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# **CD1d-iNKT Cell Axis in Dyslipidemia**

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# 1 Preface

In 2010 'Top inside of the decade' issue of Science Dr. Couzin-Frankel wrote: "Over the past decade, it has become widely accepted that inflammation is a driving force behind chronic diseases that will kill nearly all of us: cancer, diabetes and obesity, Alzheimer's disease, and **atherosclerosis**[1]. It is thus not surprising that nowadays modulation of immune responses is considered as a promising approach to treat the chronic diseases. Potent immunomodulatory properties of 'lipid sensing' natural killer T (NKT) cells have been reported over the last 15 years [2,3]. Given the lipid related 'nature' of atherosclerosis, a lot of interest has been raised toward the role of NKT cells in atherogenesis. My PhD project is driven by the idea that modulating NKT cell responses could be beneficial in treating atherosclerosis. Specifically, I characterize classical NKT cells numerically and functionally in dyslipidemic conditions and investigate the role of lipoproteins in CD1d-iNKT cell axis.

# 2 Introduction

## 2.1 What are iNKT cells?

### 2.1.1 CD1 dependent immunity.

Lipid reactive T cells represent more than 10% of all TCR- $\alpha/\beta^+$  T lymphocytes in human blood [4,5]. Unlike traditional T cells which recognize peptides presented by APCs via MHC class I or MHC class II molecules lipid reactive T cells recognize glycolipids presented via MHC class I-like non-polymorphic CD1 molecules (Figure 1 (below))[6].

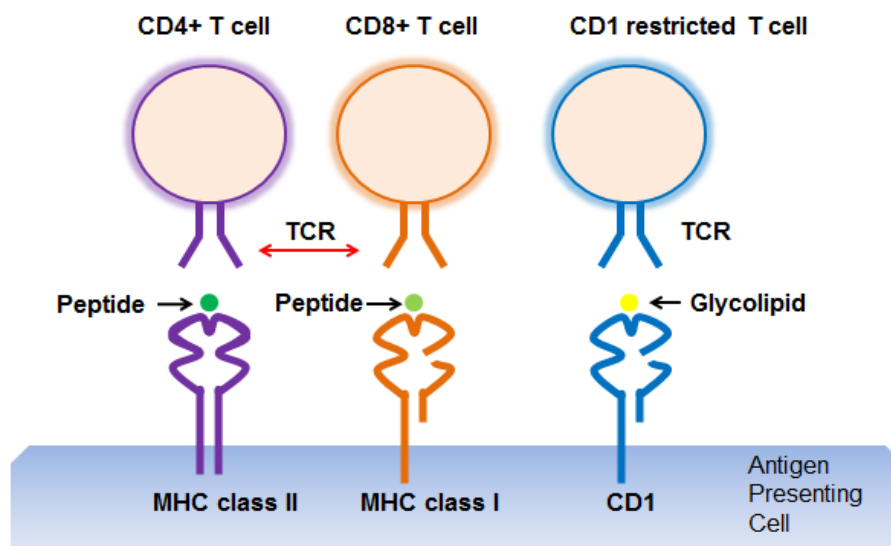


Figure 1. CD1 dependent lipid antigen presentation.

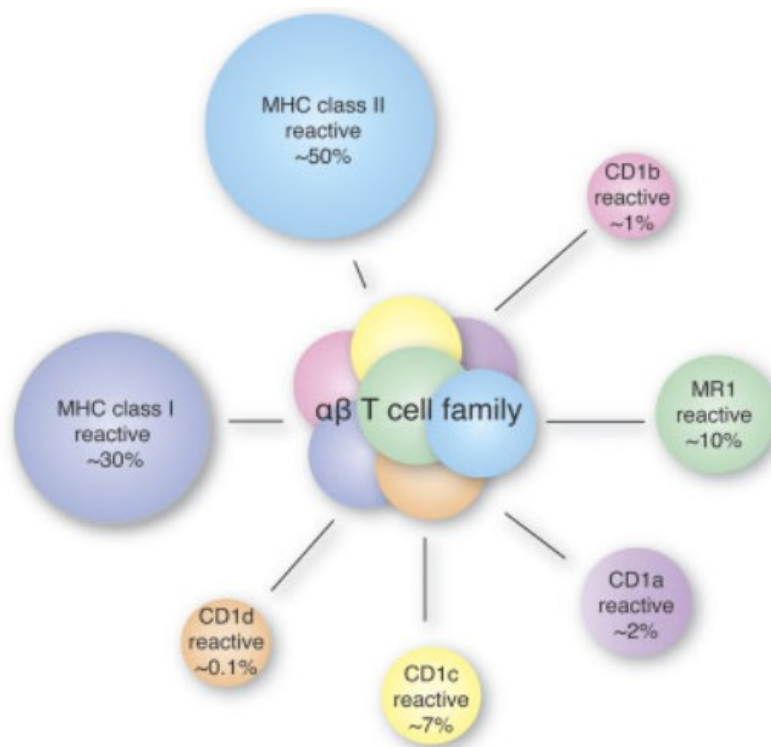
Based on sequence homology, human CD1 family proteins are divided into group I, containing CD1a, CD1b and CD1c, group II, represented by CD1d (Table 1 (below)) and group III, represented by CD1e[7].

**Table 1. CD1 family members participating in lipid antigen presentation.**

Group I (humans)			Group II (mice and humans)
CD1a	CD1b	CD1c	CD1d

Murine genome contains only group II CD1 family member (CD1d). Unlike human genome which contains a single CD1d gene murine genome contains two CD1d genes (mCD1d1.1 and mCD1d1.2) which are 95% identical. Murine CD1d isoforms are equally expressed in the thymus, but mCD1d1.1 is predominant in other tissues[8]. CD1e is the only CD1 family member that is not expressed on the surface of APCs and thus does not participate in lipid antigen presentation. Unlike CD1 a,b,c,d molecules, CD1e is polymorphic. It was suggested that CD1e participates in lipid antigen processing and loading[7].

Figure 2 (below) represents approximate frequencies of TCR- $\alpha/\beta^+$  T lymphocytes reactive to antigens presented via MHC class I, MHC class II, CD1 and MR1 molecules in human PBMC.



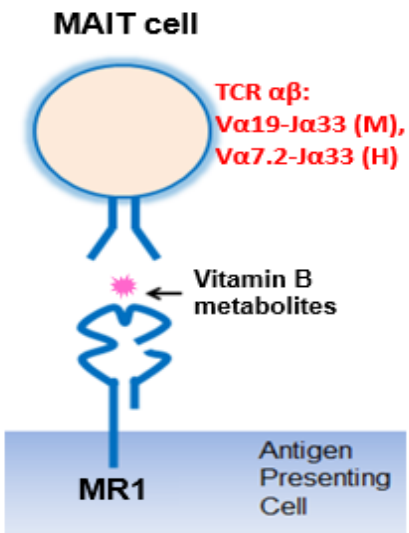
**Figure 2. Subsets of TCR- $\alpha/\beta^+$  T lymphocytes.**

Subsets of TCR- $\alpha/\beta^+$  T lymphocytes according to their reactivity against antigens presented by MHC class I, MHC class II, CD1 and MR1 molecules in human PBMC. (Adapted from Young M. et al[9]).



MR1 molecules, like CD1 molecules, also belong to MHC class I-like molecules and present vitamin B metabolites to mucosal associated invariant T cells (MAIT cells). MAIT cells derive their name from their abundance in intestinal lamina propria and from the fact that they are absent in germ free mice. Like CD1 a, b, c, d molecules, MR1 molecules are non-polymorphic. For a long time the identity of antigens presented by MR1 molecules was unknown. Only recently it was shown that MAIT cells are reactive to vitamin B metabolites [10].  $\alpha$ -chain of MAIT cells is invariant ( $V\alpha 19-J\alpha 33$  in mice and  $V\alpha 7.2-J\alpha 33$  in humans, Figure 3 (below)).

MAIT cells constitute 1-8% of T cells in human blood and 20-45% of human hepatic T cells [11,12]. Majority of MAIT cells express NK cell receptor NK1.1 (CD161 in humans) and are double negative (for CD4 and CD8). Murine MAIT cells have a naïve phenotype, while human MAIT cells have memory phenotype[11]. Due to similarities with properties of NKT cells it is possible that human MAIT cells substituted iNKT cells in evolution from mice to humans (discussed below).



