the detection of rCD1d surface expression. To confirm the presence of CD1d in different rat organs, RNA from thymus, liver, spleen and bone marrow lymphocytes was isolated and RT-PCR with CD1d specific primers was done.
According to data presented in Figure 13, rat CD1d could be detected on mRNA level in all tested organs. A strong signal for rCD1d was found in BM, weaker in spleen and liver. No or only a weak band was observed for cDNA prepared from RNA isolated from thymocytes.

3.2. Cloning and expression of ratCD1d

To characterise the specificity of rat NKRP1A^{+}TCR^{+} cells for α-GalCer, rat CD1d was cloned. The CD1d DNA prepared from F344 rat bone marrow was cloned into TOPO-cloning vector and sequenced. Alignment of cloned rat CD1d with published rat (AB029486) and mouse CD1ds (mCD1d1-NM_007639; mCD1d2-NM_007640) is shown in Figure 14.

Mouse and rat CD1d molecules were highly homologous. One nucleotide at the position 28 of cloned rCD1d was different with rCD1d sequence published in the GeneBank (NM_017079, sequence not shown), but identical with other rCD1d submitted under the number AB029486. The arginine 28 (responding to 11. aa according to numbering by Ichimiya (Ichimiya *et* al., 1994) was also found in the mouse CD1d sequence. Furthermore, the tyrosine-targeting motif (YXXZ-marked by quadrant frame), required for endosomal localisation of CD1d as well as four aa (Arg79, Asp80, Glu83 and Asp 153), important for the glycolipid presentation by mouse CD1d (Kamada *et* al., 2001) were also found in the rat CD1d sequence. Therefore, it is very possible that both mouse and rat species have the same or similar pathway of processing and Ag presentation.

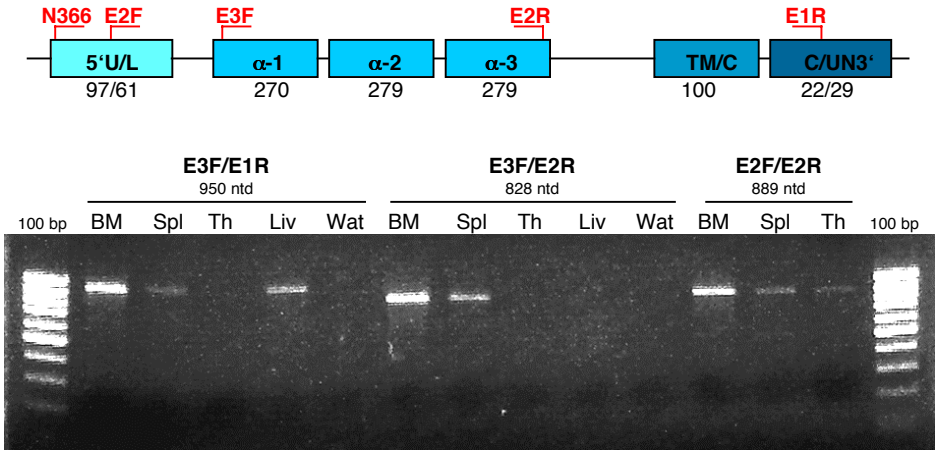


Figure 13: Detection of F344 rat CD1d mRNA by RT-PCR.
500 ng of RNA isolated from bone marrow (BM), spleen (Spl), thymus (Th) and liver (Liv) lymphocytes were used to prepare cDNA, which was further amplified with rCD1d specific primers (E3F/E1R, E3F/E2R, E2F/E2R).

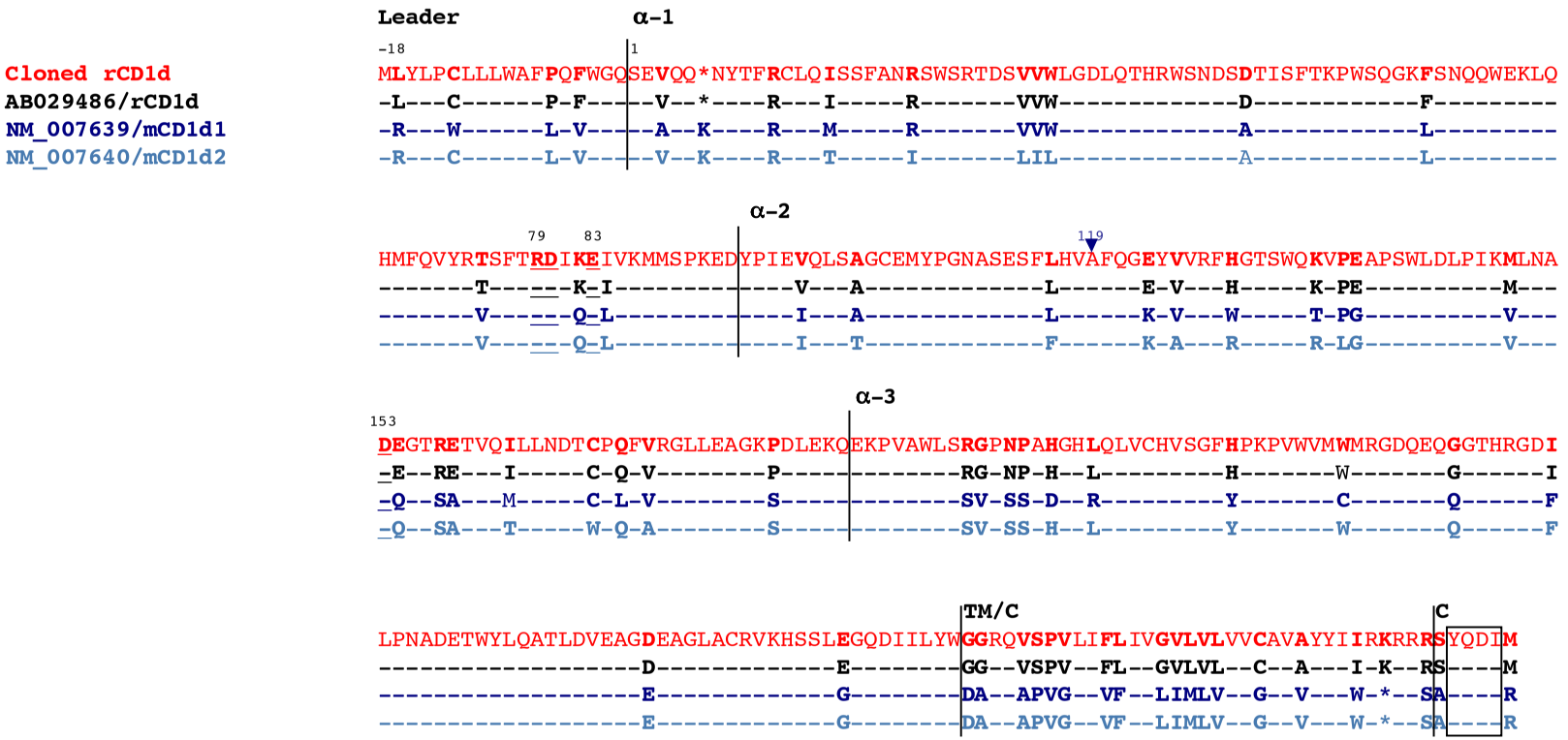


Figure 14: Comparison of cloned rat CD1d with published mouse and rat CD1d sequences.
F344 rat BM derived DNA was amplified with N366/E1R primers, cloned into TopoCloning vector and sequenced with TopoCloning vector specific primers (M13F/M13R) binding externally to the insert. The alignment of cloned rat CD1d (in red) with published rCD1d (AB029486) and mouse CD1d1 (NM_007639) and CD1d2 (NM_007640) sequences. Identical aa are presented as dashes, different aa as a bold letters, and amino acid deletions are represented by a (*). Four amino acids involved in the α-GalCer-CD1d interaction (Arg79, Asp80, Glu83 and Asp153; Kamada *et al*., 2001) are presented as underlined bold letters. Alanine at position 119 typical for F344 rat strain (Katabami *et al*., 1998) is depicted by blue arrow. The numbering of aa responds to the numbering given by Ichimiya (Ichimiya *et al*., 1994).

3.3. Functional characterisation of cloned rCD1d

The question was if the structural amino acid homology of rat and mouse CD1d corresponded to functional homology. Cloned into bicistronic retroviral vector BM derived rat CD1d was used for transfection of 293T cells and infection of different human, mouse and rat cell lines. GFP-positive cells were then used in functional assays, in which the capacity of rCD1d transduced cell lines to present α-GalCer was tested.

3.3.1. Presentation of α-GalCer by CD1d transgenic cells to KT12 hybridoma

As example, JM2-rat F344 hepatoma infected with rat CD1d was loaded with different doses of α-GalCer and was used for stimulation of mouse Vα14^{+} KT12 hybridoma. The level of IL-2 produced by KT12 was tested in supernatants after 24h co-culture. Results from this assay are presented in Figure 15.

KT12 hybridoma cells, due to high autopresentation, could secrete some IL-2 when cultured with α-GalCer only (not depicted in Figure 15). JM2 cells as hepatic cells expressed some endogenous rCD1d, therefore the wild type or mock-infected cells were able to stimulate IL-2 production by KT12 hybridoma. However, when KT12 hybridoma was co-cultured in the presence of JM2 transduced with rCD1d cells, the amounts of mouse IL-2 significantly increased. Therefore, cloned rat CD1d was present on the surface of JM2 hepatoma and efficiently stimulated IL-2 production by KT12 in α-GalCer dose dependent manner.

JM2 cells derive from F344 tissues. Therefore, it could be possible that cloned rat CD1d molecule was efficiently expressed on the surface of this hepatoma due to species/tissue specificity. To test whether rCD1d could be expressed in other cells, diverse cell lines were infected with this CD1d and were used as APCs in functional *in vitro* assays.

KT12 hybridoma could be efficiently stimulated with α-GalCer presented by mouse RMA-S (Figure 16 D), P80 (Figure 16 B), and BW (Figure 16 C), human RAJI (Figure 16 A), or rat H-II-4-E (Figure 16 E) cell lines transduced with rat CD1d. In all cases, the increase in IL-2 secretion, as compared with control wild type or GFP-vector infected cells, was especially visible for KT12 hybridoma stimulated with rCD1d transgenic cells, loaded with lower doses of α-GalCer.

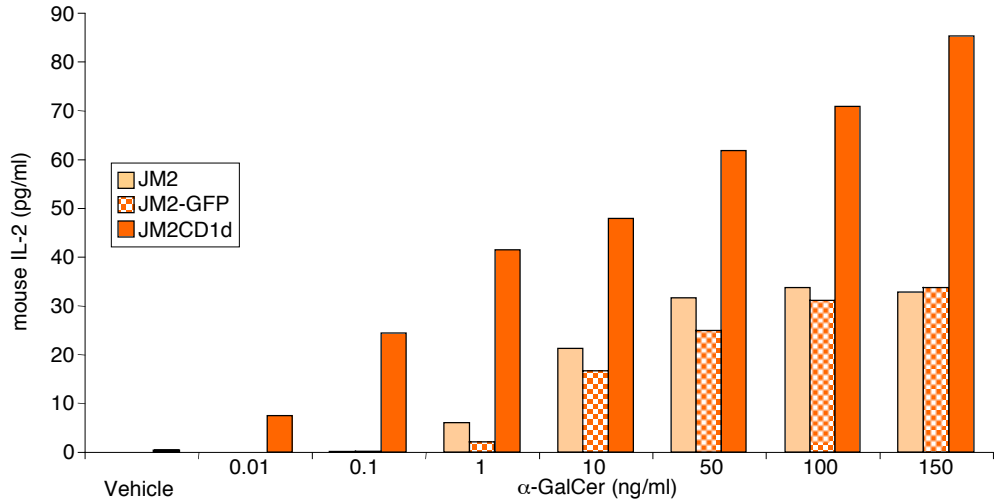


Figure 15:
IL-2 production by KT12 hybridoma after 24h stimulation with α-GalCer presented by JM2 cells transduced with rat CD1d.
$2.5*10^4$ of JM2 transgenic cells, plated onto 96 well flat bottom plate, were loaded ON with α-GalCer. Next morning $5*10^4$ of KT12 hybridoma cells were added to APCs. After 24h culture at 37°C, the IL-2 in supernatants was measured by ELISA.

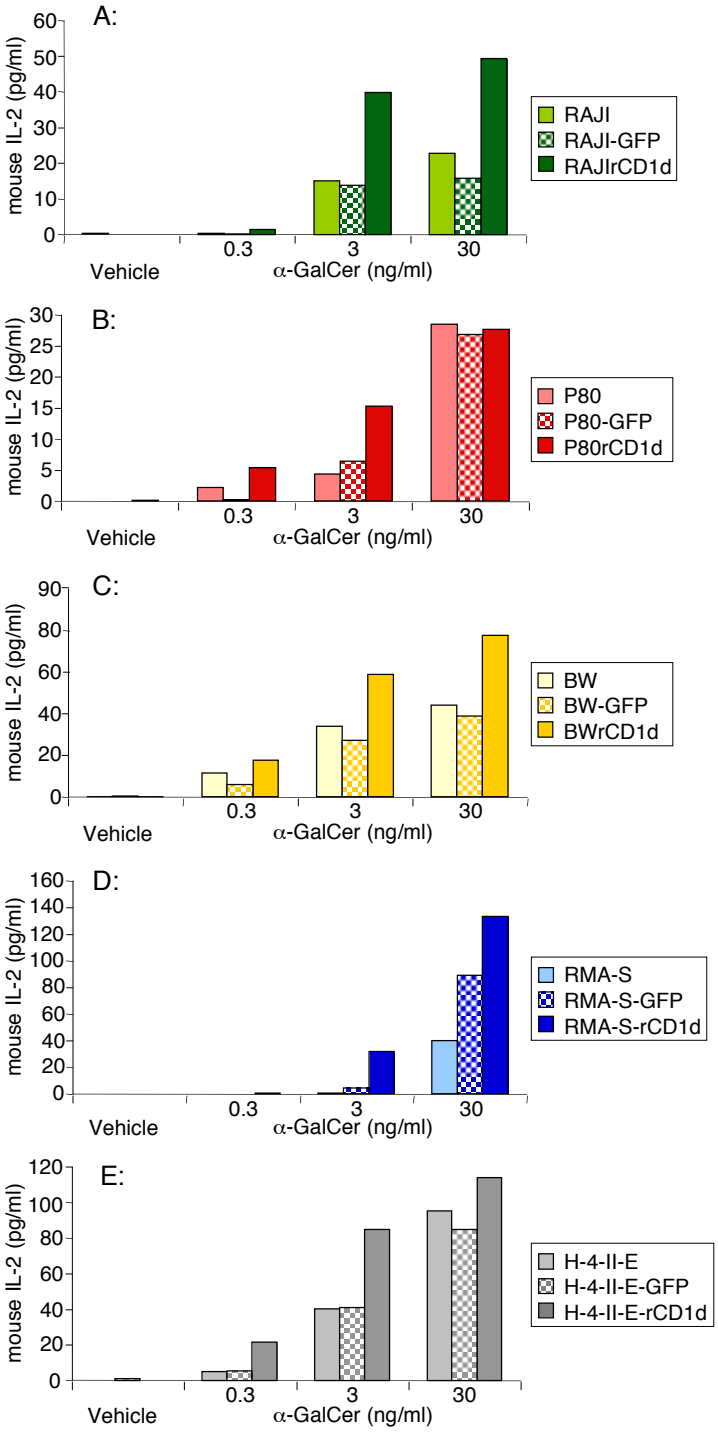


Figure 16:
IL-2 production by KT12 hybridoma after 24h stimulation with α-GalCer presented by cell lines expressing rat CD1d.
To $5*10^4$ of CD1d transgenic cells, loaded for 1-2h with α-GalCer, $5*10^4$ of KT12 hybridoma cells were added. After 24h coculture at 37°C, the IL-2 in supernatants was measured by ELISA.

The differences in terms of α-GalCer presentation to KT12 hybridoma, by CD1d$^+$ cell lines could be caused by differences in cell line origin or/and differences in α-GalCer processing. Additionally, the natural capacity of CD1d$^+$ cells for Ag presentation should be also taken into account.

3.3.2. Presentation of α-GalCer by CD1d transgenic cells to mouse TCR^{inv}. The importance of CD80-CD28 co-stimulatory pathway in the activation of Vα14$^+$ cell lines.

The presence of CD80-CD28 co-stimulatory signal is necessary for efficient activation of T cells. NKT cells express CD28 molecule, thus the interaction between CD28 and CD80 present on APC could be important for activation of NKT cells.

From previous functional assays (data not presented in the thesis), we knew that the expression of TCR^{inv} alone on the surface of BW cell line was not efficient in triggering cytokine secretion. In contrast, BW cells positive for CD28, when transduced with TCR^{inv} could be activated and could respond to α-GalCer-CD1d stimulation.

To enhance the response of BWm/rCD28$^+$ cells expressing mouse TCR^{inv} to α-GalCer, rat CD80 molecule was expressed on the surface of CD1d$^+$ cell lines, creating in this way optimal conditions for co-stimulation. In the presence of two signals: TCR-α-GalCer-CD1d and CD28-CD80, the stronger response of BWr/mCD28-mVα14$^+$ cells to α-GalCer was expected.

Different P80 CD1d$^+$rCD80$^+$ APCs, loaded with α-GalCer were used for 24h stimulation of mouse TCR^{inv} transgenic BWr/mCD28 cells. The cytokine secretion was estimated by ELISA and results are shown in Figure 17.

No or low levels of IL-2 could be detected in supernatants of BWr/mCD28-mVα14$^+$ cells incubated with P80 wild type, P80-GFP, P80mCD1d$^+$ or P80rCD1d$^+$ cells. When P80 cells positive for rCD80 and either rat or mouse CD1d were used as stimulus, the cytokine secretion increased significantly. Comparing the capability of mouse and rat CD1d to present α-GalCer, in this experiment the presentation of Ag by rat CD1d was better than the presentation by mouse counterpart- mouse Vα14$^+$ cells produced more IL-2 in the response to rat than to mouse CD1d.

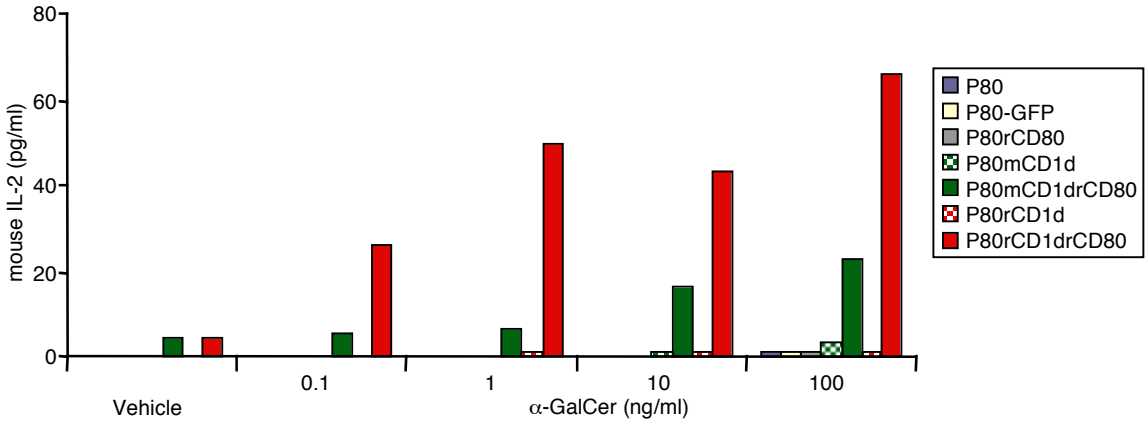


Figure 17:
Importance of CD28-CD80 co-stimulatory interactions for responses of Vα14 positive cells.
5*10⁴ APCs were loaded with different doses of α-GalCer and were used for 24h stimulation of BWr/mCD28 (5*10⁴) cells expressing mVα14mVβ8.2 TCR. After 24h IL-2 in supernatants was measured by ELISA.

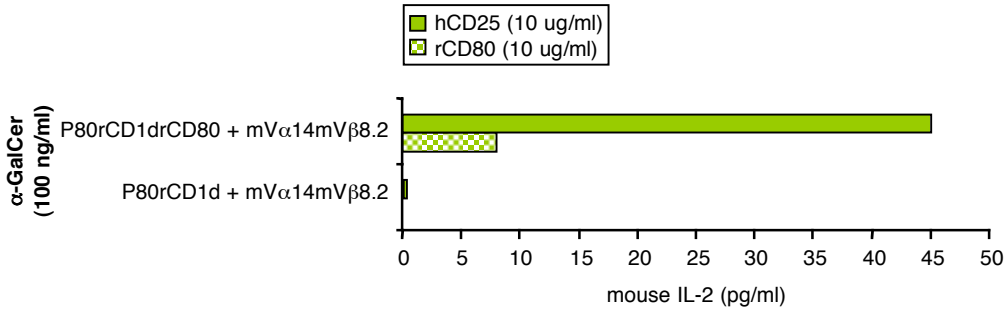


Figure 18:
Inhibition of IL-2 production by anti-rat CD80 mAb.
BWr/mCD28mVα14mVβ8.2 (5*10⁴) cells were incubated for 24h with α-GalCer loaded APCs and either hCD25 or rCD80 mAbs. IL-2 in supernatants was measured by ELISA.

Furthermore, BWr/mCD28 cell line expressing mouse TCR^{inv} could recognise α-GalCer presented by both mouse and rat CD1d but only in the presence of two stimulatory signals.

Figure 18 shows that the IL-2 production by BWr/mCD28mVα14mVβ8.2 cells could be inhibited in the presence of anti-rat CD80 mAb (10 μg/ml), while the addition of isotype control (here hCD25 Ab at 10 μg/ml) to the culture did not influence cytokine secretion.
Therefore, the presence of CD80-CD28 costimulatory pathway was not only important but rather necessary for efficient activation of mouse $Vα14^+$ cell lines by α-GalCer presented by P80CD1d transgenic cells.

In conclusion, the CD1d-lipid Ag-Vα14TCR^{inv} system shows similarities to MHC-peptides Ag-TCR system. In both cases, Ag recognition and efficient cell activation can take place only in the presence of two stimulatory signals.
The cloning and over-expression of rCD1d on the surface of different cell lines did not represent the natural situation *in vivo*. Nevertheless, it allowed confirmation that, similar to mouse, rat CD1d was functional and able to represent α-GalCer to KT12 hybridoma and BWr/mCD28 cell line expressing mouse TCR^{inv}.

3.4. Production of anti-rat CD1d monoclonal Ab

Because no anti-rat CD1d monoclonal antibody is available, we attempted to generate such an antibody. To this end, M12 lymphoma cells, known to be efficient APCs, were transduced, via retroviral infection, with genes encoding rat CD1d. GFP-positive cells were used for immunisation of BALB/c $CD1d^{-/-}$ mice (detailed protocol is written in Materials and Methods).
Fusion was done using SP2/O fusion partner and supernatants were tested for rCD1d specificity by FACS staining using rat thymocytes and splenocytes or rCD1d transgenic cells.

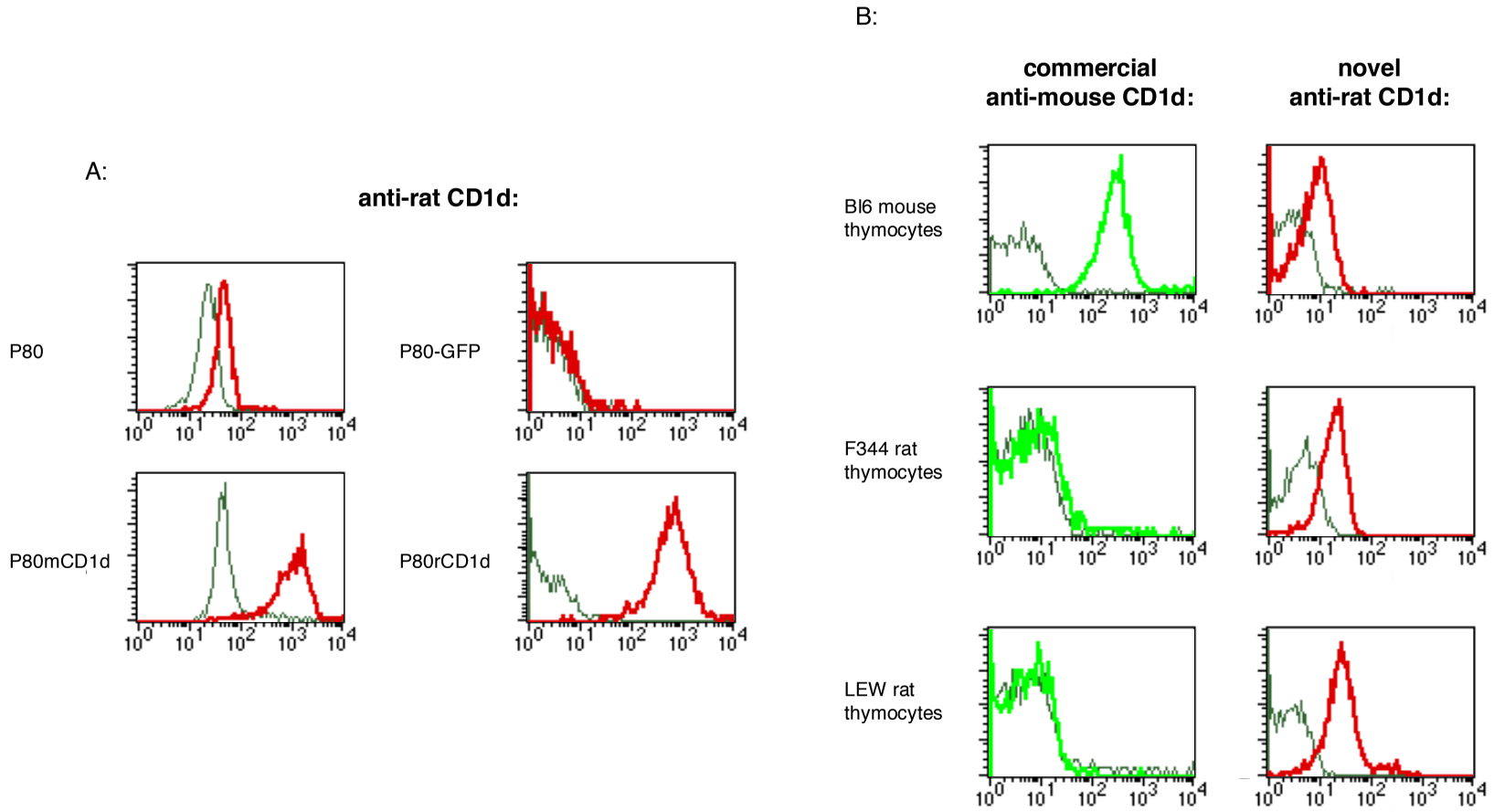


Figure 19: Detection of mouse and rat CD1d on thymocytes and cell lines using novel anti-rat CD1d monoclonal antibody.
Total mouse and rat thymocytes or P80 CD1d transgenic cells (2*10^5 cells) were preincubated for 15 min at 4°C with 2.4.G.2 hybridoma supernatant . Then cells were stained in the presence (red line) or absence (green line) of 40ml of rCD1d fusion supernatant (1h at RT) and were detected with DαMIgGPE (30 min at 4°C). As a control the staining with anti-mouse CD1d PE (1B1) mAb (bright green line) or isotype control (rIgG2a) (dark green line) were performed.

3.4.1. Screening of fusion supernatants on P80-CD1d transgenic cells

The capability of rCD1d fusion hybridomas to produce anti-rat CD1d mAb was tested on human RAJI and mouse P80 rCD1d transgenic cells.

As example, the staining of P80rCD1d cells is shown in Figure 19A. P80 wild type, as well as mock or mouse/rat CD1d infected P80 cells were incubated with the culture supernatant from one of the fusion hybridomas. The presence of anti-rat CD1d specific Abs bound to surface CD1d was detected with donkey anti-mouse IgG-PE.

Novel anti-rat CD1d Ab was specific not only for rat but also mouse CD1d. The rat antibody recognised mCD1d naturally expressed on the surface of P80 cells but when these cells were additionally transduced with cloned mCD1d much higher expression of CD1d could be detected. Furthermore, the novel rat antibody positively stained rCD1d but not mock infected P80 cells confirming it's specificity for rCD1d.

Similar staining was performed for human RAJI cell line transduced with rCD1d. Only RAJI cells expressing rCD1d, but not wild type or GFP-vector infected cells, were positively stained with fusion hybridoma supernatant and DαMIgGPE (data not shown).

3.4.2. Screening of fusion supernatants on mouse and rat primary cells

Mouse splenocytes and thymocytes are known to be CD1d-positive. Therefore, the rCD1d specificity of fusion supernatant was tested on mouse (control) and rat thymus and spleen-derived lymphocytes. Here, only FACS staining of thymocytes is presented.

Figure 19B shows that the 1B1 anti-mouse CD1d mAb, used as a control, positively labelled CD1d expressed by mouse but not rat thymocytes, excluding its cross-reactivity.

When thymocytes of both species were stained with a fusion supernatant, positive cells could be detected in both rat and mouse, confirming the species cross-reactivity of novel anti-rat CD1d Ab. Furthermore, the better binding of rCD1d

Ab/DαMIgGPE complex to LEW than F344 thymocytes could be observed. This may suggest that some inter-strain differences in the natural level of CD1d expression, among LEW and F344 rat, can be observed.
A similar pattern of the staining was found when mouse and rat splenocytes were used (data not shown).

The staining of primary rat cells and the staining of CD1d transgenic cell lines suggest that the novel T cell hybridoma produces antibody against rCD1d which is cross-reactive with mouse CD1d. The specificity of culture supernatant will be further tested in the functional assays and analysed using immunoprecipitation and Western Blot methods.

IV. The comparison of CD1d-α-GalCer-TCR^{inv} system in F344 and LEW rat

4.1. F344 and LEW splenocytes respond differently to α-GalCer stimulation

α-GalCer is the specific Ag activating mouse NKT cells but also (as presented above) lymphocytes isolated from F344 liver or spleen. In order to check whether the response of rat cells to α-GalCer stimulation was characteristic only for F344 rat strain, lymphocytes isolated from F344 and LEW spleen were stimulated with α-GalCer or its chemical analog- OCH. After 3 days culture the production of cytokines by these cells was measured by ELISA.
Figure 20 indicates that upon α-GalCer stimulation F344 but not LEW splenocytes were able to produce significant amounts of IFN-γ (Figure 20 A) and IL-4 (Figure 20 B) in an Ag-dose dependent manner. When OCH was used as stimulus, the response of F344 cells was much weaker suggesting that only α-GalCer was able to efficiently activate rat NKT cells. This is a surprising result given that Myamoto *et al.,* (2001) used OCH to treat autoimmune disease in mice.