**Figure 3. MR1 proteins present Vitamin B metabolites to MAIT cells.**

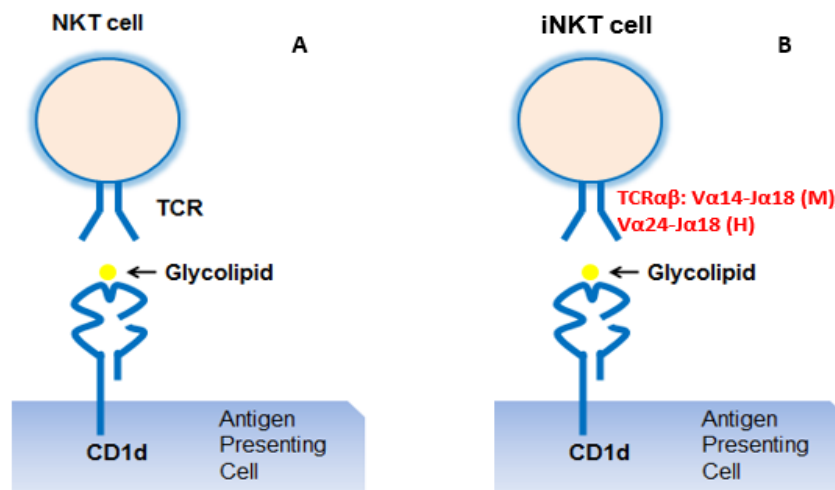
### 2.1.2 Definition of NKT and iNKT cells.

Natural killer T cells (NKT) are CD1d restricted T cells (Figure 4A (below)). There are two types of NKT cells. Type I NKT cells, defined as iNKT cells (invariant NKT, also called classical NKT cells) possess semi-invariant T cell receptors (TCRs), which recruit  $V\alpha 24-J\alpha 18$  and  $V\beta 11$  gene segments in humans and  $V\alpha 14-J\alpha 18$  and  $V\beta 8/7/2$  segments in mice (Figure 4B (below))[6]. They were combined into a separate group due to much higher frequencies of T cells expressing these particular TCRs ( $10^4$  times higher) compared to frequencies of conventional  $\alpha\beta$  T cells of any one particular TCR $\alpha$  [13]. Importantly, iNKT cell TCRs are used only by iNKT cells and not by conventional  $\alpha\beta$  T cells, that is they do not recognize peptide antigens presented by MHC class I/II molecules[14].  $\alpha$ -chain of murine iNKT cell TCRs is virtually invariant (without junctional diversity), while  $\alpha$ -chain

of human iNKT TCRs was reported to have a single amino acid substitution in 5-30% of iNKT cell clones[15]. The fact that  $\beta$ -chain in TCRs of human and murine iNKT possesses junctional diversity [16,17] (in CDR3 $\beta$  loop) and multiple (but very limited number) TCR  $\beta$ -chains ( $V\beta 8/7/2$ )[17] are used by murine iNKT led to the term “semi-invariant” iNKT.

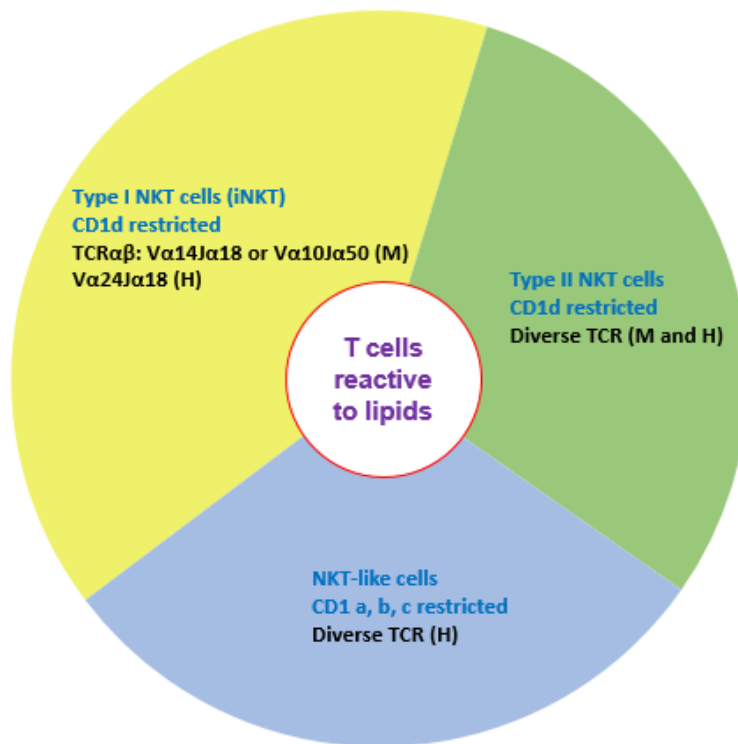
Type II NKT cells express diverse TCRs. The lack of specific markers for type II NKT cells has so far limited a clear evaluation of the frequency of these cells. Regardless, they were suggested to represent the majority of NKT cells in humans [18], but not in mice [19]. Studies on type II NKT cells showed that these cells (at least partly) represent T cell subpopulations (with identical TCRs) which are more frequent than those of traditional MHC-I/II-restricted T cells[19], but still by far less frequent than those of iNKT cells[19].

Originally NKT cells were defined as T cells expressing NK cell receptor NK1.1 (CD161), but later definition changed since large proportion of conventional T cells were also found to express NK1.1 and significant amount of NKT cells were shown to lack NK1.1. Interestingly, NK1.1 positive and (at the same time)  $\alpha$ -GalCer-CD1d-tetramer negative T cells are closer to NK cells in their genetic profile [20]. However, they remain largely unstudied. Recently one additional subset of T cells expressing  $V\alpha 10$ - $J\alpha 50$   $\alpha$ -chain was included into iNKT subset of murine NKT cells [21]. Similarly to traditional iNKT cells this subset is reactive to  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer - the most potent iNKT cell lipid antigen), expresses NK1.1 and secretes IFN- $\gamma$ , IL-4, IL-10, IL-13, IL-17 upon TCR activation. The “ $V\alpha 10$ - $J\alpha 50$ ” iNKT subset is recognized by  $\alpha$ -GalCer loaded tetramers which are used for murine iNKT cell identification. The frequency of this subset is almost 100 times lower than the frequency of iNKT cells [22]. Figure 5 (below) summarizes subtypes of T cells reactive to lipids in mice and humans.



**Figure 4. Defining of NKT and iNKT cells.**

Defining NKT (A) and iNKT (B) cells. M – mice; H – humans.



**Figure 5. T cells reactive to lipids.**

T cells reactive to glycolipid antigens. M – mice; H – humans. Note: not to scale.

## 2.2 How are iNKT cells activated?

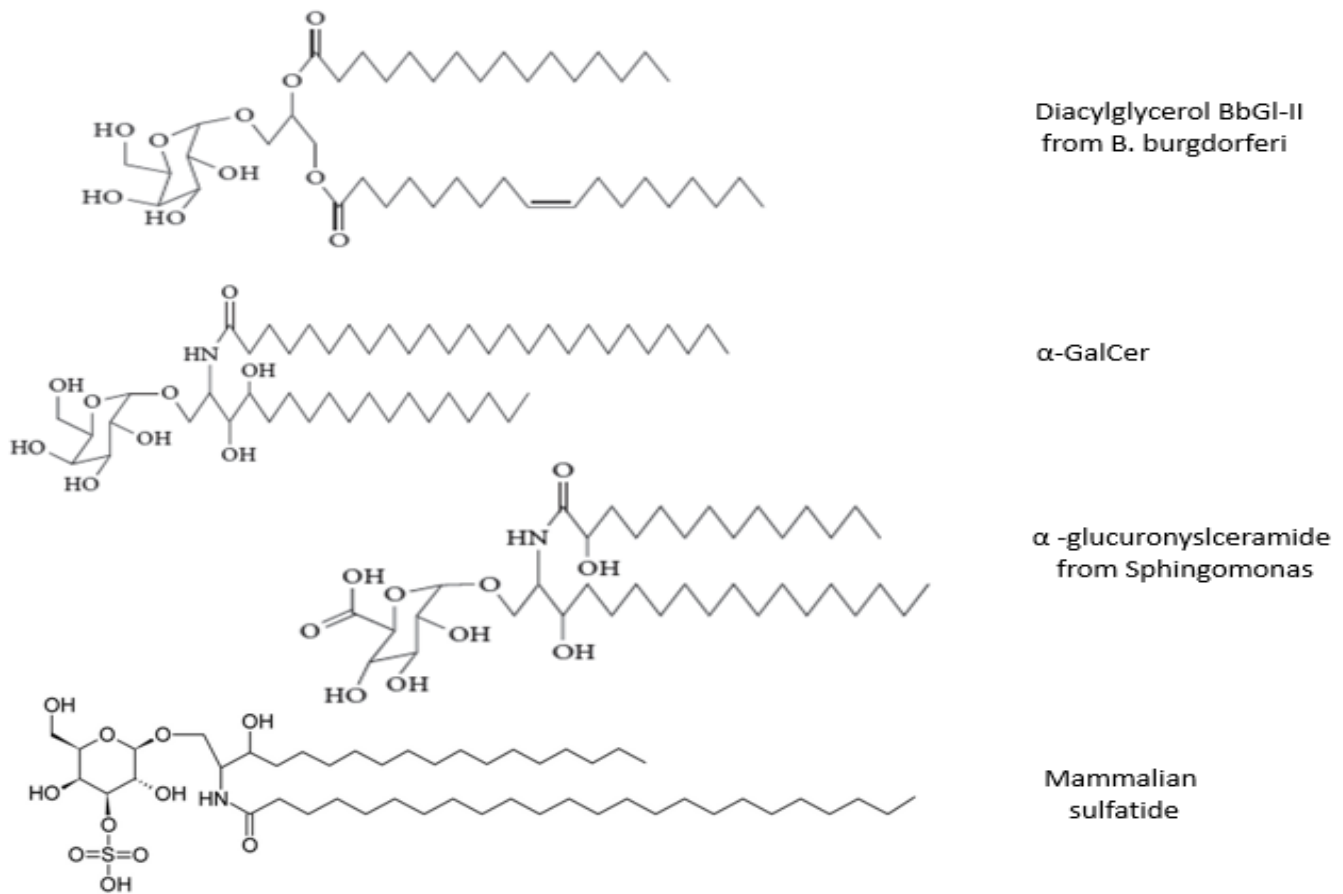
Upon activation iNKT cells produce a wide variety cytokines (Th1, Th2 and Th17 cytokines, GM-CSF, etc.) and (unlike traditional MHC I/II-restricted T cells) can produce them all at once (but at different levels, depending on iNKT subtype (discussed below))[6]. iNKT cells have been shown to be activated by several different mechanisms, which can be split into two groups: CD1d-dependent and CD1d-independent.

### 2.2.1 CD1d dependent iNKT cell activation.

CD1d dependent iNKT cell activation is mediated via interaction of iNKT TCRs with glycolipid antigens presented by CD1d expressed on the surface of APCs. Some of the glycolipid antigens can be presented by different types of CD1 family members (CD1 a, b, c, d), though with different affinities to corresponding TCRs. Sulfatide, for example, can be presented by all four CD1 family members[23]. Notably, sulfatide is not presented by CD1d to iNKT cells, but to type II NKT cells [24]. Structural features of lipid antigens which allow them to bind a particular type of CD1 molecule are under investigation, and some of them have been described in detail[25].

CD1- (a, b, c, d) presented antigens are also termed ‘lipid-linked’ antigens as (with rare exceptions, e.g. mycolic acid) they present hydrophilic head groups rather than lipid moieties. Lipid moieties instead are recruited for binding to hydrophobic binding sites within binding pockets of CD1 molecules[6]. In the case of

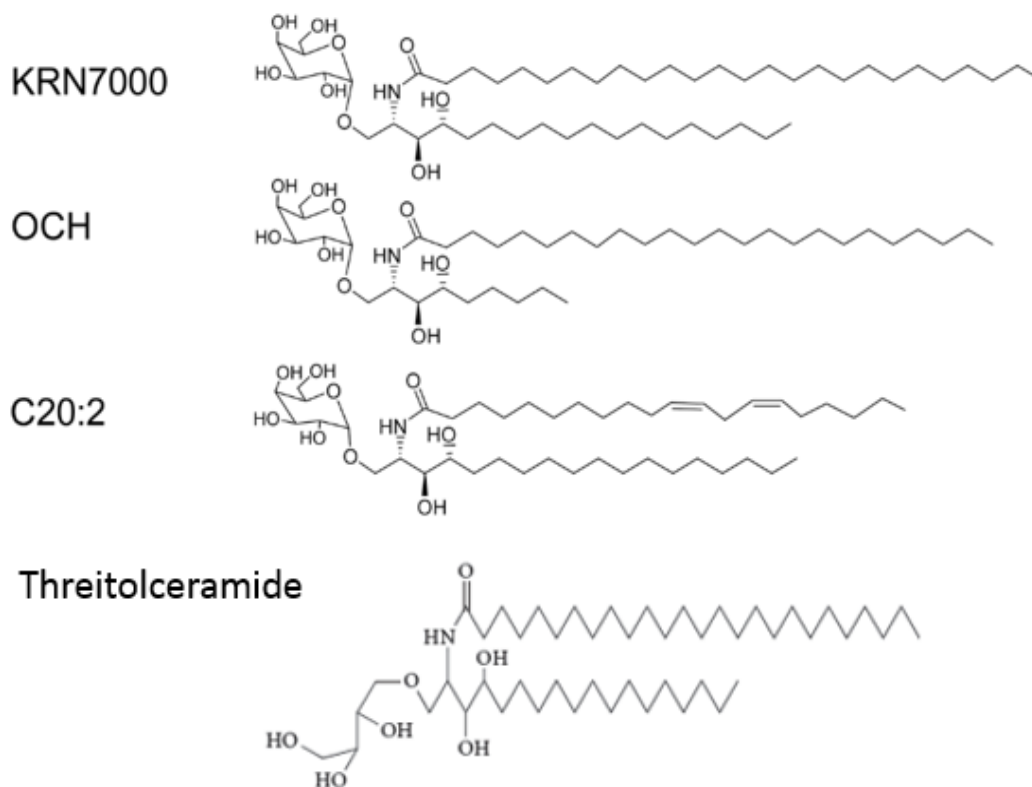
CD1d dependent presentation to iNKT in most cases the presented moiety is sugar (Figure 6 (below)). Synthetic nonglycosidic lipid antigens (such as threitolceramide) were shown to induce iNKT, but whether such compounds can be naturally synthesized is unclear [26].



**Figure 6. Lipid antigens presented by CD1d.**

Some of the lipid antigens presented by CD1d. (Modified from Cerundolo V. *et al* [27])

$\alpha$ -GalCer is the first described and the most potent iNKT cell agonist[28]. It is a synthetic glycosphingolipid. In 1995 several  $\alpha$ -linked glycosphingolipids were isolated from a marine sponge (*Agelas mauritanus*) and , based on its high potency, one of their synthetic analogs KRN7000 ((2S,3S,4R)-1-O-( $\alpha$ -D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol, herein called  $\alpha$ -GalCer) was selected as a clinical candidate to treat cancer in patients with solid tumors[29]. It is likely, that  $\alpha$ -linked glycosphingolipids isolated from a marine sponge are associated with bacterial wall of *Spingomonas* normally present in the marine sponge [30-32]. Several  $\alpha$ -GalCer analogs, which are also capable of activating NKT cells, have been synthesized. Those of them that are most studied are (2S, 3S, 4R)-1-O-( $\alpha$ -D-galactopyranosyl)-N-tetracosanoyl-2-amino nonane-1,3,4-triol (OCH)[33], C20:2  $\alpha$ -GalCer analog [34] and nonglycosidic threitolceramide [26] (Figure 7 (below)).



**Figure 7.  $\alpha$ -GalCer and its synthetic analogs.**

$\alpha$ -GalCer (KRN7000) and its most studied synthetic analogs. (Modified from Cerundolo V. *et al* [27])

Apart from glycosphingolipids derived from marine sponge *Sphingomonas* and their synthetic analogs (including  $\alpha$ -GalCer) several other bacterial lipid antigens presented by CD1d were reported to activate iNKT cells. Another interesting example of bacteria that carries glycolipid antigen that activates iNKT is *Borrelia burgdorferi*. *Borrelia burgdorferi* is the main cause of Lyme disease (affects ~ one in a thousand) which is transmitted to humans by the bite of infected ticks (most often by bite of *Ixodes scapularis* ticks)[35]. Interestingly, the glycolipid antigen from *Borrelia burgdorferi* is diacylglycerol-based (Figure 6 (above)), not ceramide-based glycolipid. It is now clear that other naturally occurring microbial lipid antigens can activate iNKT cells. Among the growing panel of bacteria which supply lipid antigens capable of stimulating iNKT cells are *Mycobacterium bovis* BCG, *Streptococcus pneumoniae*, *Helicobacter pylori*, etc.[36,37]. Notably, most bacteria, are capable of activating iNKT cells not only in CD1d-dependent but also in CD1d-independent modes via interaction with pathogen recognition receptors (discussed below).

The search for physiological lipid antigen capable to activate iNKT was a long story (mostly due to the lack of sensitivity of available analytical techniques). Isoglobotrihexosylceramide (iGb3) was a first candidate to activate iNKT [38]. Several studies confirmed that iGb3 can activate murine iNKT cells via mCD1d [39-41].

However it was later shown that mice with deleted iGb3 synthase gene exhibit no defects in either iNKT-cell repertoire or function. Most importantly, it was shown that while both human and murine iNKT cells are able to recognize iGb3 presented by murine CD1d (mCD1d), human CD1d (hCD1d), as iGb3-hCD1d, is unable to stimulate iNKT cells due to single amino acid mutation in hCD1d [42]. This illustrates that in spite high degree of conservation of CD1d-iNKT cell axis in mammals, some lipid antigens are species-selective.