Organ	C57Bl6 mouse	Rat	
		LEW	F344
LN	++	-	+/-
Thymus	++	+/-	+
Spleen	+	+/-	+
Liver	+++	-	++

Table 5:
Detection of rat Vα14 mRNA by RT-PCR.
500 ng of mRNA isolated from mouse or rat lymphocytes obtained from LN, thymus, spleen, and liver were used to prepare cDNA. 1 ml of each cDNA was amplified with Vα14TCR specific or β-actin primers. The PCR signal was estimated as follows: (+++)- very strong band, (++)- strong band, (+)- positive, (+/-)- slightly positive, (-)- negative.

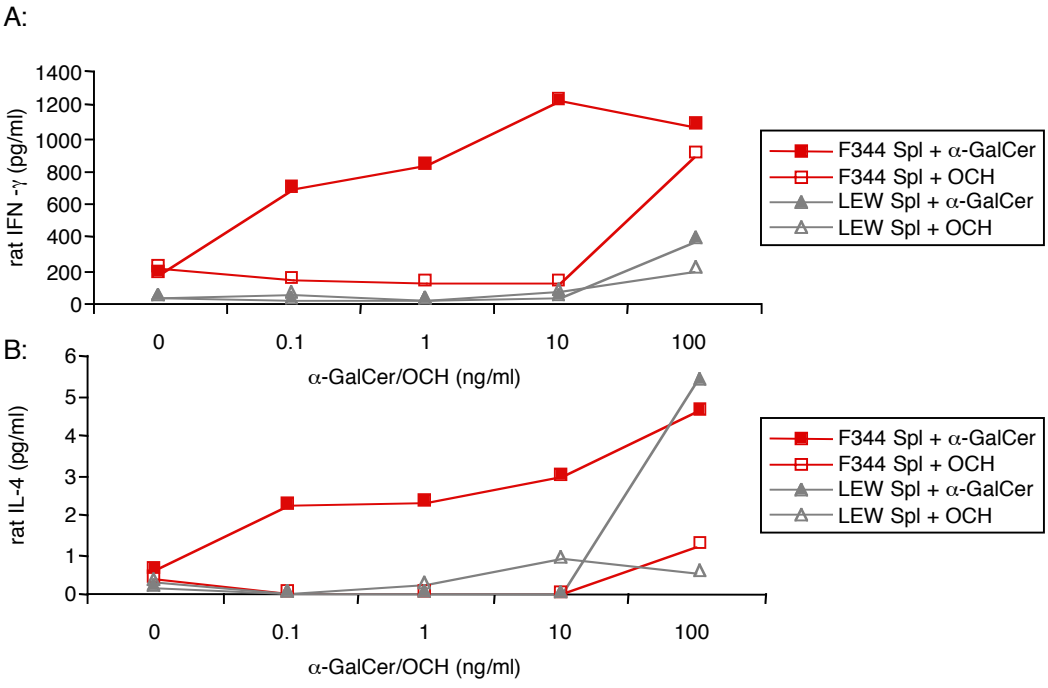


Figure 20:
Comparison of IFN-γ (A) and IL-4 (B) production by F344 and LEW splenocytes (5*10^{5}cells) after 24h stimulation with α-GalCer and OCH.

In contrast to F344, LEW rat splenocytes responded similarly to α-GalCer and OCH stimulation. In both cases no IL-4 and very low levels of IFN-γ could be detected in culture supernatants.

The results from functional assays are in agreement with data obtained from the RT-PCR analysis of Vα14 mRNA in different LEW and F344 rat tissues. As it was shown in Table 5, in all tested organs the Vα14$^+$-PCR signal was weaker in LEW than in F344 rat tissues. Therefore, it is likely that LEW rat, in contrast to F344 rat, did not contain or contained only a small proportion of α-GalCer responding cells.

4.2. Characterisation of rat CD1d$^+$ APCs

As it was previously shown, rat thymocytes, similar to mouse cells, were able to present α-GalCer and efficiently stimulate the IL-2 production by BWr/mCD28 cell line expressing mouse TCR^{inv}. Given the different response of LEW and F344 rat splenocytes to α-GalCer stimulation it was important to check if these two stains have also different capacities of Ag presentation. To this end, mouse (as a control) and LEW and F344 rat CD1d$^+$ thymocytes were loaded with different doses of α-GalCer and were used for stimulation of Vα14$^+$ KT12 hybridoma. The level of IL-2 released by activated KT12 cells, after 24h coculture, was determined by supernatant ELISA and was presented in Figure 21.

Both F344 and LEW thymocytes were able to activate KT12 hybridoma almost as well as mouse cells. This could suggest that the rCD1d was expressed in rat thymus at comparable level with mCD1d in mouse organ. Secondly, rat CD1d was functional and could stimulate α-GalCer-dependent IL-2 production by mouse KT12 cells.

Because KT12 hybridoma possesses a high level of autoreactivity and may produce significant amounts of IL-2 even in the absence of APCs, presented results can be questioned. However, when KT12 hybridoma cells were stimulated with Ag-loaded APCs, the cytokine secretion was more pronounced especially at lower doses of α-GalCer. To confirm whether IL-2 production by KT12 hybridoma was due to Ag presentation by F344 or LEW rat cells but not due to KT12's autoreactivity, additional assays with hybridoma expressing rat TCR^{inv} would be needed.

Comparing F344 and LEW rat CD1d-α-GalCer-TCR^{inv} system it might be concluded that both strains had similar capability of α-GalCer presentation but they differed in terms of this Ag recognition.

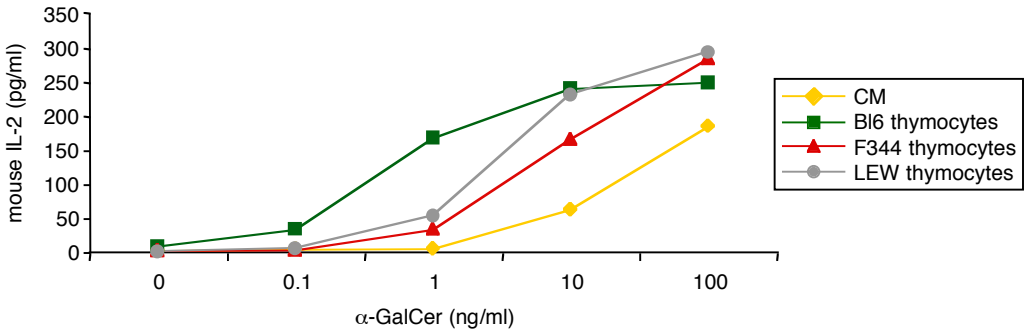


Figure 21:
IL-2 production by KT12 hybridoma (5*10^{4}cells) after 24h stimulation with α-GalCer presented by mouse and rat thymoctes (1*10^{6}cells).

Chapter 4:
DISCUSSION

I. Properties of rat NKRP1A$^+$TCR$^+$ cells

1.1. Phenotype, frequency and α-GalCer-mCD1d tetramer staining of rat splenocytes and IHLs

Mouse and human NKT cells are well characterised, while this population remains poorly characterised in rat. It has been difficult to clone NKRP1$^+$ T lymphocytes from rat and only a few reports describing these cells are available.

Firstly, Knudsen has identified rat NKRP1$^+$TCR$^+$ cells, which phenotypically could correspond to mouse NKT cells. The DN or CD4$^+$ NKRP1$^+$TCR$^+$ clones, obtained from PVG rat splenocytes cocultured with MIP-1α (macrophage inflammatory protein-1α) *in vitro,* expressed Vβ8.2$^+$ TCR and secreted various cytokines (Knudsen *et al*., 1997). By contrast, freshly isolated rat splenic NKRP1$^+$TCR$^+$ cells are mostly CD8$^+$ with no skewing in the Vβ repertoire. Therefore, it is likely that Knudsen cloned rare rat NKT cells.

The presence of rat NKRP1$^+$TCR$^+$ cells was additionally confirmed for allo- and xenogeneic (rat-mouse) chimeras, in which approximately 80-90% of NKRP1$^+$ cells expressed α/βTCR (Kaufman *et al.,* 1993).

In order to determine whether rat spleen-derived NKRP1$^+$TCR$^+$ clones, generated by Knudsen, corresponded to mouse or human NKT cells, the phenotype, distribution, frequency and functions of NKRP1$^+$TCR$^+$ cells were characterised in this thesis. All experiments were done on F344 rat, since this strain was previously reported to express an invariant Vα14$^+$ TCR (Matsuura *et al*., 2000).

Firstly, the phenotype and distribution of F344 rat NKRP1$^+$TCR$^+$ lymphocytes in diverse tissues were determined by FACS analysis.

It is well known that mouse Vα14$^+$ and human Vα24$^+$ NKT cells express several NK-locus-encoded C type lectins (Bendelac *et al*., 1997; Exley *et al*., 1998).

In mice, the NK locus encodes three NKRP1 molecules: NKRP1A, B and C. NKRP1C has been reported to be an activating receptor, whereas NKRP1B is a killer cell inhibitory receptor (Carlyle *et al.,* 1999).

A human homologue of the mouse NK1.1 (NKRP1C) is NKRP1A, which may be expressed on NK and T lymphocytes (Lanier *et al.*, 1994) or on a subset of human immature thymocytes (Poggi *et al.*, 1996). Although human NKRP1A is not the exact homologue of mouse NK1.1 (NKRP1C), this marker is commonly used to identify NKT cells in the liver or perypherial blood (Ishihara *et al.,* 1999; Exley *et al.,* 1998).

In rats, there are two genes encoding NKRP1 molecules. Similar to mouse, rat NKRP1A functions as an activation receptor, while NKRP1B contains immunoreceptor tyrosine-based inhibitory motif and therefore is an inhibitory receptor (Brissette-Storkus et al., 1994; Ryan *et al.*, 1995; Li *et al.*, 2003). Interestingly, similarly to anti-NK1.1 PK136 mAb that recognises both mouse activating NKRP1C and inhibitory NKRP1B markers (Kung *et al.,* 2001), anti-NKRP1 mAb, used in the study, has a specificity for both A and B forms of rat NKRP1 (Li *et al.*, 2003).

By analogy to the human system, NKRP1A in rats corresponds to mouse NK1.1 and is expressed by NK and NKT cells, to lesser extent by rat neutrophils (Kalia *et al.*, 2003; Chambers *et al.,* 1992). Since the expression of NKRP1A is not exclusive only for NKT cells, to identify F344 rat invariant T cells, anti-NKRP1A mAb together with anti-TCR or anti-CD3 mAbs were used in the study. Only lymphocytes coexpressing TCR/CD3 and NKRP1A marker were identified as rat NKT cells.

As tested by FACS staining, rat $NKRP1A^+TCR^+$ lymphocytes were the most frequent in the liver. However, their frequency in this organ was 4-8 fold lower than the frequency of mouse IHLs. Generally, $NKRP1^+TCR^+$ cells represented around 0.1-6% of total lymphocytes present in various rat tissues, while mouse counterparts were present at higher frequencies (2-40%).

Interestingly, both species differed in the proportion of liver and splenic NKT cells. Rat IHLs and splenocytes were expressed at the similar or comparable frequencies. Liver contained 6.39% of $NKRP1^+CD3^+$ cells, while in rat spleen 4.04% of cells with this phenotype could be found. By contrast, mouse NKT cells were around 10 fold more frequent in liver (32.18%) than in spleen (2.89%).

In contrast to PVG NKT cell clones generated by Knudsen, hepatic and splenic F344 rat NKRP1A^{+}TCR/CD3^{+} lyphocytes were more of CD8^{+} than of CD4^{+} phenotype. However, the CD8 phenotype of F344 cells is consistent with data presented by others. Matsuura and coworkers showed that the majority of F344 rat splenic and hepatic NKT cells expressed CD8 (Matsuura *et al.*, 2000). Similarly, CD8^{+} cells have been found to be the main subset of NKT cells present in F344 rat lymph nodes (LN) or submandibular salivary glans (SMG) (O'Sullivan *et al.*, 2001).

The existence of CD8αβ^{+} rat NKT cells may contradict dogma on positive selection of CD1d-α-GalCer specific TCR^{inv} T cells (Eberl *et al.*, 1999-c; Bendelac *et al.*, 1994; Benlagha *et al.*, 2002; Gapin *et al.*, 2001). It has been postulated that during thymic development, NKT cells expressing CD8αβ, due to their high binding avidity to CD1d are deleted (MacDonald 2000; Pellicci *et al.*, 2002; Bendelac *et al.*, 1994; Eberl *et al.*, 1999-b). The mechanisms of negative selection of NKT cells are unknown and have been proven only indirectly. Data from experiments using transgenic mice showed that CD1d-dependent CD8^{+} cells are absent, and Vα14Jα18^{+} NKT cells can not develop in CD8α/β transgenic animals. Similarly, in Vα14Jα18^{+} transgenic mice the CD8 compartment is depleted of Vβ7 and Vβ8 cells (Bendelac *et al.*, 1994; Lantz and Bendelac *et al.*, 1994). Therefore, the canonical NKT (Vα24^{+} TCR^{inv} in human and Vα14^{+} TCR^{inv} in mouse) cells can be either CD4^{-} CD8^{-}(DN) or CD4^{+} but they never express heterodimeric CD8 (Davodeau *et al.*, 1997; Bendelac *et al.*, 1994). However, the existence of CD8^{+} NKT cells in various mouse organs has been previously reported. For instance, Hammond *et al.* as well as Ohteki *et al.* suggested that the total population of hepatic NKT cells contains around 4% of CD8α^{+}β^{-} T lymphocytes, which developed in a thymic- and Vα14Jα281-independent manner (Hammond *et al.*, 1999; Ohteki *et al.,* 1994). Mouse CD8α^{+}β^{-} as well as CD8α^{+}β^{+} T cells have been also found in large intestine (Bannai *et al.*, 2001). However, in contrast to classical NKT cells, these CD8^{+} cells possessed diverse and not invariant TCRα chain (Bannai *et al.*, 2001).

Since rat CD8αβ^{+} cells represent the majority of splenic and hepatic NKRP1^{+}TCR^{+} lymphocytes, it is tempting to suggest that CD8 marker may be more broadly expressed as it has been previously appreciated. It would be interesting to determine whether rat CD8^{+} NKT cells express Vα14^{+} TCR^{inv} and whether they are generated

according to the same or different developmental and selection mechanisms of classical NKT cells.

The phenotype and low frequency of rat NKT cells may suggest more similarity to human than to mouse NKT cell sub-populations.

It has been reported that the frequency of human NKT cells is much lower than the frequency of mouse Vα14$^+$ T cells and the phenotype and functions of these cells may vary depending on the tissue distribution (Kim *et al.*, 2002-a). Thus, the CD4$^+$ or DN Vα24$^+$NKRP1A$^+$ NKT cells constitute only a small (between 0.01 and 0.1%) proportion of human PBMCs, express low levels of NK cell markers and share the functional features with mouse Vα14$^+$ NKT cells (Dellabona *et al.*, 1994; Porcelli *et al.*, 1993; Lee *et al.*, 2002; Exley *et al.*, 1997). By contrast, hepatic Vα24$^+$ T cells represent 0.5% of IHLs, express high levels of CD56, CD161 or CD69 and comprise CD4$^+$ as well as CD8$^+$ cells (Kenna *et al.*, 2003; Doherty *et al.*, 1999; Ishihara *et al.*, 1999; Takahashi *et al.,* 2002). Therefore, since human Vα24$^+$ T cells may be CD8$^+$ and are less frequent than Vα14$^+$ T cells in mice F344 rat NKRP1A$^+$TCR$^+$ cells would possibly correspond more to human than to mouse NKT cells.