In 2011 it was reported that several forms of  $\beta$ -D -glucopyranosylceramide ( $\beta$ -GlcCer) are endogenous iNKT cell antigens [43].  $\beta$ -GlcCer, as  $\alpha$ -GalCer, is a glycosylceramide (that is any glycosyl derivative of a ceramide, synonym – sphingolipids). However the same group later showed that previously published activity of  $\beta$ -GlcCer was not contained within the bulk of  $\beta$ -GlcCer molecular species, but in a minor lipid species that copurifies with  $\beta$ -GlcCer [44]. The authors proposed that  $\alpha$ -linked glycosphingolipid (not  $\beta$ -GlcCer compound) activates iNKT in their experiments. Importantly, the levels of this unknown lipid antigen are upregulated upon stimulation of APCs via pathogen recognition receptors (PRRs)[43]. The search for physiological lipid antigen capable of activating iNKT cells continued and recently it was confirmed that  $\alpha$ -linked glycosylceramides are indeed present in mice and only  $\alpha$ -linked glycosylceramides (not  $\beta$ -linked) are the main endogenous ligands of iNKT cells in mice (and probably in humans). Surprisingly, one of the isolated physiological  $\alpha$ -linked glycosylceramides turned out to be  $\alpha$ -GalCer [45]. Thus,  $\alpha$ -GalCer, previously thought to be non-physiological synthetic iNKT cell ligand, is an endogenous (and thus physiological) iNKT glycolipid ligand.

Several lysophospholipids (such as lysophosphatidylcholine (LPC), ether-bonded versions of plasmalogen lysophosphatidylethanolamine and lysophosphatidic acid) were also reported to activate iNKT cells and serve as physiological iNKT cell ligands. However, lysophospholipids were not antigenic for all iNKT cell clones (e.g. 75% of iNKT clones were antigenic for LPC) [46].

Although lipid antigens can clearly activate iNKT cells following CD1d-mediated presentation, there is evidence that NKT cells carrying TCRs with specific sequences of CDR3 $\beta$  loop can be activated simply by binding to CD1d, regardless of the nature of the lipid loaded on CD1d. Moreover, a number of lipid antigens loaded onto CD1d may decrease the affinity of this binding and thus downregulate NKT cell stimulation [47].

It is commonly assumed that presentation of antigenic glycolipid is required for iNKT cell stimulation and that the major physiological result of CD1d-dependent interactions between iNKT cells and APCs depends on the following iNKT cell proliferation and secretion of cytokines. However recent findings suggest that this may not be the case. One study shows that iNKT cells induce release of antibodies by B cells in the absence of  $\alpha$ -GalCer but in CD1d dependent manner. iNKT cells induced expression of CD40, CD69, CD83, and CD86 on B cells [48]. Furthermore, it was demonstrated that iNKT cells secrete IL-2 upon stimulation by hepatocytes in the

absence of  $\alpha$ -GalCer (exogenous) but in CD1d dependent manner [49]. Although this secretion was 5 fold lower than the one in the presence of exogenous  $\alpha$ -GalCer, it might be important physiologically. It is noteworthy that both observations are associated with cell types (B cells and hepatocytes) which (unlike other APCs such as DCs) express CD1d at high levels. It would be interesting to find out whether physiological lipids or costimulatory molecules are responsible for CD1d-dependent iNKT cell activation in the mentioned examples (in the absence of exogenous  $\alpha$ -GalCer).

### *2.2.2 CD1d-independent iNKT cell activation.*

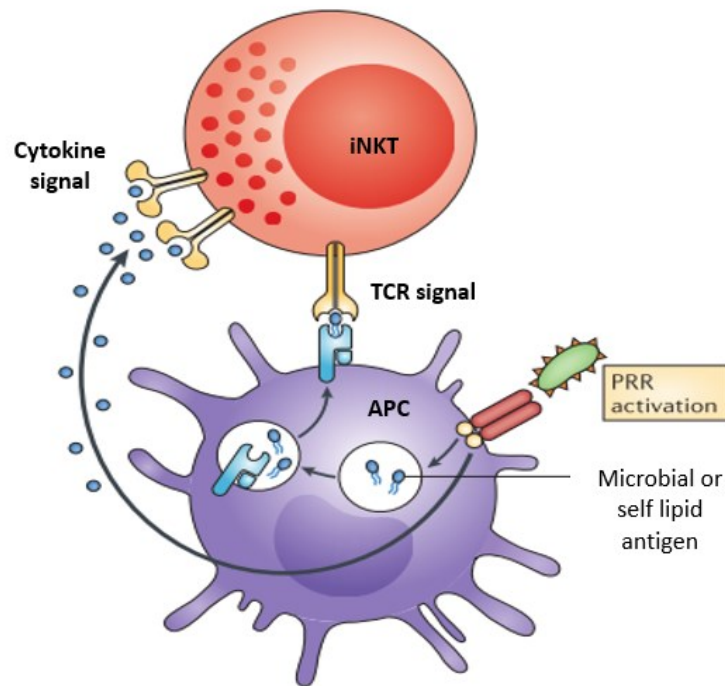
NKT cells can be activated in a CD1d-independent manner. Firstly, a combination of cytokines (interleukin (IL)-12 and IL-18) was reported to activate iNKT cells independently of CD1d-mediated antigen presentation[50]. However it should be considered whether cytokine-mediated iNKT cell activation reflects an effector response similar to that of natural killer (NK) and effector T cells. Secondly, it was reported that iNKT cells can be stimulated via interaction of T cell Ig-like mucin-like-1 (Tim-1) expressed on the surface on iNKT with phosphatidylserine, a lipid exposed by apoptotic cells[51]. This interaction promotes iNKT cell activation with a polarization toward an immunosuppressive phenotype characterized by increased IL-4 and decreased IFN- $\gamma$  production [52].

### *2.2.3 The role of costimulatory signals in iNKT cell activation.*

As in the case of traditional T cells, costimulatory signals between iNKT cells and APCs also play important role in amplifying and/or polarizing iNKT-mediated responses. CD40-CD40L interaction was shown to enhance IL-12 production by DCs, thus providing an adjuvant effect to proinflammatory NKT cell responses [53]. Homotypic interaction between signaling lymphocytic activation molecules (SLAMs) expressed on DCs and iNKT cells promotes the expression of T helper type 2 (Th2) cytokines by iNKT cells [54]. PD1-PD1L interactions were suggested to play a role in monocyte differentiation into regulatory APCs [55]. CD28:B7 costimulation releases iNKT cells from the inhibitory effect of PD1:PD1L interactions [56]. 4-1BB-4-1BBL interaction promotes iNKT cell development and survival (similar to traditional T cells of memory phenotype) [57]. One of the most studied iNKT costimulatory interactions, is OX40-OX40L signaling, as interaction between OX40 expressed by iNKT cells and OX40L expressed by plasmacytoid dendritic cells (pDCs) leads to type I IFN (such as IFN- $\alpha$ ) secretion by pDCs[58]. These and other cell surface receptor mediated interactions have been recently reviewed [59].

All these findings point to the relevance of the cell surface receptor mediated costimulatory interactions in modulating NKT cell responses. However, cytokine driven costimulation also significantly modifies iNKT cell responses. In fact it was suggested that cytokine mediated costimulation plays more important role in iNKT cell activation than in the activation of traditional T cells as in many cases cytokine driven costimulation is required

for iNKT cell activation upon recognition of CD1d presented lipid antigen by iNKT TCR[3]. It was recently reported that weak TCR stimulation with self-lipid antigens induces histone H4 acetylation at specific regions near the IFN- $\gamma$  gene loci of iNKT rendering the iNKT cells able to produce IFN- $\gamma$  in an innate manner (without concurrent TCR stimulation) upon exposure to IL-12 and IL-18. Thus TCR-mediated and cytokine driven pathways of iNKT cell activation work together for robust iNKT cell activation [60]. Relative contribution of each mechanism is probably context dependent [3] (Figure 8 (below)).



**Figure 8. Mechanisms of iNKT cell activation.**

TCR-mediated and cytokine driven pathways of iNKT cell activation. (Modified from Brennan P.J. *et al*[3]).

## 2.3 iNKT cell frequencies in mice and humans

### 2.3.1 Circulating iNKT frequencies.

In humans circulating iNKT cell frequencies (herein, unless specified, always presented as a percentage of CD3+ lymphocytes) remain relatively stable over time, but vary greatly between individuals (from undetectable to 1%, with an average of 0.14%) [61,62]. The record frequency reported is 5% [63]. Aging is associated with decreased iNKT cell frequencies [64]. In mice circulating iNKT cell frequencies are strain dependent. For most popular strains (C57BL/6 and BALB/c) the numbers vary between 0.2%-1% with average of 0.5%. Interestingly, circulating iNKT cell frequencies are very high (3-4%) in hypotonic mice BPL/J1[65].