The expression of NK1.1 on NKT cells may vary depending on the maturation level- immature NKT cells usually lack the NK1.1 (Pellicci *et al.*, 2002; Benlagha *et al.*, 2002), their activation state (Chen *et al.*, 1998), as well as culture conditions- upon *in vitro* activation NKT cells can lose NK1.1 expression (Chen *et al.*, 1997-a). Furthermore, a subpopulation of NKT cell may be present in strains negative for NK1.1 (like BALB/c) (Hammond *et al.*, 2001) or NK1.1 can be expressed by lymphocytes, which are not CD1d-reactive like human and mouse activated CD8$^+$ CTLs (McMahon *et al.,* 2001; Assarsson *et al.*, 2000) or mouse NK1.1$^+$ cells that neither express Vα14$^+$ TCR nor are CD1d-dependent (Hammond *et al.,* 1999; Eberl *et al.,* 1999-c).

Since NK1.1 is not exclusively expressed by NKT cells, the FACS staining with anti-NK1.1 mAb seems to be not the most optimal method for the detection of CD1d-restricted Vα14$^+$ TCR positive cells. This problem has been overcome by generating α-GalCer-CD1d tetramers. These tetramers are more sensitive than monoclonal antibodies and are able to specifically recognise CD1d-restricted TCR^{inv} positive T cells within mixed lymphocyte populations (Matsuda *et al.*, 2000; Karadimitris *et al.,* 2001; Benlagha *et al.,* 2000-b). The usage of CD1d oligomers

has an advantage- they can be used for both surface and intracellular phenotyping, as well as for the *in situ* staining of Ag-specific T cells in tissues. Additionally, they might be combined with functional assays (Klenerman *et al.*, 2002).

CD1d-α-GalCer tetramers bind selectively to NKT cells, which express the invariant TCR α-chain: Vα14-Jα18 in mice and Vα24-JαQ in humans, even if these cells are present at very low frequencies (Gumperz *et al.*, 2002; Matsuda *et al.*, 2000; Benlagha *et al.,* 2000-b). The cells isolated from TCRJα18$^{-/-}$ or CD1d$^{-/-}$ mice are tetramer negative (Matsuda *et al.,* 2000; Benlagha *et al.,* 2000-b).
Commonly used mouse and human CD1d tetramers are cross-reactive and recognise mouse, human or rhesus macaque TCR^{inv} positive NKT cells (Karadimitris *et al.*, 2001; Benlagha *et al.,* 2000-b). However, the CD1d tetramer staining of Vα14^{+}/Vα24^{+} T lymphocytes can differ depending on the protocol used for CD1d synthesis and Ag loading, or depending on the type of CD1d-α-GalCer multimer used. The staining of Vα14^{+} cells with α-GalCer-loaded CD1d monomer usually does not lead to labelling. CD1d dimers are more effective than monomers. The best staining can be obtained when CD1d-α-GalCer tetramers are used (Benlagha *et al.,* 2000-b; Matsuda *et al.*, 2000).

From the sequence analysis of mouse and rat CD1d we knew that these molecules were highly homologous. Therefore, it appeared likely that due to structure and amino acid homology, rat NKRP1A^{+}TCR^{+} cells would be positively stained with mCD1d-α-GalCer-tetramer, assuming that they express TCR^{inv}.
When rat liver or spleen-derived lymphocytes were stained with mCD1d-α-GlaCer-tetramer, only a few tetramer-binding cells could be detected. Because the staining with mCD1d tetramer control gave also little reactivity, it was difficult to judge if the binding of mCD1d-α-GlaCer-tetramer to rat cells was really specific. By contrast, mouse IHLs or splenocytes, were positive in tetramer and clearly negative in the control staining. It has been previously shown that around 80% of thymic or liver and only 50% of spleen or BM NKT cells are α-GalCer-reactive and Vα14Jα18 expressing T cells (Benlagha *et al.,* 2000-b; Matsuda *et al.,* 2000). When the frequency of CD3^{+}NK1.1^{+} mouse liver and splenic T cells was compared with their affinity for CD1d-α-GalCer complex, 83% of IHLs and 73% of splenic CD3^{+}NK1.1^{+} T lymphocytes could bind to α-GalCer-loaded mouse CD1d tetramer.

This relatively high number of tetramer positive mouse splenic cells may be suprising, given that the spleen is an organ where mostly CD1d-independent NKT cells are expressed. However, mouse spleen and BM may contain various cells with different phenotype and functional features (Eberl *et al.*, 1999-c; Hammond *et al.*, 2001). Therefore, apart from CD1d-independent cells, some CD1d-dependent NKT cells were also present, and could bind to mouse CD1d-α-GalCer tetramer.

In both cases, when mouse or rat cells were labelled, a small population of CD3 or CD4 negative cells that did bind to CD1d-αGalCer tetramer could be identified. The presence of $TCR^-NK1.1^-tetramer^+$ cells in mouse spleen has been also described by Matsuda and colleagues (Matsuda *et al.*, 2000). Because the majority of these TCR^- $NK1.1^-$ cells expressed also CD19 marker, they concluded that these cells were splenic B lymphocytes, which bound to tetramer in α-GalCer independent manner.
Therefore, it is likely that "false tetramer positive" cells, detected in mCD1d tetramer staining, represented $TCR^-NK1.1^-tetramer^+$ splenic B lymphocytes or other cell types, present in the cell preparation and unspecifically binding to mouse CD1d tetramer. Relatively high background tetramer staining was also obtained for human NKT cells. Lee and coworkers showed that among 0.01-0.1% of freshly isolated PB Vα24/CD1d-α-GalCer NKT cells around 0.01-0.05% of unspecifically stained noncanonical Vα24 cells could be found (Lee *et al.*, 2002).
To exclude an unspecific binding and wrong interpretation of results, in future FACS and tetramer stainings the anti-rat NKPR1A mAb will be used together with additional markers such as CD3, CD19, rat homolog of mouse DX5 or human CD56. The DX5 mAb was used for the staining of mouse NKT cells by Yang *et al.* (2003), while the usage of anti-CD56 Ab for the detection of NK receptor positive population of hepatic VαJαQ TCR cells was recommended by Doherty *et al.* (1999).

It is generally accepted that α-GalCer tetramers stain only TCR invariant, $Vα24^+$ in human and $Vα14^+$ in mice, NKT cells (Matsuda *et al.*, 2000; Benlagha *et al.*, 2000-b). According to this, mouse thymus and liver $CD4^+$ or DN NKT cells are α-GalCer-reactive and are positively stained with CD1d tetramers. By contrast, CD1d-independent $CD8^+$ $NK1.1^+$ T lymphocytes, present in mouse bone marrow and spleen, do not express invariant TCR and do not bind to mCD1d-α-GalCer tetramer

(Matsuda *et al.*, 2000; Benlagha *et al.,* 2000-b; Hammond *et al.,* 2001). However, some deviations from this rule have been observed. For example, Gadola *et al.* suggested that the specific recognition of CD1d/α-GalCer in human can be mediated by T cells expressing a TCR other than Vα24 and Vβ11 (Gadola *et al.*, 2002). According to Gadola, the population of PBMC-derived NKT cells is heterogenous and may contain not only classical invariant $V\alpha24^+V\beta11^+$ NKT cells, but also $CD8\alpha/\beta^+$ or $CD4^+$ (but never $CD4^-CD8^-$) CD1d-α-GalCer specific $V\alpha24^-$ T cells (Gadola *et al.*, 2002). The subset of $CD8\alpha/\beta^+$ $V\alpha24^-$ T cells exhibited CD8-dependent cytotoxicity, rarely expressed CD161 and expressed non-invariant TCR (diverse TCRVα frequently paired with TCRVβ11) other than $V\alpha24^+$, with low affinity for α-GalCer-CD1d complex. Nevertheless, human $CD8\alpha/\beta^+V\alpha24^-$ lymphocytes could be stained with CD1d-α-GalCer tetramer (Gadola *et al.*, 2002). The presence of these $CD8\alpha\beta^+$ $V\alpha24^-$ tetramer-binding human NKT cells may be surprising given that only T cell clones bearing $V\alpha24^+$TCR were shown to be α-GalCer-CD1d-reactive (Couedel *et al.,* 1998).

In contrast to human $CD8\alpha\beta^+$ NKT cells, introduced by Gadola and coworkers, rat $CD8^+$ NKT cells, tested in this thesis, coexpressed $NKRP1A^+$ and did not have specificity for α-GalCer-loaded mCD1d-tetramer. So, how do F344 rat $CD8\alpha\beta^+$ $NKRP1A^+$ cells correspond to human $CD8\alpha\beta^+$ NKT cells?

Since human NKT cells may express TCR with other than $V\alpha24^+$chain, which still shows the specificity, although low, for α-GalCer-CD1d complex, it is likely that rat $CD8\alpha\beta^+NKRP1A^+$ cells possessed analogous TCR. Therefore, at least two hypothesis explaining the lack of mouse CD1d-α-GalCer tetramer binding to rat liver or spleen dervived $NKRP1A^+$ lymphocytes are possible. Firstly, rat $CD8\alpha\beta^+$ $NKRP1A^+$ lymphocytes, similarly to human $CD8\alpha\beta^+$ NKT cells expressed α-GalCer-reactive non-invariant TCR and therefore they could produce IL-4 and IFN-γ when stimulated with α-GalCer *in vitro*. However, due to very low α-GalCer affinity of this non-invariant TCR, rat $CD8\alpha\beta^+$ $NKRP1A^+$ cells could not be stained with mouse CD1d-α-GalCer tetramer.

The second possibility, $V\alpha14^+$ invariant TCR was expressed only by a small subset of rat NKT cells (perhaps $CD4^+$ cells), making detection of such cells with mCD1d-α-GalCer tetramer infrequent.

Alternatively, the lack of the mouse CD1d-α-GalCer tetramer binding to rat splenic and hepatic $TCR^+NKRP1A^+$ lymphocytes could have been caused by the lack of cross-reactivity of their TCR with mouse CD1d molecule. This hypothesis is plausible only if the TCR of rat liver and spleen lympocytes had specificity only for rat CD1d and was not able to recognise Ag presented by CD1d from other species. In order to identify if rat TCR can recognise α-GalCer presented by mouse/rat CD1d, the response of primary cells and TCR^{inv} positive cell lines to α-GalCer presented by mouse or rat $CD1d^+$ APCs, in the presence or absence of blocking anti-mouse and novel anti-rat CD1d mAbs, will be tested. Furthermore, the generation of chimeric mouse/rat CD1d molecules will allow the identification of regions in the rat/mouse CD1d sequence responsible for Ag presentation and the interaction with TCR^{inv} of mouse or rat origin. Both methods will help to better characterise the model of the CD1d-α-GalCer-TCR^{inv} interaction.

Additionally, since mCD1d-α-GalCer tetramer, which binds to human and mouse NKT cells, seems to be of limited usage for the staining of rat cells, the generation of rat CD1d-α-GalCer tetramers should be considered as a new tool for testing of the differences in the recognition of α-GalCer–CD1d complexes by mouse and rat NKT cells.

Because the population of rat $NKRP1A^+TCR^+$ cells was much smaller than the population of mouse NKT cells, we took a few approaches to expand these cells *in vitro*.

There are some reports presenting methods for the expansion of rare human or rhesus macaque $V\alpha24^+$ T cells by cultivating them with α-GalCer or α-GalCer loaded APCs, and cytokines (Gansuvd *et al.,* 2003; Takahashi *et al.,* 2002; van der Vliet *et al.*, 2001; Maeda *et al.*, 2001; Okai *et al.,* 2002).

Additionally, rat $NKRP1^+\alpha/\beta TCR^+$ cells could be successfully expanded using syngeneic or xenogeneic bone marrow transplantation (BMT) models (Kaufman *et al.*, 1993) or by *in vitro* co-culture in the presence of MIP-1α (Knudsen *et al.*, 1997).

Neither protocols for the expansion of rat $NKRP1^+TCR^+$ clones nor methods adapted for the expansion of human NKT cells did work in our system. We were not able to activate the proliferation of rat liver or spleen derived lymphocytes *in vitro*.

It is well known that the *in vivo* or *in vitro* α-GalCer stimulation of mouse NKT cells may trigger anergy (Fuji *et al*., 2002-1) or apoptotic death of these cells (Osman *et al*., 2000; Leite-de-Moraes *et al*., 2000; Matsuda *et al*., 2000). Because α-GalCer was used for stimulation and expansion of rat liver and splenic lymphocytes, an apoptotic death triggered by coculture conditions could be the main reason why we failed to expand these cells.

Since the expansion of rat $NKRP1^+TCR^+$ cells was not possible, we decided to characterise their capability to produce cytokines upon Ag stimulation.

1.2. Stimulation of rat IHLs and splenocytes with α-GalCer *in vitro*

Human and mouse CD1d are not able to present pathogen-derived lipids but they may efficiently present self lipid antigens to NKT cells (Porcelli *et al*., 1999; Gumperz *et al*., 2000; Park *et al*., 1998-a; Joyce *et al*., 1998; Burdin *et al*., 1998). Therefore, human, mouse, and macaque NKT cells, recognising self-lipid antigens *in vivo*, can be stimulated with α-GalCer in various *in vivo* or *in vitro* systems. α-GalCer as well as α-Glucosylceramide have been shown to selectively activate only NKT cells, which express TCR invariant: Vα14/Vβ8.2 TCR in mice and Vα24/Vβ11 TCR in humans (Kawano *et al*., 1997; Nieda *et al*., 1999; Spada *et al*., 1998; Couedel *et al*., 1998; Porcelli *et al*., 1996).

The CD1d-restricted and TCR^{inv}-dependent activation of NKT cells usually stimulates their proliferation and cytokine secretion (Metelitsa *et al*., 2001; Nieda *et al*., 1999; Spada *et al*., 1998; Brossay *et al*., 1998-c; Kawano *et al*., 1997). Activated human and mouse NKT cells are able to produce various cytokines including IFN-γ, TNF-α, GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10 and IL-13 (Kim *et al*., 2002-a; Hayakawa *et al*., 1992; Burdin *et al*., 1999-a; Lee *et al*., 2002).

Since mouse NKT cells are activated upon α-GalCer stimulation, there was a need to check whether rat $NKRP1A^+TCR^+$ cells possessed the same features.

Similar to mouse NKT cells or human CD1d-restricted clones cultured *in vitro* (Gumperz *et al*., 2002), rat spleen or liver-derived lymphocytes were able to release cytokines like IL-4 or IFN-γ in response to α-GalCer stimulation. However, it is not clear if cytokines were produced by rat $NKRP1^+TCR^+$ cells or by other cells, present

in liver or spleen cell preparations. To determine the phenotype of cytokine producing cells, the intracellular FACS staining of α-GalCer stimulated rat lymphocytes will be performed.

F344 rat hepatic and splenic lymphocytes, when stimulated with α-GalCer *in vitro,* produced both IL-4 and IFN-γ in Ag-dose dependent manner. Some background levels of IFN-γ could be already detected for rat IHLs or splenocytes cultured in the presence of medium or vehicle (DMSO). Since liver lymphocytes are known to be very sensitive to stress, the long cell preparation and purification procedure could be a source of stress triggering an unspecific IFN-γ production. Alternatively, this α-GalCer-independent, unspecific IFN-γ production could be driven not by rat NKT cells but rather by other cell types present in cell preparations. For instance, NK cells, which are usually abundant in liver, may release large amounts of IFN-γ but not IL-4 when triggered (Winnock *et al.,* 1995). Furthermore, the detailed analysis of cell types responsible for IFN-γ secretion in the response to *in vivo* stimulation with α-GalCer showed that at least in mouse system, NK cells indeed served as the major source of this cytokine (Carnaud *et al.,* 1999). Therefore, it is very possible that IFN-γ, detected in supernatants of rat liver or splenic lymphocytes incubated with medium or vehicle was produced by NK cells present in the cell preparations (Matsuda *et al*., 2000; Eberl *et al.,* 2000).

When rat liver and splenic lymphocytes were stimulated with α-GalCer, the level of IFN-γ in supernatants increased. The Ag-dependent IFN-γ production could be driven by activated NKT cells alone or more possibly by NKT and NK cells together. It is likely that especially in a long period culture, rat NKT cells were activated as first and then they stimulated the IFN-γ production by NK cells. The last hypothesis is in agreement with the fact that in natural *in vivo* situation both populations are in constant "cross-talk" and they cooperatively affect immune responses (Carnaud *et al*., 1999; Metelista *et al*., 2001; Eberl *et al.,* 2000).

The phenotype of mouse NKT cells and their functions may vary in relation to the presence of APCs, tissue distribution or culture and stimulation conditions. Additionally, human $V\alpha24^+V\beta11^+$ T cells have been shown to be phenotypically and functionaly even more heterogenous than mouse counterparts. However, the stimulation of human and mouse NKT cells with α-GalCer (Brossay *et al*., 1998-c) as well as usage of $V\alpha14/V\alpha24^+$ TCR-specific CD1d-α-GalCer tetramers (Matsuda

et al., 2000; Lee *et al.*, 2002) allow the general classification of NKT cells according to their cytokine secretion profile. Activated invariant $CD4^+$tetramer$^+$ T cells secrete both Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-5, IL-10, IL-13) cytokines and are biased to Th0 phenotype, whereas DN NKT cells secrete mostly IFN-γ and TNF-α and have Th1-like cytokine profile (Gumperz *et al.,* 2002; Hammond *et al.*, 1999; Kim *et al.*, 2002-a; Lee *et al.*, 2002).

In contrast to human or mouse NKT cells, very little is known about the capacity of rat invariant T cells to release cytokines. Among $NKRP1^+V\beta8.2^+$ TCR rat clones generated upon *in vitro* culture with MIP-1α, the DN and $CD4^+$ cells producing diverse cytokines (IL-4, IL-2, IL-12, or IFN-γ) could be distinguished (Knudsen *et al.*, 1997).

Tested in our system, F344 rat IHLs and splenocytes stimulated with α-GalCer *in vitro* produced both IL-4 and IFN-γ, although IFN-γ seemed to be preferentially secreted by rat hepatic lymphocytes. The preferential IFN-γ production was also observed for rare human liver NKT cells (Takahashi *et al.*, 2002; Kenna *et al.*, 2003; Exley *et al.*, 2002). The hepatic $CD161^+$, $CD56^+$, and/or $CD69^+$ $CD8^+$ $V\alpha24^+V\beta11^+$ T cells, which exist as a minor NKT population, when stimulated with α-GalCer or PMA/ionomycin, produce IFN-γ and TNF-α but not IL-2 or IL-4 (Kenna *et al.*, 2003; Takahashi *et al.,* 2000). However, the dominant IFN-γ secretion by activated F344 IHLs differs from results presented by Ohkawa, suggesting that rat $CD8^+$NKT cells in rat hepatic allografts preferentially produced IL-4 promoting the natural tolerance (Ohkawa *et al.*, 1999).

Several groups have reported that NKT cells produce cytokines in a developmental and age-dependent manner (Benlagha *et al.,* 2002). Immature mouse $NK1.1^-$ NKT cells, as well as neonatal human cord blood NKT cells preferentially secrete IL-4, while mature NKT cells are source of IL-4 and IFN-γ after polyclonal activation (Pellicci *et al.,* 2002; Kadowaki *et al.,* 2001). The age-dependent increase in cytokine production by mouse NKT cells upon α-GalCer stimulation has been also reported (Inui *et al.,* 2002). By contrast, the correlation between the age of animals and the cytokine secretion pattern by NKT cells has never been tested in the rat system. However, there are a few reports showing age-related changes in the frequency of rat $NKRP1A^+$ T lymphocytes. In an experimental model of chronic marginal vitamine A status (the influence of vitamine A on ageing), the number and

percentage of rat $CD3^{inter}NKRP1^{dim}$ cells in PBMC and spleen increased in older animals (Dawson and Ross, 1999). This age-related increase in rat NKT cells correlated with increased percentages of $CD8^+$ T cells, confirming previous reports by Brissette-Storkus *et al.* (1994), showing rat NKT cells to be $CD8^+$ (Dawson and Ross, 1999). The similar correlation between the number of mouse liver $V\alpha14^+$NKT cells and the age of animals has also been reported (Tsukahara *et al.*, 1997). By contrast, age-associated decreases in the percentage of human peripheral blood $V\alpha24^+$ cells have been described by others (DelaRosa *et al.*, 2001).
To exclude the maturation-related differences in the frequency of NKT cells or amounts of released cytokines, in all experiments animals of the same age were used. Furthermore, cells prepared from male and female animals had the same pattern of cytokines released. Therefore, we assume that the preferential IFN-γ production by rat hepatic lymphocytes could not be a result of differences in the sex or maturation stage of analysed animals.

To sum up, upon *in vitro* α-GalCer stimulation F344 rat liver and spleen derived lymphocytes were able to release cytokines but it was difficult to define a correlation between the phenotype of these cells and their cytokine secretion pattern. Furthermore, since human hepatic lymphocytes have been reported to be phenotypically and functionally more diverse than their mouse counterparts (Doherty *et al.,* 1999), it might be possible that the population of rat NKT cells is heterogenous and consists of cells with different phenotype and cytokine production capacities.

The level of cytokines produced by α-GalCer activated F344 rat liver and spleen-derived T lymphocytes was usually lower than the levels of mouse cytokines. One exception from this finding were rat IHLs, which seemed to be better IFN-γ producers than their mouse counterparts. However, as it has been already discussed, it is likely that this IFN-γ was secreted not by NKT but rather by other cells present in liver preparations.
Since $NKRP1A^+TCR^+$ cells represented the only a minor subset of rat liver or spleen lymphocytes, smaller amounts of secreted rat cytokines could be expected.

Data from functional assays showed that the presence of CD1d in the culture was required for efficient activation of rat intrahepatic and splenic lymphocytes. Total splenocytes or preparations from rat liver contained some $CD1d^+$ APCs. In the absence of these APCs, the cytokine secretion was reduced. The same correlation between the presence of CD1d and amounts of released cytokines has been previously found in human and mouse CD1d-NKT cell systems (Fujii *et al.*, 2003-b; Exley *et al.*, 1997; Kawano *et al.*, 1997). Therefore, α-GalCer or α-Glucosylceramide-pulsed DCs may be effectively used to induce the proliferation of splenic Vα14Jα18 NKT cells (Kawano *et al.,* 1997) or the activation and expansion of human PBMC-derived $V\alpha24^+$ NKT cells (Nieda *et al.*, 1999; Fujii *et al.,* 2003-b).

The cytokine secretion by human and mouse NKT cells depends on the tissue location, stimulation conditions, cytokine balance in the microinveronment, as well as cell-cell interactions (Ikarashi *et al.,* 2001; Kawano *et al.,* 1997; Yang *et al.,* 2003; Hayakawa *et al.,* 2001).

Additionally, the cytokine profile and phenotype of NKT cells can be modulated by APC from different tissues (Yang *et al.*, 2003). Thus, mouse thymic NKT cells have been shown to preferentially produce high amounts of IL-4, whereas splenic NKT cells were better IFN-γ producers. These differences in the cytokine profiles probably reflected the differences in the requirements of these cells for costimulatory signals. According to this, $CD8^+$ CD1d-independent splenic lymphocytes expressing high level of NK cell markers produced high levels of IFN-γ and required both TCR and CD28-mediated signals to be fully activated. By contrast, the engagement of CD28 had no effect on the activation of CD1d-dependent thymic NKT cells, which had an activated T cell phenotype and expressed low levels of NK cell markers (Eberl *et al.,* 1999-c; Yang *et al.*, 2003).

Furthermore, the nature of Ag as well as the maturation status of APC may influence the activation of NKT cells (Fujii *et al.,* 2003-b). DCs have been shown to be superior to monocytes as APCs for presenting α-GalCer and activating human NKT cells. The maturation of DCs may further enhance NKT cell activation and cytokine production (Fujii *et al.,* 2003-b).

Since the NKT cell cytokine profile is strictly regulated by culture conditons, it might be interesting to investigate whether different subpopulations of APCs present in F344 rat liver and spleen may differently influence the cytokine secretion by rat NKT cells. The usage of blocking anti-adhesion molecules or anti-cytokine

antibodies will allow the definition of role CD40-CD40L (CD80/CD86-CD28) costimulation and cytokines contribution in the activation of rat NKT cells.
Concluding, upon α-GalCer stimulation F344 rat liver and spleen-derived lymphocytes were able to produce IL-4 and IFN-γ in an Ag- and CD1d-dependent manner. However, it remains to be determined whether rat NKRP1$^+$TCR$^+$ cells used for stimulation are the same cells which produce cytokines and whether they express invariant Vα14$^+$ TCR. It is also not clear whether such activities found *in vitro* represent the behaviour and features of rat Vα14$^+$ T cells naturally present *in vivo*. To this end, additional experiments with administration of α-GalCer *in vivo* would be needed, but because of limited amounts of Ag they were not performed.

II. Characterisation of rat Vα14$^+$TCR

Mouse and human NKT cells express α-GalCer-specific TCR^{inv}. In order to determine whether a homologous TCR might be expressed by F344 rat cells, the frequency of lymphocytes expressing TCR with Vα14$^+$Jα18$^+$ rearrangement was estimated by RT-PCR.
The Vα14$^+$ signal was much weaker in rat than in mouse organs. It could be clearly detected in rat liver and spleen but was hardly found in thymus and LN. This may suggest that the population of Vα14$^+$ TCR NKT cells does exist in F344 rat but is much smaller than the population of mouse NKT cells. The low expression of TRAV14 gene products in F344 rat liver, spleen and thymus was also reported by Matsuura (Matsuura *et al.,* 2000). The data from the RT-PCR analysis were consistent with FACS staining results. Liver and spleen were organs, where rat NKRP1A$^+$TCR$^+$ cells were the most frequent and the Vα14$^+$ RT-PCR signal was the strongest.
In addition to RT-PCR analysis more quantitative methods for the estimation of the frequency of Vα14$^+$ TCR NKT cells in different rat organs will be utilised in future.

When PCR products were cloned and analysed in terms of Vα14-JαC expression, the Vα14Jα18 rearrangements could be detected but they were very rare.

Furthermore, the rat Vα14-region could be joined not only with rat Jα18 region, corresponding to mouse Jα18, but also with other diverse Jα-segments.

It has been postulated that Vα14Jα18 TCR is selected during T cell maturation and therefore some differences in the expression of Vα14Jα18 rearrangements, depending on maturation status, are possible in mice. The expression of Vα14Jα18 in $CD4^+CD8^+$ (about 50% of $V\alpha14^+$ chain) immature thymocytes is low and become high in mature single positive T cells (85% of $V\alpha14^+$ chain) (Koseki *et al.,* 1990). At very early stages of the thymus maturation, the $V\alpha14^+$ TCR may be joined with Jα other than Jα18, and then the frequency of invariant Vα14Jα18 TCR expression increases reaching maximum levels in adult animals at around 5-8 weeks after birth (Koseki *et al.,* 1991; Koseki *et al.,* 1990).

However, because Vα14Jα18 α-chain was found in the spleen of athymic mice, it has been suggested that Vα14Jα18 T cells may be positively selected in the periphery (in BM, liver or intestine) without thymic influence (Makino *et al.,* 1994).

Since F344 rat $NKRP1^+TCR^+$ cells were less frequent than mouse NKT cells, we conclude that the subset of cells expressing $V\alpha14^+$TCR should be also smaller and the $V\alpha14^+$PCR signal in rat organs might be weaker that this found in mouse tissues. Therefore, the low number of rat clones expressing the right Vα14Jα18 rearrangement could be result of limited frequency of TCR^{inv}-positive cells rather than, as it was observed for mouse NKT cells, the differences correlated with the thymic or extrathymic maturation.

2.1. Cloning of rat $V\alpha14^+$ TCR^{inv}

It has been reported that the expression of α/βTCRs in TCR-deficient recipient cells may confer the specificity for Ag and CD1d isoforms (Grant *et al.,* 1999). In this thesis rat TCR^{inv} was expresses in BWr/mCD28 cells and its α-GalCer reactivity was tested in various *in vitro* functional assays. Unfortunately, none of two the cloned rat TCRs corresponded to mouse TCR^{inv}. The first rat $V\alpha14^+$ TCR, obtained from F344 rat liver, was successfully expressed on the surface of BWr/mCD28 cells, however it did not have specificity for α-GalCer.

The comparison of the rat liver $V\alpha14^+$ TCR sequence with published rat and mouse $V\alpha14^+$ TCR sequences revealed the presence of three amino acid alterations. It is not

clear whether these differences in the sequence were mutations or PCR errors and whether they influenced α-GalCer-CD1d complex recognition. When compared to published rat Vα14$^+$ sequences, the cloned rat TCR contained lysine (K) instead of arginine (R) at position 22 (1 according to IMGT). Since lysine 22 was also found in the mouse Vα14$^+$ TCR sequence, it is likely that this amino acid was not critical for the Ag recognition.

The second "mutation"- the exchange of threonine for lysine at position 72 (51 according to IMGT) occurred in the CDR2 region of cloned rat Vα14$^+$ TCR.

The amino acid composition of the CDR2 region is crucial for Ag recognition. However, Sim *et al.* showed that some polymorphism in the mouse Tcra locus (a three aa substitution in the CDR2 region between mouse TRAV11 alleles) does not affect the interaction with the mCD1d/α-GalCer complex (Sim *et al.,* 2003). Matsuura *et al.* also reported that the CDR2 region (amino acids from 70 to 77) of rat TCR^{inv} contains some amino acid diversity. However, the threonine at position 72 is one of six amino acids which are conserved for TRAV14S1, TRAV14S2 and TRAV14S4 sequences (Matsuura *et al.,* 2000), implying that the appearance of K at this position could negatively influence the αGalCer-CD1d recognition by rat TCR^{inv}.

The third aa alteration in rat liver Vα14$^+$ TCR sequence was found in the CDR3 region, in the position, where Vα and Jα segments are joined. The amino acid sequence of Vα14TCR V-J-junction is conserved between species and is composed of:

Human: ICVVSDRGS**T**LG**R**

Rhesus macaque: ICVVSDRGS**T**LG**K**

Mouse: ICVVSDRGS**A**LG**R**

Rat: ICVVSDRGS**A**LG**K** (Kashiwase *et al.,* 2003).

This homogeneity of CDR3 regions may suggest that the binding of lipid to CD1d and their presentation to TCR^{inv} are constant among various species (Matsuura *et al.,* 2000; Lantz and Bendelac 1994).

The nucleotide composition of V-J joining of mouse canonical TCR^{inv} is well known. The invariant TCR α gene, encoded by germline Vα14 and Jα18 segments, contains one-nucleotide N region, in which any one of four nucleotides can be present, resulting in the synthesis of glycine (Koseki *et al.,* 1990; Shimamura *et al.,* 2001;

Lantz and Bendelac 1994; Shimamura *et al.*, 1997). However, in some cases, alanine or other residues can be expressed at this position. For instance, for NKT cells generated by *in vitro* culture of fetal liver precursors, several sequences, in which the 3'-end codon of Vα14 (GGN) was converted to GCC, GCG, GTC or ATA, could be found (Shimamura *et al.,* 2001). The conversion from Gly to more hydrophobic amino acid residues such as Ala, Val, or Ile did not alter the interaction between the mouse TCR α-chain CDR3 region and CD1d (Shimamura *et al.,* 1997; Shimamura *et al.,* 2001). Therefore, it has been postulated that the conversion from canonical sequence may be a marker of the conversion from fetal to adult form. According to this, the fetal-form sequence contains GCC, whereas in the adult-form GCG, GTC or ATA at 3' codon of Vα14-region can be found (Shimamura *et al.,* 2001).