### 2.3.2 *Where are iNKT most abundant in human and murine organs?*

In mice, iNKT cells are most abundant in the liver, where they represent 10–40% of hepatic T cells (strain dependent[66]). In humans iNKT cells are not as abundant in the liver as murine iNKT. As I already mentioned, it is possible that human MAIT cells and/or type II NKT cells substituted iNKT cells in evolution from mice to humans. Nevertheless, human iNKT cell frequencies in the liver are 5-10 fold higher than those in the human peripheral blood, representing on average 0.5% of CD3+ T cells[67].

It has been recently reported that in humans iNKT cells are most abundant in omentum, where they represent on average 10% of T cells (with frequencies as high as 70%)[68,69]. iNKT cells are also enriched in murine adipose tissue (most abundant in epididymal adipose tissue) representing 2-20% of adipose tissue T cells [70,71]. It was shown that iNKT cells are protective in diet-induced obesity. They improve insulin sensitivity[70], protect from weight gain and adipocyte hypertrophy and reverse obesity-associated metabolic disorders [69]. The protective effect of adipose tissue iNKT is associated with (and probably result of) regulatory phenotype of adipose tissue iNKT cells. Adipose tissue iNKT were shown to express much more IL-10 than iNKT from other organs [72]. However there remains significant controversy concerning these data (reviewed in [73]), as there are reports showing detrimental effects of iNKT in obesity (including weight gain, tissue inflammation, decreased insulin sensitivity and exacerbation of obesity-associated metabolic disorders)[74,75].

### 2.3.3 *iNKT cell frequencies in other organs.*

Most iNKT cell studies in humans are limited to peripheral blood. There are very few data on human iNKT cell frequencies in other organs (in addition to already mentioned reports on human hepatic and omental iNKT). They are enriched in human maternal-fetal interface (in decidua) representing 0.48% of T cells [76]. Interestingly, human bone marrow and peripheral blood iNKT cell frequencies are increased from average 0.1%-0.2% to average 1% in visceral leishmaniasis patients (the second largest parasitic killer after malaria), suggesting that *Leishmania donovani* parasite carries iNKT cell lipid ligands [77].

In mice iNKT represent 0.5–2% of T lymphocytes in thymus, spleen, bone marrow and colonic lamina propria[3,78]. Notably, murine lymph nodes (mediastinal, mesenteric, popliteal, inguinal[79] and axillary (unpublished observation)) are poor (compared even to peripheral blood) in iNKT cells (0.1–0.4% of T cells). Normal murine skin contains very few iNKT (0.03% of all skin cells), but they are increased to 0.6% in hyperplastic skin and suppress skin graft rejection [80].

Recently iNKT cells have been found in knee joints of both mice and humans. Unlike hepatic iNKT cells which are found literally in blood vessels (sinusoids) joint iNKT cells are located in extravascular tissue surrounding blood vessels of knee joints. These iNKT prevented dissemination of *Borrelia burgdorferi* into the

joints by granzyme mediated killing of the bacteria. Since fewer iNKT cells were detected in human joints (compared with murine joints) it was suggested that reduced numbers of extravascular iNKT cells in humans explain why humans, and not mice, are subjected to Lyme arthritis [81,82].

#### 2.3.4 *iNKT frequencies in human diseases.*

In humans, reduced levels and functions of circulating iNKT cells have been reported in several conditions, including cancer[83-85], obesity[69], infectious diseases[86] and autoimmune diseases such as systemic lupus erythematosus[87], rheumatoid arthritis[88], systemic sclerosis[89] and type 1 diabetes[90]. In the latter case (type 1 diabetes), however, iNKT cell numbers were also reported to be unchanged, and even increased [91]. Since methods for iNKT cell identification improved in the last few years it would be of great value to confirm previously published results with usage of two different positive markers (6B11 and anti-V $\alpha$ 24 antibodies recognize different epitopes on the TCRs of iNKT cells) and usage of a dump channel to remove 'sticky cells' for unambiguous identification [92].

### 2.4 Subsets of iNKT cells

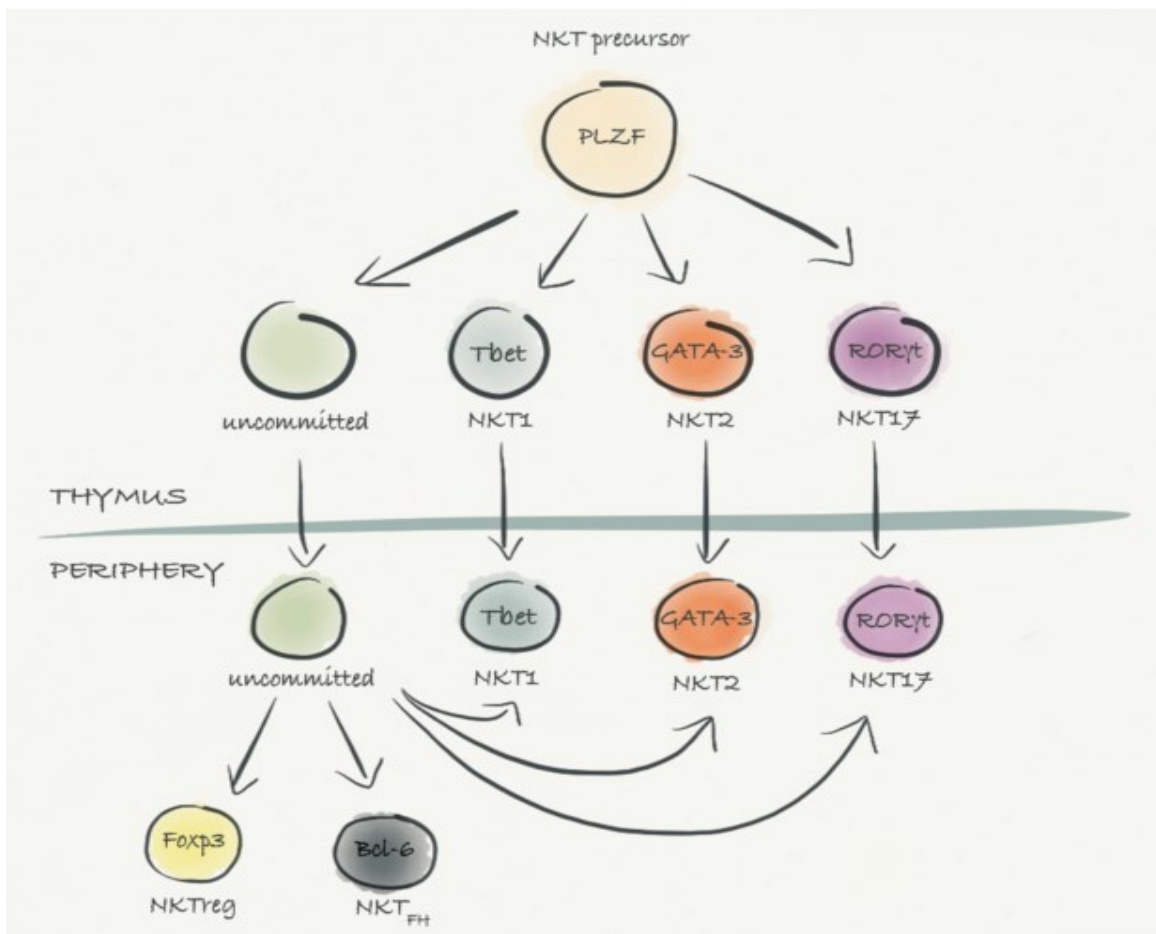
Based on its cytokine production (upon activation) iNKT cells (similar to traditional CD4+ T cells) can be divided into NKT1 (Th1-like), NKT2 (Th2-like), NKT3 (Th17-like), NKTreg (Treg-like) and NKT<sub>FH</sub> (T follicular helper - like) subsets, although the plasticity of these iNKT cell subsets has not been properly investigated. Characteristic feature of all iNKT cells which distinguish them from traditional T cells is the expression of transcription factor PLZF (promyelocytic leukemia zinc finger protein) [93]. Interestingly, MAIT cells also express PLZF [94].

iNKT cells develop in the thymus. However, unlike in the case of traditional T cells, CD1d expressing double-positive thymocytes (rather than thymic epithelial cells) mediate positive selection of iNKT [3]. The current model of iNKT cell development suggests that most iNKT cells acquire specialized effector functions already in the thymus, while some of them leave the thymus uncommitted and acquire specialized functions in peripheral microenvironment which supports acquisition of certain specialized functions (Figure 9 (below))[95].