In contrast to mice, the rat germline sequence is unknown. The amino acid composition of Vα14-Jα18 junction was predicted and could contain either alanine (A) or valine (V) (Matsuura *et al.,* 2000). Since, as discussed above, some amino acid variability in Vα14Jα18 joining site of mouse Vα14TCR is possible, we conclude that the third amino acid difference- the presence of valine at the Vα/Jα-joining site of rat TCR^{inv} probably could not be responsible for the lack of rat TCR specificity for α-GalCer-CD1d complex.

The second rat $Vα14^+$ TCR invariant was generated using molecular biology methods. It possessed the amino acid sequence corresponding to germline sequences but, for unknown reasons, it could not be successfully expressed on the surface of BWr/mCD28 cell line. The new $Vα14^+$ TCR could be paired with neither mouse nor rat VβTCR chain. Because rat NKT clones generated by Knudsen preferentially expressed $Vβ8.2^+$ TCR, it is unlikely that rat $Vα14^+$ TCR might be paired with other than Vβ8.2 chain. Furthermore, rat Vβ8.2 and Vβ8.4 TCR, when expressed together with mouse $Vα14^+$ TCR, equally contributed to the recognition of α-GalCer-$rCD1d^+$APC complexes *in vitro*. Since both rat Vβ8 chains could be successfully paired with mouse $Vα14^+$ TCR, it was possible that the lack of surface expression of rat TCR^{inv} was due to the inability of $Vα14^+$ TCR chain to combine with rat TCRβ-chains.

The lack of the pairing of cloned rat $Vα14^+$ TCR with rat Vβ8 TCR can be supported by personal communication with H. U. Weltzien (Max-Planck-Institute for

Immunobiology, Freiburg, Germany), who observed the same phenomenon in his experimental system. The TCR of murine $CD8^+$ CTL clone, specific for hapten TNP was expressed in BW cell line. Only one (Vα17/Vβ16), from two possible TCR rearrangements, could appear on the surface. The Vα3/Vβ16 combination functionally expressed on CTLs could not be expressed at the surface of hybridoma cells. However, the Vα3 TCR, which could not be transported to the surface in combination with Vβ16 was successfully expressed with other unrelated TCR-β chain. Therefore, it might be possible that similar to Weltzien's observation, we failed to express rat TCR^{inv} because cloned $V\alpha14^+$ TCR could not recognise a particular CDR3 region of rat Vβ8.2 or Vβ8.4 TCR.

2.2. Stimulation of $V\alpha14^+$ cell lines with α-GalCer

The high-affinity interaction of TCR^{inv} with CD1d-α-GalCer (but not with unloaded CD1d molecule) is stronger than that observed for α/βTCR–peptide-MHC class I interaction (Cantu *et al.*, 2003; Sidobre *et al.*, 2002). The long half-life of CD1d-α-GalCer-Vα14TCR complex creates optimal conditions for contacting of α- and β-chain of TCR^{inv} with CD1d and Ag, leading to efficient activation of NKT cells.
The mechanisms of α-GalCer recognition by TCR^{inv} are not well known. It has been reported that the α-chain of TCR^{inv} is necessary but not sufficient without specific Vβ chains for α-GalCer/CD1d recognition. The α-GalCer/CD1d reactivity can be modulated by β-chain, which stabilises the interaction with an antigenic epitope (Gui *et al.*, 2001).

To show the specificity of TCR^{inv} for α-GalCer, mouse $V\alpha14^+$ cell lines were tested in *in vitro* functional assays. When mock infected BWr/mCD28 cells were stimulated with α-GalCer, some background level of IL-2 could be measured in supernatants. Because these cells did not express TCR, as tested by FACS analysis, they could not be triggered by Ag stimulation. By contrast, mouse thymocytes, used in the assay as APCs, could be activated under stimulation conditions and by themselves they could have been a source of cytokines. This observation is in agreement with a finding, showing that mouse thymic NKT cells, which are unresponsive to *in vivo* α-GalCer stimulation (which is incapable of reaching the

thymus tissue) may be stimulated with this Ag in suspension culture, even in the absence of exogenous APCs (Matsuda *et al.*, 2000). Additionally, no IL-2 could be detected in supernatants of $CD1d^{-/-}$ mouse thymic lymphocytes cultured with α-GalCer, confirming above hypothesis.
When BWr/mCD28 cells transduced with mouse Vα14 TCR were stimulated with α-GalCer-loaded $CD1d^{+}$ mouse or rat thymocytes, some differences in amounts of released IL-2, depending on TCR α/β-chain combination, could be observed.

It has been previously described that mouse $V\alpha14^{+}$ TCR can be paired with random (either Vβ8.2, Vβ7 or Vβ10) β-chains, and still maintain high specificity binding to CD1d (Matsuda *et al.*, 2001; Gui *et al.*, 2001; Sidobre *et al.*, 2002; Park *et al.*, 1998-b). We showed that TCR comprising mouse Vα14Jα18 chain and either mouse or rat Vβ-chain (Vβ8.2 or Vβ8.4) could bind to α-GalCer-CD1d. However, the recognition of Ag-CD1d complexes occurred with different avidity resulting in different levels of IL-2 secretion.

In contrast to peptide-specific T cells, which show strong selection for CDR3β aa (Wang *et al.,* 1998), α-GalCer reactive T cells have polyclonal CDR3β sequences (Matsuda *et al.,* 2001). Furthermore, some aa diversity in CDR3β region of human or mouse NKT cell TCR may be possible without influencing the CD1d-α-GalCer interaction (Dellabona *et al.,* 1994; Porcelli *et al.,* 1996; Exley *et al.,* 1997; Sidobre *et al.,* 2002). Thus, it is very likely that the $V\alpha14^{+}$ TCR transgenic cells tested in this thesis, even if they had various aa composition of the CDR3β regions, could be activated and could secrete IL-2 in the response to α-GalCer/CD1d complex stimulation.

It has been shown that the Vβ-chain may determine the affinity of TCR^{inv} to the physiologic self ligand or may regulate the specificity for α-GalCer by recognising a sugar moiety of this antigen (Kawano *et al.,* 1999; Burdin *et al.*, 2000; Gui *et al.*, 2001; Sidobre *et al.*, 2002). Furthermore, the TCRβ may be responsible not only for T cell reactivity for mCD1 but also for certain selection mechanisms. For instance, the transgenic expression of rearranged β-chain isolated from a thymus-derived Vα14Jα18-expressing $NK1.1^{+}$ T hybridoma promoted the positive selection of thymocytes carrying canonical Vα14Jα18 α-chain and increased frequency of

NK1.1$^+$ T cells in the thymus of these TCR β-chain transgenic mice (Viret *et al.*, 2000). The influence of β-chain rearragements on the TCR sequence and specificity of developing thymocytes has been also confirmed by Dudley *et al.* (1994).

Therefore, the results from *in vitro* functional assays as well as data presented by others suggest that the β-chain of TCR^{inv} may be much more critical for Ag recognition as it has been previously appreciated. It might be possible that the Vβ-chain influences the α-GalCer reactivity and IL-2 production by TCR^{inv} positive cells by discriminating between mouse and rat CD1ds. However, this hypothesis needs to be confirmed and the mechanisms of the β-chain function and influence on TCR^{inv} specificity remain to be determined.

2.3. Tetramer staining of TCR^{inv} transgenic cell lines

In order to confirm the specificity of cloned TCR^{inv} for α-GalCer, BWr/mCD28 cells expressing these receptors were stained with mCD1d-α-GalCer-tetramer. Because tetramers themselves might form higher order oligomers (Klenerman *et al.*, 2002), or they might unspecifically bind to biotin or streptavidin labelled Abs, used for double labelling, single colour staining was performed, to exclude unspecific binding. TCR transgenic cells were stained with mCD1d-α-GalCer-tetramer PE and, in parallel, with anti-mouse CD3 mAb, to control the level of the TCR expression.

As previously shown the tetramer staining is not restricted to particular Vα14Vβ combinations. NKT cells with the Vα14$^+$ TCR paired with diverse β-chains were tetramer positive, while the staining hybridomas expressing Vα chains other than Vα14$^+$ with tetramer has been never observed (Matsuda *et al.*, 2000).

In this thesis three mouse Vα14$^+$ TCR transgenic cell lines expressing diverse Vβ-chains were positive in mCD3 staining but they bound to mCD1d-α-GalCer-tetramer with different avidity. The pattern of tetramer staining correlated with the capacity of these cells to produce cytokines upon α-GalCer stimulation. TCR transgenic cell lines secreting less IL-2 could bind to mouse tetramer with intermediate affinity, even if they had a high level of CD3 expression, whereas good IL-2 producers were very positive in both mCD3 and tetramer staining.

Presented data suggest that only mouse but not rat Vα14$^+$ TCR transgenic cell lines were fully functional and capable of cytokine secretion upon *in vitro* α-GalCer

stimulation, as well as of binding to mCD1d-α-GalCer-tetramer. The cytokine production correlated with the pattern of the tetramer staining and it was dependent on the presence of $CD1d^+$ APCs. BWr/mCD28 cells expressing rat liver $V\alpha14^+$ TCR were able to produce IL-2 upon Ab but not Ag stimulation and they did not have reactivity to the mCD1d-α-GalCer-tetramer.

2.4. Comparison of IL-2 production by BWr/mCD28-$V\alpha14^+$ transgenic cells and KT12 hybridoma in the response to α-GalCer stimulation

KT12 hybridoma expressing canonical mouse Vα14Vβ8.2 TCR was used as positive control in many of our experiments. This hybridoma was also a source of cDNA used for cloning of mouse Vα14 and Vβ8.2 chains, done in parallel as a control for the cloning of rat $V\alpha14^+$ TCR invariant. Therefore, the Vα14Vβ8.2 TCR cloned from KT12 hybridoma was expressed in BWr/mCD28 cells.

When KT12 hybridoma and BWr/mCD28 transgenic cells, both expressing the same TCR^{inv}, were stimulated with α-GalCer *in vitro,* some differences in amounts of released IL-2 could be observed. KT12 hybridoma produced high amounts of IL-2 in the response to α-GalCer presented by $CD1d^+$ mouse/rat thymocytes and splenocytes as well as by CD1d transgenic cell lines. In contrast, upon the same stimulation conditions mouse $V\alpha14^+$ TCR transgenic cells secreted much less IL-2 and recognised α-GalCer, with different avidity, depending on APCs used for stimulation.

NKT cells express inhibitory or/and stimulatory receptors, which keep the triggering of activation in the check (Davodeau *et al.*, 1997; Brossay *et al.*, 1998-d). Thus, it is likely that the differences in the level of secreted cytokines by KT12 and BWr/mCD28 TCR transgenic cells might have reflected differences in the expression level of their coreceptors.

BW cell line is known to be negative for NK inhibitory receptors. The expression of other molecules may be down modulated. Therefore, in the absence of additional receptors the costimulatory interactions were not strong enough to enhance the

TCR^{inv}-α-GalCer signal and therefore BWr/mCD28-mVα14$^+$ transgenic cells produced only limited amounts of IL-2 upon α-GalCer stimulation.
KT 12 hybridoma was generated by fusion of NK1.1$^+$ T cell clone with BW5147αβ$^-$ cells (Makowska *et al.*, 2000). It has been shown that the expression of many inhibitory receptors (like Ly49) on T hybridomas may be lost upon cell fusion (Bendelac *et al.*, 1995-c; Park *et al.*, 1998-b). However, because NKT cells express various NK markers and adhesion molecules, it is hard to believe that all these receptors are lost during fusion. Therefore, we think that some of inhibitory and activating receptors, absent on BWr/mCD28 cells, were still present on the surface of KT12 cells and could positively influence triggering of the KT12 hybridoma activation.

Concluding, the response of BWr/mCD28 cell line expressing mouse TCR^{inv} and KT12 hybridoma to α-GalCer-CD1d stimulation may have been different due to differences in the expression level of their stimulatory/inhibitory receptors. KT12 hybridoma had low activation threshold and was able to recognise α-GalCer presented by CD1d expressed at low level by thymocytes or splenocytes as well as CD1d over-expressed in CD1d transgenic cell lines. The response of KT12 hybridoma to α-GalCer stimulation could be triggered easier than the response of mVα14$^+$ cell lines.
It has been shown that the lower activation threshold of T cell hybridomas may be caused by the lack of inhibitory NK receptors, which are normally expressed by freshly isolated NKT cells (Bendelac *et al.*, 1997; Park *et al.*, 1998-b**).** Therefore, the comparison of the stimulatory/inhibitory receptors expression on freshly isolated NKT cells, KT12 hybridoma and BWr/mCD28 transgenic cells, with their capability to produce cytokine upon α-GalCer stimulation will allow the definition of optimal conditions necessary for efficient activation of various Vα14$^+$T cells.

2.5. Importance of CD80-CD28 costimulatory pathway in the activation of Vα14$^+$ cell lines

α-GalCer-mediated stimulation of mouse Vα14$^+$ and human Vα24$^+$ NKT cells is CD1-d restricted and TCR/costimulatory molecule-dependent. CD1d and other

molecules expressed by APCs as well as costimulatory receptors on NKT cells are required for the activation of NKT cells and cytokine production (Kawano *et al.,* 1997; Hermans *et al.,* 2003; Hayakawa *et al.*, 2001**;** Kitamura *et al.*, 1999). The activation and proliferative responses of Vα14^{+}NKT cells may be inhibited by monoclonal antibodies against CD1d, B7, CTLA-4, CD28, CD40 or CD40L (Kawano *et al.,* 1997). Furthermore, experiments done on CD28 and CD40-deficient mice showed that the response of NKT cells stimulated with α-GalCer *in vitro* or *in vivo* could differ depending on which costimulatory pathway was involved (Hayakawa *et al.*, 2001). The blockade of CD28-CD80 interaction by anti-CD80 and anti-CD86 mAbs inhibited IFN-γ and IL-4 production by splenic Vα14^{+} NKT cells, while the inhibition of CD40-CD154 interaction influenced only the IFN-γ production. Therefore, CD28-CD80 and CD40-CD154 stimulatory pathways seem to be important for the regulation of Th1 and Th2 functions of NKT cells (Hayakawa *et al.*, 2001).

Interestingly, various subset of NKT cells may differ in their phenotype, cytokine profiles and requirements for costimulatory signals, depending on the tissue localisation, maturation state of APCs, or differences in the expression levels of CD1d, CD86 or CD40 molecules (Yang *et al.,* 2003; Fuji *et al.,* 2002-2).

Since costimulation plays such a crucial role in the activation of human and mouse NKT cells *in vivo*, we attempted to test if this situation could be mimicked in *in vitro* system. Upon α-GalCer-CD1d stimulation BWr/mCD28 cells expressing mouse TCR^{inv} were able to produce IL-2 in Ag-dose dependent manner. To enhance the response of these cells to α-GalCer, rat CD80 molecule was expressed together with CD1d on the surface of P80 cell line. Over-expression of CD28 on BW and CD80 on APCs created optimal conditions for costimulation.

BWr/mCD28 cells expressing mouse TCR^{inv}, stimulated with α-GalCer-loaded P80rCD80CD1d transgenic cells, secreted IL-2 in a CD1d and CD80 dependent manner. The incubation of these cells with anti-CD80 mAb inhibited the IL-2 production, suggesting that, similar to the situation *in vivo,* CD28-CD80 interaction was necessary for efficient *in vitro* activation of Vα14^{+} cells. By contrast, the CD28-CD80 costimulation was not so critical for activation KT12 hybridoma, since the IL-2 production by hybridoma cells was decreased by anti-rat CD80 Ab only to some extent. Therefore, the CD28-CD80 interaction seems to be not only one mechanism

responsible for the activation of KT12 hybridoma. Other stimulatory or inhibitory receptors present on hybridoma cells (as discussed earlier) could have probably additionally contributed to the activation. One good candidate for such costimulatory molecule might be NK1.1/NKRP1A. It has been shown that mouse NK1.1 and human NKRP1A have costimulatory functions and their ligation may additionally influence the cytokine secretion by human or mouse invariant NKT cells (Exley *et al.*, 1998; Arase *et al.*, 1996). It would be interesting to check whether rat NKRP1A, similarly to human NKRP1A or mouse NK1.1 may serve as a costimulatory molecule or perhaps as alternative pathway for activation of rat NKT cells. Furthermore, the determination of NKRP1A/NK1.1 as well as costimulatory receptor expression levels on primary T cells and $V\alpha14^+$ cell lines might be helpful in the defining a molecular basis of their activation.

The *in vitro* model, used to test the role of CD28-CD80 interaction in the activation of $V\alpha14^+$ TCR cells, is rather artificial and does not represent the natural situation *in vivo*, where several receptors are involved in Ag recognition and where there is constant feed-back interaction between NKT cells and APCs (Gillessen *et al.*, 2003; Ikarashi *et al.*, 2001), T (Hermans *et* al., 2003) and B cells (Kitamura *et al.,* 1999; Galli *et al.*, 2003) or NK cells (Metelitsa *et al.,* 2001; Carnaud *et al.,* 1999). Activated through TCR^{inv}-α-GalCer-CD1d and CD28-CD80 stimulation, NKT produce cytokines, which promote the IL-12 secretion by APCs (Kawamura *et al.,* 1998; Tomura *et al.,* 1999) or induce upregulation of costimulatory molecules (like CD86) on APCs (Fujii *et al.*, 2003-a; Hermans *et al.,* 2003). These interactions between several molecules and cytokines, which usually accompany the activation of NKT cells are missing in our *in vitro* system. Nevertheless using a cell line model we were able to show that costimulation through CD28, which is required for the activation of T cells (Chambers *et al.,* 1997; Bennett *et al.,* 1998) is also important for the activation of NKT cells.

III: Rat CD1d

3.1. Rat CD1d as Ag presenting molecule:

The CD1d molecule is expressed in various lymphoid and nonlymphoid rat organs including spleen, thymus, liver, lung, kidney and skin (Kasai *et al.,* 1997; Ichimiya *et al.,* 1994).

To confirm the co-localisation of both CD1d transcript and protein, the tissue distribution of rat CD1d was analysed by Northern blot and RT-PCR analysis, *in situ* hybridisation (IHS) or by immunohistochemistry (Ichimiya *et al.,* 1994; Kasai *et al.,* 1997). In some of these techniques 1H1 and 3C11 rat anti-mouse CD1 mAbs were used. However because of their reactivity with limited epitopes on rat CD1d, these antibodies were successfully used in immonoblotting but failed to positively stain rat thymocytes (Burke *et al.,* 1994; Kasai *et al.,* 1997). Similarly, 1H1 and 3C11 Abs could not be used for flow cytometric or immunohistochemisric staining of human thymic CD1d although they positively stained CD1d on transfected cells, B cells or intestinal epithelial cells (Blumberg *et al.,* 1991).

Since 1H1 and 3C11 anti-mouse CD1d antibodies appeared to be not cross-reactive with rCD1d, another anti-mouse CD1d (clone 1B1) Ab was used for the immunofluorescence staining of rat thymus or spleen derived T lymphocytes. 1B1 positively labelled mouse cells, used as control, but similarly to two other anti-mouse CD1d antibodies, it did not show cross-reactivity with rat CD1d. Additionally, the personal communication with Matsuura, who tested polyclonal and monoclonal anti-mCD1d antibodies against rat CD1d transgenic cell lines, allows the assumption that none of the commercially available anti-mouse CD1d antibodies may be used for the FACS analysis of rat CD1d surface expression.

To confirm the presence of CD1d in rat organs and to elucidate the tissue distribution, RT-PCR and cloning methods have been undertaken. In order to characterise functions of rat CD1d, the capability of rat $CD1d^+$ primary cells and rCD1d transgenic cell lines to present Ag to $V\alpha14^+$ cells were tested in detail. Since, no anti-rat mAb is available we took attempts to generate such an antibody, as a tool

for the determination of CD1d surface expression as well as for the identification of mechanisms of α-GalCer recognition by rat NKT cells.

3.2. Cloning of rat CD1d

To closer characterise the CD1d-Ag-TCR interactions, rat CD1d was cloned. The comparison of F344 rat bone marrow-derived rCD1d sequence with mouse counterpart revealed that, similarly to data presented by Ichimiya *et al.* (1994), these molecules were highly homologous, especially in their α-1 and α2 domains. Additionally, similar to mouse, the rat CD1d sequence contained in the cytoplasmic tail a YXXZ- tyrosine targeting motif, required for endosomal localisation of CD1d (Cernadas *et al.,* 2003; Chiu *et al.,* 2002; Brossay *et al.,* 1998-d).

The high level of structural homology might have suggested that rat and mouse CD1d molecules would also have the same functional features. Data from *in vitro* assays showed that the activation of rat splenic and hepatic lymphocytes and their cytokine secretion depended on the presence of $CD1d^+$ APCs. Similarly, BWr/mCD28 cells transduced with mouse TCR^{inv} could produce IL-2 upon α-GalCer stimulation but only when mouse or rat $CD1d^+$ thymocytes were present in the culture. This confirms that the CD1d naturally present on rat thymocytes and splenocytes is expressed as a functional molecule able to present α-GalCer to $V\alpha14^+TCR$.

3.3. Presentation of α-GalCer by CD1d transgenic cells to KT12 hybridoma

NKT cells and cell lines with restricted Vα14Vβ8.2 TCR are able to recognise α-GalCer presented by $CD1d^+$ tissues or CD1d transfected cells (Brossay *et al.,* 1998-a; Burdin *et al.,* 1998; Couedel 1998; Makowska *et al.,* 2000). However, the CD1d recognition is not limited only to $NK1.1^+$ T cells. The CD1d-autoreactive hybridomas expressing diverse TCR α-chain or hybridomas generated from $CD4^+$ T cells of MHC class II-deficient mice recognise CD1d and produce high amounts of

cytokines but they do not show specificity for α-GalCer (Cardell *et al.*, 1995; Gumperz *et al.,* 2000; Chiu *et al.,* 1999; Makowska *et al.*, 2000). Also in humans, the pairing of the invariant Vα24 with Vβ11 is not absolutely required for CD1d recognition. The expression of $V\alpha24^+$ chain with other Vβs can generate CD1d-reactive TCRs (Exley *et al.*, 1997).

Because of high homology between human and mouse CD1d, mouse NKT cell hybridomas produce IL-2 in the response to mCD1d or hCD1d loaded with Ag (Brossay *et al.,* 1998-a). By analogy, rat CD1d shows similarity with mouse CD1d molecule and therefore we predicted that mouse KT12 hybridoma expressing invariant Vα14Vβ8.2 TCR would have specificity for α-GalCer presented by rat CD1d. To check this possibility, rat CD1d was expressed in different cell lines which, after loading with α-GalCer, were used to stimulate $V\alpha14^+$ KT12 hybridoma. Autoreactive KT12 hybridoma, due to high self-presentation, could respond to α-GalCer stimulation even in the absence of APCs. However, when rCD1d transgenic cells were used as APCs, the amounts of IL-2 released by KT12 hybridoma increased significantly in an Ag-dose dependent manner. Despite the fact that transfectants had similar levels of CD1d expression, KT12 hybridoma produced different amounts of IL-2 depending on the APCs used. These discrepancies in the response of KT12 cells to α-GalCer stimulation might have reflected the differences in organ or/and species origin of tested APCs (Kronenberg, 2002). However, because KT12 hybridoma was able to recognise, although with different efficiency, α-GalCer presented by CD1d transgenic cells of human, mouse and rat origin, the diversity in the amounts of released IL-2 could be possibly caused by tissue rather than species specificity. This finding is in agreement with data presented by Park, who showed that mouse NKT cell hybridomas, when screened on mouse, rat and human cell lines transfected with CD1d cDNA, could distinguish between CD1d expressed by cells of distinct tissue types (Park *et al.,* 1998-b). The high level of tissue specificity of CD1d recognition might suggest that $V\alpha14^+$ hybridomas recognise diverse CD1d-bound ligands of cellular origin (Park *et al.,* 1998-b; Brossay *et al.,* 1998-d). Alternativelly, the differences in the ability to recognise CD1d, depending on tissue microenvironment, could reflect differences in CD1d processing (Balk *et al.,* 1994; Exley *et al.,* 2000). In line with this, Makowska has shown that the variability in the $V\alpha14^+$ T hybridoma activation reflected the

differences in the Ag-presenting mechanisms between the different APCs used (Makowska *et al.*, 2000).

Additionally, it could be possible that KT12 hybridoma by itself had some preferences in CD1d recognition, conversely that CD1d transgenic cells had different capabilities of α-GalCer presentation. Thus, the activation of KT12 hybridoma could be easier triggered by rCD1d expressed by professional "APCs", while CD1d transgenic cells unable to present Ag could not stimulate KT12 hybridoma. According to this, it is likely that the variability in the response of KT12 hybridoma to α-GalCer presented by different $CD1d^+$ cell lines was caused by differences in the Ag processing or/and the differences in the expression level of costimulatory molecules.

Because the discrepancies in the responces of KT12 hybridoma to different APCs were large, the interpretation of results was difficult. Therefore, in future experiments the α-GalCer loaded CD1d transgenic cells will be used for stimulation of various $V\alpha14^+$ cells including mouse/rat NKT cells, $V\alpha14^+$ transgenic cells lines and KT12 hybridoma. Furthermore, to confirm the specificity of TCR^{inv} for α-GalCer, anti-mouse and novel anti-rat CD1d monoclonal antibodies will be used to block the α-GalCer-dependent cytokine production.

3.4. Production of anti-rat CD1d mAb

Thymus and spleen are organs, where human, mouse or rat CD1d seem to be constitutively expressed. However the level of CD1 expression might be quantitatively different among species. It may also differ depending on the sensitivity of the techniques used. (Brandbury *et al.,* 1990; Balk *et al.,* 1991). Additionally, some heterogeneity in the level of the CD1d expression between various tissues within one organism or even between various cells within the same organ are possible. For instance, cortical thymocytes, which are predominantly immature T cells, express high levels of CD1 protein during thymic development, while medullary thymocytes are negative for CD1 staining (Brandbury *et al.,* 1990; Exley *et al.,* 2000). Similarly, the CD1d in the spleen can be expressed at variable

levels depending on the CD1d expression by different subsets of splenic lymphocytes (Brossay et al., 1997).

To elucidate the tissue distribution of rat CD1d, we took an approach to produce anti-rat CD1d monoclonal antibody.

Both mouse and rat splenic or thymic lymphocytes were positively stained with culture supernatant from CD1d fusion hybridomas. The specificity of supernatants for CD1d was confirmed in the staining of rCD1d transgenic cell lines (P80rCD1d, RAJIrCD1d) and the staining of lymphocytes, isolated from $CD1d^{-/-}$ mouse. The novel anti-rat CD1d mAb was cross-reactive, specifically recognising CD1d expressed by rat and, to a lesser degree, by mouse thymocytes and splenocytes. This might suggest that, similarly to a previous report (Kasai *et al.,* 1997; Ichimiya *et al.,* 1994), the expression of CD1d on mouse lymphocytes may be lower than the expression of CD1d on rat cells.

Furthermore, some tissue or inter-strain differences in the levels of CD1d expression could be observed. Firstly, the expression of CD1d in LEW rat tissues seemed to be higher than in F344 rat. Secondly, F344 and LEW thymocytes stained with fusion supernatants were more positive than rat splenocytes (data not shown), suggesting different levels of CD1d expression in these two rat tissues. This finding, however, is in contrast to the staining of mouse cells, in which splenic APCs expressed significantly higher levels of CD1d and CD86 than thymic APCs did (Yang *et al.*, 2003; Park *et al.*, 1998-b).

To confirm the differences in CD1d distribution on the species, strain and tissue levels, a purified, concentrated and titrated anti-rat CD1d monoclonal antibody will be used. The rCD1d-specificity of this antibody will be further confirmed by immunoprecipitation and Western-Blot techniques, as well as by immunohistochemistry of $CD1d^{+}$ tissues.

Data presented in the thesis imply that rat CD1d can be expressed as a functional molecule able to present α-GalCer to $V\alpha14^{+}$ cells. On the other hand, the activation of TCR^{inv} transgenic cell lines and to some extent KT12 hybridoma requires the presence of CD1d and CD80 positive APCs. Therefore, the usage of anti-rat CD1d mAb to block CD1-d-α-GalCer-TCR^{inv} interaction will allow the determination of CD1d expression, on primary rat or rCD1d transgenic cells, necessary for triggering the acivation and IL-2 production by $V\alpha14^{+}$ cells.

Concluding, no anti-rat CD1d mAb has been available so far, although many attempts in this direction have been undertaken. During this study 6 fusion hybridomas, producing anti-rat CD1d specific monoclonal antibodies were generated from $CD1d^{-/-}$ mice. Some of these antibodies are cross-reactive with mouse CD1d and probably recognise different rat/mouse CD1d epitopes, which makes them a precious tool for the comparison of CD1d expression in various mouse and rat tissues/organs.

IV. The comparison of CD1d-α-GalCer-TCR^{inv} system in F344 and LEW rat

Mouse strains may differ in terms of NK1.1 expression (some of them express others lack the NK1.1 molecule) and the frequency of NKT cells (Hammond *et al.*, 2001). It was possible that such variability would be also present among different rat strains.

The F344/Crj and LEW/Crj- two rat strains of different genetic background were compared in this thesis. When the distribution and the frequency of $NKRP1A^{+}TCR^{+}$ lymphocytes isolated from both strains were estimated by Vα14-specific RT-PCR, the Vα14 TCR-positive PCR signal was much weaker in LEW than in F344 rat organs. It remains to be determined whether the differences in the expression level of Vα14 TCR-positive cells correlate with the differences in the frequency of F344 and LEW NKT cells. Therefore, in addition to RT-PCR analysis the FACS staining of F344 and LEW rat lymphocytes isolated from different organs will be performed.

Only α-GalCer has been postulated to be a specific Ag activating NKT cells. Other lipid antigens, even if they bind to CD1d, are not antigenic and do not stimulate $Vα14^{+}$ cells (Sidobre *et al.,* 2002; Naidenko *et al.,* 1999). However, a chemical analog of α-GalCer, called OCH has been recently described (Miyamoto *et al.,* 2001). This lipid has a shorter sphingosine base (C5 vs. C14) and acyl chain (C24 vs. C26) compared with α-GalCer, and has a different antigenic capacity to α-GalCer. OCH used for stimulation of mouse NKT cells, was able to trigger

preferential IL-4 and almost no IFN-γ production (Miyamoto *et al.,* 2001). Since the usage of two lipid antigens differentially influenced the cytokine secretion by mouse NKT cells, we used α-GalCer and its chemical analog for stimulation of LEW and F344 rat cells. When the capacities of F344 and LEW splenocytes to release cytokines upon *in vitro* α-GalCer/OCH stimulation were tested, LEW lymphocytes could respond only to a limited extent to α-GalCer but not OCH stimulation. By contrast, F344 rat cells were more responsive to both α-GalCer and OCH activation. Interestingly, the stimulation of F344 rat splenocytes with OCH triggered the secretion of both IL-4 and IFN-γ, not only IL-4 as was found by Miyamoto for mouse NKT cells (Miyamoto *et al.,* 2001). These discrepancies in the pattern of released cytokines could reflect the differences in the stimulation protocols. Additionally, it might be possible that mouse and rat NKT cells differ in their response to OCH stimulation.