In humans iNKT cells can be CD4+ (~50%), double negative (DN) (~40%) and rarely (<10%) CD8+. The latter (CD8+) subset is enriched in CD8 $\alpha$ + $\beta$ - cells (looks like CD8 $\alpha$  intermediate after staining with anti-CD8 $\alpha$  antibodies) [96]. Often CD8 $\alpha$ + $\beta$ - fraction is included into DN subset [97]. Murine iNKT cells are segregated only into 2 subsets (based on the expression of CD4 and CD8): CD4+ and DN.

Expression of specific cell surface receptors was associated with some of the functional iNKT cell subsets. In humans (but not in mice) DN iNKT cells preferentially produce Th1 cytokines while CD4+ iNKT cells produce both Th1 and Th2 cytokines [97,98]. But recently this type of iNKT cell segregation have been challenged,

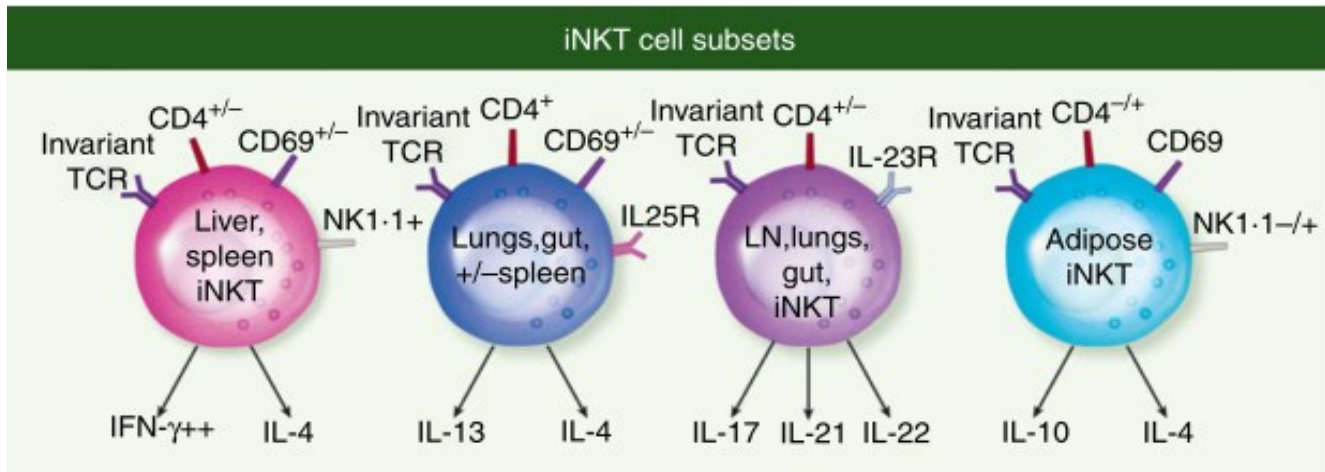
suggesting the opposite: that CD4+ iNKT cell subset is Th1 biased and DN iNKT cell subset is Th2 biased [99]. The controversy could arise due to the different type of *in vitro* iNKT cell stimulation. The model of Th2 bias of CD4+ iNKT, however, holds in a condition that is more close to physiological one: particularly, it was shown that only CD4+ human iNKT cells are able to suppress the expansion of antigen-specific cytotoxic T cells (CTLs)[100]. The fact that the generation of tolerance in several murine models of autoimmune diseases was shown to depend on CD4+ (but not DN) iNKT, also supports the model in which CD4+ iNKT are Th2-like or Treg-like[101,102] (if one assumes that murine data can be extrapolated to humans). Interestingly, it was shown that in long term Type 1 diabetes CD4+ iNKT cell fraction is decreased, although the total iNKT cell numbers are similar to that of control healthy patients (importantly two positive markers were used for iNKT identification) [103]. Lastly, CD4+ (but not DN) iNKT cells were shown to induce expansion of cells with phenotype of regulatory B cells (Bregs) [48].



**Figure 9. Transcription factors drive the acquisition of iNKT cell effector functions.**

Transcription factors which are coexpressed with PLZF during acquisition of effector functions by iNKT cells during thymic development and in the periphery (adapted from [95]).

One should remember however, that iNKT cell phenotype is also location dependent and, thus, the presence of CD4 marker expression is not enough to identify iNKT cell functional status. It is particularly evident in the case of adipose tissue iNKT [72]. Anti-tumor iNKT cell action was shown to depend only on hepatic CD4<sup>-</sup> (CD4 negative) iNKT cell subset [104]. Figure 10 (below) shows functional subsets of murine iNKT cells which are classified based on location and cell surface receptors.



**Figure 10. Functional subsets of murine iNKT cells.**

Functional subsets of murine iNKT cells which classified based on location and expression of cell surface receptors (Adapted from [105]).

## 2.5 The role of iNKT cells in immunity and disease

### 2.5.1 Immunomodulatory properties of iNKT cells.

Despite the fact that iNKT cells express TCRs, suggesting a role in adaptive immunity, iNKT cells were initially included in the innate immune arm due to their ability to rapidly (within hours) release large amounts of cytokines upon activation by  $\alpha$ -GalCer. Furthermore, NKT cells share common features with other participants of the innate immune system, NK cells, since both express NK1.1 and possess anti-tumoral activity. Later, as the field developed, iNKT cells were shown to induce the activation of cells belonging to the adaptive immune arm, thus suggesting, that components of innate and adaptive immune systems interact and that NKT cells may serve as a bridge between the systems [3,106,107].

A characteristic feature of iNKT cells which distinguishes them from traditional MHC-restricted T cells and group I CD1-restricted T cells (but not from MAIT cells) is their effector memory phenotype. They are all CD45RO<sup>+</sup> in peripheral blood in adults (there are no naïve CD45RA<sup>+</sup> NKT cells), they lack CCR7 and CD62L (lymph node homing receptors), and, most importantly (to be considered as effector cells), possess effector function (upon stimulation of TCRs with PMA and ionomycin) [108].

Homeostatic regulation of iNKT cells in humans is distinct from that in mice [61]. Homeostasis of murine iNKT cells (except murine NKT-17) depends on IL-15 [109]. Murine NKT-17 cells depend exclusively on IL-7 for their homeostasis and survival [110]. In humans, homeostasis of CD4<sup>+</sup> iNKT depends on IL-7 and homeostasis of CD4<sup>-</sup> (CD4 negative) iNKT depends on IL-15 [61]. Human CD4<sup>-</sup> iNKT cells undergo extensive peripheral expansion after their exit from the thymus (they recycle like most memory T cells), while peripheral expansion of CD4<sup>+</sup> iNKT cells is limited (they imitate naive T cells in their recycling behavior) [61].

As mentioned above, CD4<sup>+</sup> iNKT cell fraction is decreased in long term Type 1 diabetes. Since total iNKT cell frequency does not change, it means that DN iNKT cell frequency increases [103]. Similar result was obtained in our lab in the case of familial hypercholesterolemia (will be discussed below). We hypothesize that the increase in DN iNKT cells could be due to the increase in circulating IL-15 levels. Indeed elevated circulating IL-15 levels were reported in diabetes patients [111,112] and in murine models of diabetes [112] and atherosclerosis[113] (IL-15 levels in human atherosclerosis were not estimated). Of note, IL-15 is produced mainly by monocytes and dendritic cells. This is in contrast to IL-2 (another cytokine that promotes differentiation and proliferation of T cells) which is synthesized mainly by T cells. IL-2 have been shown to maintain Tregs, while IL-15 maintains CD8<sup>+</sup> memory T cells [114].

Immunomodulatory properties of iNKT cells arise from their effector phenotype. They include: (1) maturation of dendritic cells (DCs) leading to strong adaptive immunity [115], (2) noncognate and cognate help to B cells to boost antibody production[116,117]; (3) activation, expansion and polarization of T cells [6]; (4) direct cytotoxicity (killing not only tumor cells, thymocytes, activated T cells and APCs, but also bacteria, parasites and fungi) [6,82,118]; (5) differentiation and activation of APCs (e.g. iNKT promote differentiation of monocytes into DCs, an activity related to their ability to produce granulocyte-macrophage colony-stimulating factor (GM-CSF)), (6) polarization of immune responses (e.g. iNKT promote macrophage polarization into M2 phenotype [70]); (7) activation of NK cells [119]; and (8) alternative licensing of DCs for cross-presentation[120].

Despite such strong immunomodulatory properties, these properties still were not applied for therapeutic use since, depending on the experimental settings, modulation of iNKT cell responses can either lead to beneficial responses (protection against diet-induced obesity and metabolic abnormalities, amplification of anti-microbial, anti-viral, anti-tumoral responses and (paradoxically) promotion of immuno-suppression (and thus protection against autoimmunity)), or harmful responses (amplification of autoimmune responses, exacerbation of atherosclerosis and metabolic abnormalities, promotion of tumor growth).