Rat has only one CD1 gene, however some polymorphism on the nucleotide level among different rat strains can be present. A single nucleotide substitution, at codon 119, resulting in amino acid alteration (alanine into valine) has been described thus generating two allelic CD1d forms. The alanine is expressed in seven rat strains including F344Crj, while the valine can be found in other five strains, including LEW/Crj (Katabami *et al.,* 1998). Thus, according to this classification, F344 and LEW express distinct allelic forms of CD1d.

The exchange of alanine to valine takes place within the α-2 domain of CD1d, which together with the α-1 domain form antigen-binding groove. Therefore, it was probable that LEW and F344 rat belonging to two different CD1d allelic groups would have different capability of Ag presentation. However, when α-GalCer-loaded F344 and LEW $CD1d^+$ thymocytes or splenocytes were used for stimulation of KT12 hybridoma, no differences in the response of KT12 hybridoma cells could be observed. Furthermore, F344 and LEW thymocytes presented α-GalCer to BWr/mCD28 cells expressing mouse $V\alpha14^+$ TCR equally well. These results suggest that F344 and LEW rat expressed two different allelic forms of CD1d but the aa alteration did not influence α-GalCer presentation to $V\alpha14^+$ cells.

Since $CD1d^+$ APCs from both rat strains presented α-GalCer to $V\alpha14^+$ cells as well as mouse APCs did, we conclude that the differences in the CD1d-α-GalCer-TCR^{inv} system, observed between LEW and F344 rats, were probably caused by the

differences in the frequency and functions of NKT cells. Mechanisms of α-GalCer presentation by CD1d were the same in both rat strains. It remains to be determined whether the differences in the proportions of NKT cells in LEW and F344 rat correlate with the differences in their development and functions. Because the population of Vα14$^+$ NKT cells in LEW rat is possibly smaller than in F344 rat, it is not clear if the smaller proportion of LEW NKT cells correlates with the higher susceptibility of this rat strain to autoimunne diseases (like EAE) (Blankenhorn *et al.*, 1991). Nevertheless, the usage of α-GalCer stimulated rat NKT cells might provide a new tool for treatment of EAE, experimental autoimmune uveitis (Shao *et al.*, 2003) and other immune disorders in animal models.

Additional experiments on rat strains other than LEW and F344 will allow the better characterisation of rat NKT cell functions and their contribution in the regulation of immunity.

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Summary:

Originally, NKT cells have been defined by their expression of T-cell receptor (TCR) and NK cell markers NKRP1A in human and NK1.1 (NKRP1C) in mouse. Most of these cells express CD1d-restricted TCR with a characteristic rearrangement- Vα24JαQ/Vβ11 in human and Vα14Jα18/Vβ8.2 in mouse, and have been implicated in playing an important role in first line defence and immunoregulation. The subject of this thesis was the characterisation of the hypothetical rat NKT cell population.

In the mouse system, CD1d-restricted NK1.1$^+$ T cells represented around 30% of intrahepatic and around 3% of splenic lymphocytes and could be visualised by staining with α-GalCer-loaded mouse CD1d tetramer. Rat NKRP1A$^+$TCR$^+$ cells, similar to mouse NKT cells, were predominantly expressed in the liver. However, their frequency was around 5 fold lower than the frequency of mouse intrahepatic lymphocytes. F344 rat NKT cells, in contrast to mouse CD4$^+$ or DN NK1.1$^+$ T lymphocytes, were of CD8 rather than CD4 phenotype, and did not bind to mCD1d-α-GalCer-tetramer.
Since human hepatic CD1d-restricted Vα24JQ$^+$ T cells are not as frequent as their mouse counterparts and may express CD8- a marker not expressed by mouse CD1d-restricted cells, it is possible that the phenotype of F344 rat NKT cells corresponds more to the phenotype of human than mouse NKT cells.

Similar to mouse NKT cells, F344 rat liver- and spleen-derived lymphocytes were able to produce IL-4 and IFN-γ when stimulated with the synthetic ligand α-GalCer *in vitro*. Therefore, the lack of binding of rat lymphocytes to mouse CD1d tetramer could not be due to their inability to respond to α-GalCer.

To better characterise the reactivity of rat NKRP1A$^+$TCR$^+$ cells to α-GalCer, the rat invariant TCR was analysed. RT-PCR of liver lymphocytes with Vα14-specific primers and subsequent cloning revealed a much weaker PCR signal for rat lymphocyte cDNA than for mouse cDNA. Furthermore the analysis of rat AV14JA18 sequences showed that the rat Vα14$^+$TCR invariant could be rearranged

not only with AJ18 but also with other AJ segments. The low number of clones with in frame Vα14Jα18 rearrangement could suggest that only a small proportion of liver lymphocytes were CD1d restricted NKT cells.

Mouse and human NKT cells are able to recognise α-GalCer presented by the CD1d-β2 microglobulin complex, leading to their activation, proliferation and cytokine secretion. In order to compare the capacity of mouse and rat CD1d to present α-GalCer, rat CD1d was cloned. Sequence analysis and functional tests *in vitro* confirmed the structural and functional homology of rat CD1d with mouse CD1d.

In parallel, to characterise the reactivity of rat NKRP1A^{+}TCR^{+} cells to α-GalCer, rat Vα14^{+}TCR invariant was cloned and expressed in the TCR^{-} T cell hybridoma BWr/mCD28. Rat Vα14TCR^{+}CD28^{+} transgenic cells secreted IL-2 upon αTCR/CD3 antibody stimulation, but were not specific for α-GalCer. Such cells were also negative in staining with mCD1d-α-GalCer tetramer. The lack of reactivity to α-GalCer and the lack of binding to mouse tetramer were probably caused by amino acid alterations, particularly at position 72 (51 according to IMTG nomenclature) of cloned rat TCR^{inv}. Reversal of these "alterations" using molecular biology techniques was performed but the expression of this TCR on the surface of BWr/mCD28 cells could not be achieved.

In contrast to rat TCR^{inv}, mouse Vα14^{+}TCR was fully functional and was specific for mouse CD1d tetramer. KT12 hybridoma and BWr/mCD28 cells expressing mouse TCR^{inv}, when stimulated with α-GalCer presented by primary CD1d^{+} cells or rCD1d transgenic cell lines, produced IL-2 in an Ag- and CD1d-dependent manner. Transgenic lines expressing TCR comprising mouse Vα14 and rat Vβ8.4 responded to α-GalCer presented by rat and mouse CD1d, and bound mCD1 tetramer. By contrast, cell lines expressing TCR comprising mouse Vα14 and rat Vβ8.2 responded only to α-GalCer presented by rCD1d and bound weakly to mCD1d tetramer. This suggests that germ line encoded regions of the β-chain (CDR2 or CDR4) bind to species-specific determinants of CD1d. The cytokine secretion of the cell lines was inhibited by anti-CD80 mAb, indicating the importance of CD80-CD28 costimulation in their activation.

To check whether rat NKT cells may exist in other rat strains, the frequency and functions of $NKRP1A^+TCR^+$ in F344 and LEW rat were compared. F344 and LEW, two rat strains expressing different allelic CD1d forms, varied slightly in the level of CD1d expression, as assessed by staining with a newly generated CD1d specific monoclonal antibody. By contrast, these rat strains differed in terms of α-GalCer recognition. $NKRP1A^+TCR^+$ cells were less frequent in LEW than in F344 rats, and did not respond to α-GalCer or the analogue OCH *in vitro*, a result which is of special interest considering the susceptibility of LEW but not F344 rats to experimentally induced organ specific autoimmune diseases.

In summary, the rat and mouse CD1d-invariant TCR systems show a high degree of structural and functional homology, but it seems that invariant NKT cells in rat, similar to such cells in human, occur at lower frequency than in mice. TCR transgenic cell line species-specific patterns of CD1d α-GalCer reactivity will provide a valuable tool for the mapping of CD1d/TCR contacts. Also monoclonal antibodies specific for rat and mouse CD1d, generated in this study, provide valuable tools to determine CD1d protein expression in various rat tissues and will help to better characterise functions of CD1d-restricted rat T cells.

Zusammenfassung

NKT Zellen wurden ursprünglich über die gleichzeitige Expresion eines T-Zellantigenrezeptors (TZR) und den NK-Zellmarkern NKRP1A im Menschen bzw. NK1.1. (NKRP1C) in der Maus definiert. In Mensch und Maus exprimieren die meisten NKT Zellen CD1d restringierte TZR mit charakteristischen Genumlagerungen- Vα24JαQ/Vβ11 im Menschen und Vα14Jα18/Vβb8.2 in der Maus. Den NKT Zellen werden außerdem wichtige Funktionen in der „first line defence“ und der Immunregulation zugesprochen. Gegenstand der Doktorarbeit war die Charakterisierung eines hypothetischen Gegenstückes in der Ratte.

In der Maus wurden rund 30% der intrahepatischen Lymphozyten (IHL) und 3% der Milzlymphozyten als CD1d restringierte NK T Zellen identifiziert und konnten mittels α-GalCer beladenen Maus-CD1d Tetramer visualisiert werden. Wie in der Maus wurden in der Ratte $NKRP1A^{+}TZR^{+}$ Zellen vorwiegend in der Leber gefunden, waren aber fünfmal weniger häufig. F344 Ratten NKT Zellen waren darüber hinaus im Gegensatz zu den $CD4^{+}$ oder $CD4^{-}CD8^{-}$ Maus NKT Zellen meistens CD8 positiv und banden kein mCD1d Tetramer. Da in der menschlichen Leber CD1d-restringierte $Vα24JQ^{+}$ T Zellen ebenfalls viel seltener als in der Maus sind, scheint es nun möglich, daß der Phänotyp der Ratten NKT Zellen eher dem des Menschen als dem der Maus entspricht.

Ein Test der Fähigkeit von F344 Leber- und Milzlymphozyten nach Kultur mit α-GalCer Cytokine zu produzieren, ergab ähnlich wie in der Maus eine Produktion von IL-4 und IFN-γ. Aus diesem Grund kann eine fehlende Reaktivität von Ratten NKT Zellen für α-GalCer nicht der Grund für eine fehlende mCD1d Tetramerbindung sein.

Um die Reaktivität der $NKRP1A^{+}TZR^{+}$ Rattenzellen auf α-GalCer besser zu verstehen, wurde der Ratten TZR analysiert. RT-PCR von Leberlymphozyten mit Vα14-spezifischen Primern und die Analyse der klonierten PCR Produkte ergab ein viel schwächeres Signal für Ratten als für Maus cDNA. Darüber hinaus zeigten Sequenzanalysen, daß das Vα14 auch mit anderen J als dem für TCR^{inv} typischem Jα18 rearrangiert war. Die niedrige Anzahl von Vα14Jα18 „in frame“

Umlagerungen legt Nahe, daß nur ein kleiner Anteil der Leber-lymphozyten CD1d restringierte NKT Zellen sind.

Maus und humane NKT Zellen erkennen durch CD1d-β2m Komplexe präsentiertes α-GalCer und reagieren mit Aktivierung, Proliferation und Cytokinproduktion. Um die Fähigkeit von Maus und Ratten-CD1d α-GalCer zu präsentieren, zu testen, wurde das CD1d Molekül der Ratte kloniert. Sequenzanlyse und funktionelle Tests bestätigten die strukturelle und funktionelle Homologie des CD1d beider Spezies. Gleichzeitig wurde zur Analyse der Reaktivität von $NKRP1A^{+}TZR^{+}$ Zellen auf α-GalCer ein Ratten $V\alpha14^{+}$ invarianter TZR kloniert und in einem TZR^{-} T-Zellhybridom (BWr/mCD28) exprimiert. Zellen die transgenen Ratten $V\alpha14^{+}TZR$ und CD28 exprimierten, sezernierten IL-2 nach Stimulation mit αTZR/CD3 Antikörper aber zeigten keine Spezifität für α-GalCer. Die fehlende Reaktivität für α-GalCer und die fehlende Bindung von mCD1-α-GalCer Tetramer waren wahrscheinlich durch Aminosäuresubstitionen insbesondere an Position 71 (51 nach IMGT Nomenklatur) der klonierten TZRα Kette begründet. Eine „Umkehrung" dieser Änderung wurde mittels molekularbiologischer Techniken durchgeführt aber Expression dieses TZR auf BWr/mCD28 wurde nicht erreicht.

Im Gegensatz zum invarianten $V\alpha14^{+}$ Ratten TZR war der Maus $V\alpha14^{+}$ TZR voll funktional und spezifisch für mCD1d Tetramer. KT12 Hybridom und Maus TZR^{inv} exprimierende BWr/mCD28 Zellen wurden sowohl durch Ratten als durch Maus CD1d präsentiertes α-GalCer aktiviert. Dasselbe galt für TZR, die eine Maus Vα14 TZR Kette und eine Ratten Vβ8.4 TZR Kette enthielten. Im Gegensatz hierzu antworteten Linien mit mVα14 und Ratten Vβ8.2 nur auf durch Ratten und nicht auf durch Maus CD1d präsentiertes α-GalCer und banden nahezu kein mCD1d Tetramer. Dies legt Nahe, daß Keimbahn kodierte der β-Kettenbereiche (CDR2 oder CDR4) speziesspezifische Bereiche des CD1d erkennen. Weiterhin wurde gefunden, das die Zytokinsekretion der Zellinien durch CD80 spezifische monoklonale Antikörper inhibiert wurde, was eine wichtige Rolle der CD80-CD28 Interaktion bei der Aktivierung dieser Zellen nahelegt.

Um zu sehen ob NKT Zellen auch in anderen Rattenstämmen als F344 existieren, wurde Häufigkeit und Funktion von $NKRP1A^{+}TZR^{+}$ Zellen in F344 und LEW

Ratten miteinander verglichen. F344 und LEW, zwei Rattenstämme die unterschiedliche CD1d Allele tragen, zeigten in der Analyse mit einem neu generierten rCD1d spezifischen monoklonalen Antikörper nur geringe Unterschiede in der Expressionsstärke. Hingegen, unterschieden sich beide Stämme in der Reaktivität für α-GalCer. NKRP1A$^+$ Zellen waren in der LEW Ratte weniger häufig als in der F344 Ratte und antworteten *in vitro* nicht auf α-GalCer oder sein Analogon OCH. Ein Resultat, das insbesodere angesichts der besonderen Empfänglichkeit von LEW Ratten für experimentell induzierte organspezifische Autoimmunerkrankungen von besonderem Interesse ist.

Zusammgefasst kann gesagt werden, daß das Maus und Ratten CD1d/TZR^{inv} NKT Zellsystem hohe strukturelle und funktionale Homologie aufweist, aber daß es wie im Menschen weniger invariante NKT Zellen in der Ratte als in der Maus gibt. TZR transgene Zelllinien wiesen ein speziesspezifisches Muster in der α-GalCer Erkennung auf, das für die Analyse von CDd/TZR-Kontaktbereichen von großem Nutzen sein wird. Dasselbe gilt für den Ratten und Maus-CD1d-spezifischen monoklonalen Antikörper, der im Rahmen der Studie generiert wurde. Dieser kann bei der Charakterisierung der CD1d Proteinexpression in verschiedenen Geweben und der besseren funktionellen Charakterisierung von CD1d restringierten T Zellen der Ratte eingesetzt werden.

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