To obtain a benefit from modulation of iNKT cell responses one should consider a more sophisticated therapy than just injections of  $\alpha$ -GalCer. To date, it is still unclear how iNKT cells are polarized in the context of

cancer, autoimmune diseases and other chronic diseases (including atherosclerosis). It is clear that the type of iNKT cell response (inflammatory or tolerogenic) may depend on costimulatory signals and on the subtype of iNKT cells considered. In addition, PRR (e.g. TLR4) engagement on DCs promotes the polarization of iNKT cells from tolerogenic to proinflammatory phenotype [53]. A similar effect is observed with lipid-enriched DCs, which are able to shift NKT cell effector function from suppressive to inflammatory, while lipid-poor DCs induce tolerogenic NKT cell responses[121].

Most studies on the role of iNKT cells in human disease address their role in cancer. Clinical studies using iNKT cell stimulation to treat cancer are ongoing. Since iNKT cell numbers are reduced in cancer investigators use (along with iNKT cell stimulation) induced pluripotent stem (iPS) cells to generate unlimited numbers of NKT cells with adjuvant activity [13].

Future therapies may include injection of artificially polarized DCs pulsed with an antigen of interest (e.g. tumor antigen or autoimmune antigen) and with  $\alpha$ -GalCer, thus, making iNKT-mediated cell therapy antigen specific.

#### *2.5.2 Dual function of iNKT cells: iNKT cells in protective immunity and in peripheral tolerance.*

The widely accepted model of thymus-independent peripheral tolerance is based on the ability of DCs (termed tolerogenic DCs) to induce T cell anergy or elimination when DCs present or cross-present peptide antigens to T cells in the absence of PRR ligation. PRR ligation results in the increased MHC mediated antigen presentation and increased costimulation signaling (CD80/CD86-CD28 and CD40-CD40L) leading to the activation of an antigen specific T cell [122]. Additionally in the absence of PRR ligation DCs are able to promote the development of adaptive regulatory T cells (aTregs, also called inducible Tregs (iTregs)). In contrast to natural Tregs (nTregs, thymus dependent) iTregs develop in extrathymic sites, such as LNs, spleen, inflamed tissue, GALT. The mechanisms of iTreg development are not well investigated. One study shows the importance of IL-10 expressing DCs (termed DC10) in type 1 regulatory T cell (Tr1) differentiation [123]. Another study showed that TGF $\beta$ -producing Bregs promote differentiation of iTregs [124]. iTregs comprise a very heterogeneous T cell population (Tr1, Th3, CD8+ Tregs,  $\gamma\delta$ T cells), can be both CD4+ and CD8+. Many of them (but not all) express Foxp3. The markers to separate iTregs (even from nTregs) are still under development [125,126]. Importantly DCs distinguish between an antigen received in PRR-ligand dependent manner from an innocuous one received in PRR-ligand independent manner on a single cell level (by segregating their cargo) [127].

However this model is not complete. It does not explain how physical form of antigen, its dose and route of administration effect the outcome of immune response (inflammatory vs tolerogenic). Importantly, it does not explain why liver allograft suppresses immune responses of other solid tissue grafts (discussed next).

An increasing amount of evidence suggests that liver plays important role in the induction of peripheral tolerance as well as in the regulation of inflammatory immune responses.

The first striking data (known as “strange English pigs” experiment) showing the participation of liver in the induction of tolerance were published in 1969. It was reported that simultaneous transplantation of kidney and liver from the same donor enhanced the acceptance of the kidney graft [128]. Afterwards it was established that antigen administration via portal vein and transplantation of liver from antigen-fed mice into antigen-naïve mice result in the induction of peripheral tolerance to the antigen [129-131]. In another experiment proving the participation of liver in peripheral tolerance Yang R. *et al.* (1994) showed that portacaval shunt results in the loss of oral tolerance [132]. In this experiment the blood flow from intestine (by portal vein) was redirected into inferior vena cava (IVC), so that it bypasses the liver and flows directly to IVC. The functionality of liver after the shunt is still maintained since blood also enters the liver by hepatic artery. In spite such a strong evidence for the contribution of liver to the establishment of peripheral tolerance still no model of peripheral tolerance which includes participation of liver was suggested.

More recent findings show that liver participates also in the generation of a regular inflammatory immune responses, particularly in the generation of memory T cells. The classical model in which after antigen-APC mediated activation in the lymph nodes (LNs) activated T cells undergo proliferation in the LNs, leave LNs and travel according to the acquired differentiation state (effector memory (EM), central memory (CM) or (more recently discovered) resident memory (RM) [133]) was called into question. It was shown that liver contains focal structures, named iMATEs (intrahepatic myeloid-cell aggregates for T cell population expansion), in which previously activated in the LNs CD8<sup>+</sup> T cells undergo second round of proliferation in the absence of antigen presentation (driven by OX40-OX40L and PRR-PAMPs costimulatory signals) [134,135].

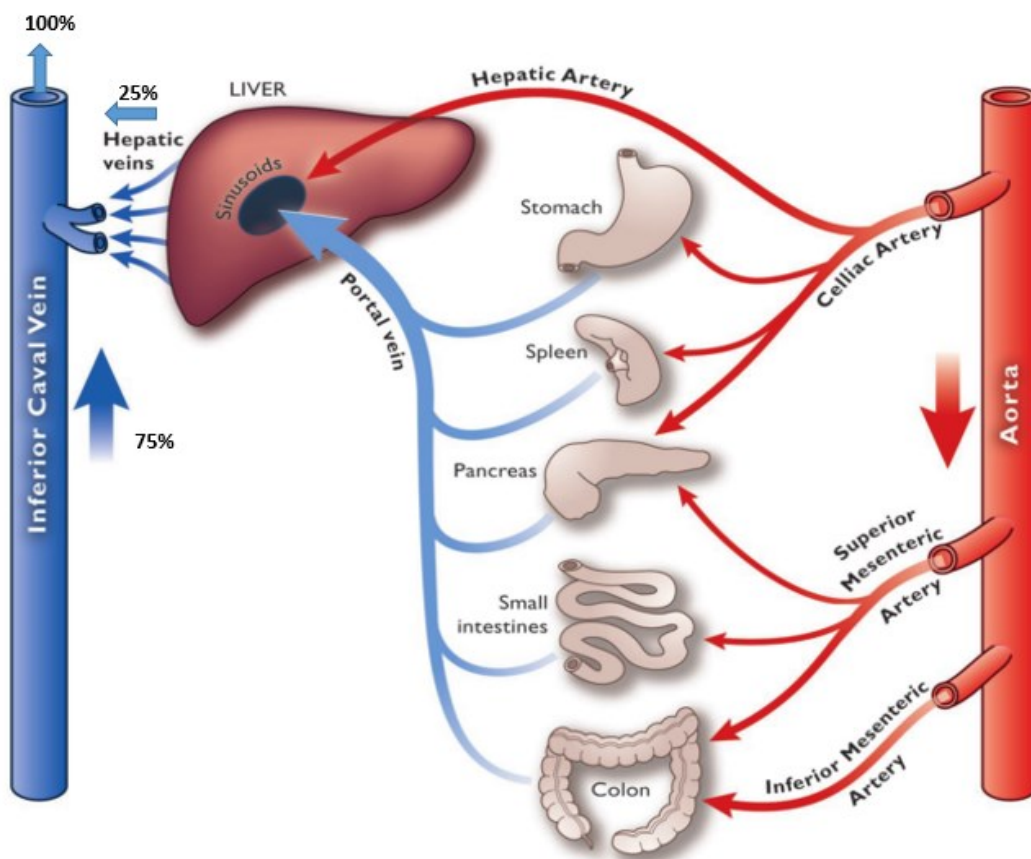
Lastly, it was established that the absence of populations of non-parenchymal cells (pDCs and iNKT cells) which are known to be enriched exclusively in the liver leads to the inhibition (or loss) of peripheral tolerance [101,102,136-139].

These data prompted me to suggest the following model of immune responses.

Most infections are ‘local’, meaning that antigens do not enter the systemic circulation, since LNs filter them [140]. Antigens are processed by local APCs (at the site of infection) and get transferred (by APCs) to the LNs or they can also travel directly to the LNs by themselves through the lymph [141]. In the LNs antigens are retained forever (in the case of ‘local’ infection) and induce activation and proliferation of naïve T cells in an antigen specific manner.

Importantly, the possibility of antigen to enter the systemic circulation (to pass through the LNs unfiltered) increases with increased dose of antigen and with increased solubility of antigen (aggregate-free, small, less complex molecules).

Activated T cells leave LNs and enter the systemic circulation. Some of them travel to non-lymphatic tissues (here I exclude liver), including the sight of infection. They mainly die with small fraction differentiating into CM, EM or RM T cells. We will return to them. Here I would like to focus on activated T cells which enter the liver. 25% of time (on every round of systemic recirculation) activated T cells diverge to splanchnic circulation and have a chance to enter the liver (Figure 11 (below)) [142]. In the liver activated T cells are retained. The mechanism of retention involves  $Ca^{2+}$  dependent LFA-1/ ICAM-1 interaction. The expression of LFA-1 is elevated on activated T cells compared to naïve T cells and ICAM-1 is constitutively expressed on sinusoidal endothelial cells and Kupffer cells. Importantly, the degree of retention is greater for CD8+ T cells (since LFA-1 expression is higher on activated CD8+ T cells compared to activated CD4+ T cells) [143,144].



**Figure 11. Diversion of aortic blood to splanchnic circulation.**

Diversion of aortic blood to splanchnic circulation (Adapted from [142]).



I speculate that two different fates (Figure 12 (below)) are possible for the liver-retained activated CD8+ T depending on whether (1) the antigen entered the systemic circulation and was cross-presented in the liver to activated CD8+ T cells or (2) not (that is if the antigen did not pass through 'LN filter' and/or was not cross-presented in the liver).

In the first case (1) the entry of activated CD8+ T cells to the liver results in their death and/or acquisition of anergic state [136,145,146]. A new mechanism of activated T cell death in the liver, termed 'suicidal emperipolesis', was recently described. It is based on the rapid destruction of activated T cells in lysosomes of hepatocytes following hepatocyte invasion [145,147]. Importantly, when antigen was cross-presented in the liver almost all activated T cells accumulated exclusively in the liver, and not in other non-lymphoid organs [145] suggesting that activated T cells which do not enter the liver die and not many of them, if any, survive to differentiate into memory T cells. Interestingly, in another work it was shown that if antigen is expressed exclusively in the liver naïve T cells specific for the antigen are also activated in the liver (and thus get retained by similar LFA-1/ICAM-1 mechanism [148]), proliferate, distribute systemically and later also die[149]. Thus naïve T cells can be activated not only in the LNs, but also in the liver and liver participates in the deletion of naïve autoreactive T cells. It is likely that these systemically distributed CD8+ T cells are CD8+ Tregs which contribute to the major mechanism of induction of tolerance (deletion of activated T cells). There is evidence showing that cross-presentation of antigen in the liver leads to the generation of iTregs in the celiac lymph nodes which collect lymph from the liver [150,151]. In the case of oral tolerance mesenteric lymph nodes (mLNs) also contribute to the generation of iTregs as their deletion results in the inhibition/loss of oral tolerance[152]. Presentation of oral antigens in mLNs by CD103+ DCs induces iTregs [153]. Thus mechanisms of deletion of activated T cells in the liver and generation of iTregs might operate together to establish peripheral tolerance.

The mechanisms of iNKT cell-mediated peripheral tolerance are unknown. One can speculate that the ability of iNKT to license APCs for cross-presentation plays a role [120]. It was shown in an *in vitro* experiment that hepatocytes can prime IL-10 producing CD8+ T cells in iNKT dependent manner (due to expression of IFN- $\beta$  by iNKT) [154]. IL-10 production in the liver itself was shown to be important for the development of peripheral tolerance [155]. Another study showed that pDC-iNKT cross-talk with DCs can lead to the generation of Tregs which promote tolerance in type 2 diabetes [156].

In the second case (2) (when antigen is not cross-presented in the liver) entry of activated T cells into the liver leads to the generation of memory T cells in iMATEs. These cells distribute systemically, help in the clearance of infection and build the defense for future antigen encounters. As already mentioned, iNKT-pDC crosstalk (via OX40/OX40L interaction) with conventional DCs leads to a more efficient memory CD8+ T cell

induction [58]. Thus both cell types (pDC and iNKT) depending on the context (presence of cross-presented antigen in the liver) contribute not only to peripheral tolerance, but also to the inflammatory immune response. It was already suggested that one has to discriminate between memory cells generated outside of liver (I promised to return to them) and memory cells generated in the liver [157]. It is an open question whether the former or latter cells play a major role in immunity.

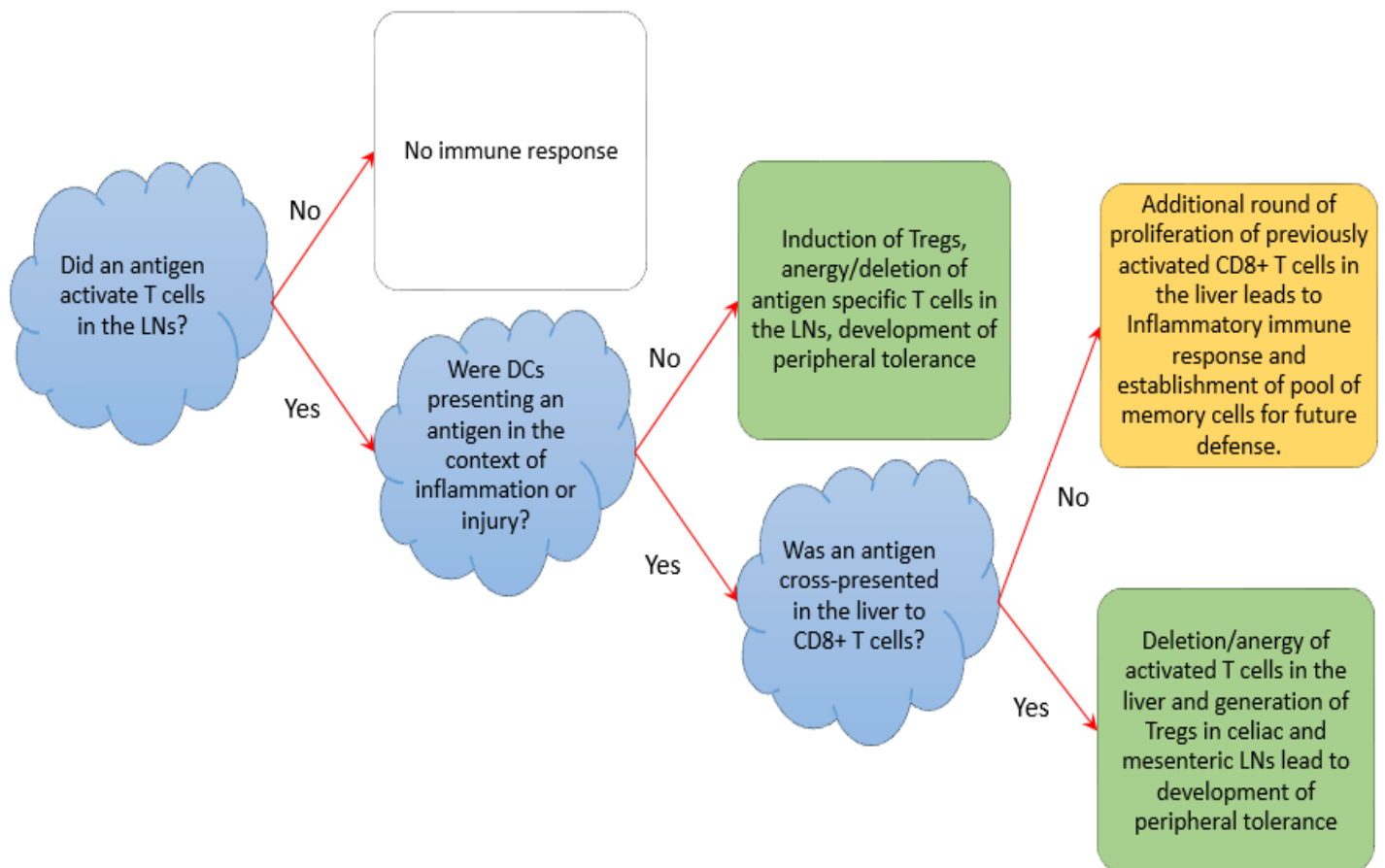
This model also explains why in the case of local infections (such as produced by sub-cutaneous or intramuscular injections) inflammatory immune responses are generated, while if antigen is delivered systemically (IV) and/or repeatedly it finally reaches the liver and gets cross-presented by liver APCs, thus leading to the induction of tolerance. In a sense liver acts as a checkpoint at which a decision is made: to promote immune response (protective or autoimmune) or peripheral tolerance.

Interesting example (although non-physiological one) of iNKT cell functioning was observed in one of the animal models of immune mediated liver injury, that is concanavalin A (Con A) mediated hepatitis. Although Con A is not an autoantigen this model adequately reflects autoimmune hepatitis [158]. Con A is a mitogenic plant lectin which can be extracted from jack beans and often used to activate T cells (both *in vitro* and *in vivo*). It binds mannose residues of different glycoproteins and glycolipids and thus activates T cells in the TCR independent manner (and thus antigen independent manner). Sublethal Con A injection (IV) into mice induces liver damage that is repaired by mechanisms of liver regeneration (liver can regenerate even if only a quarter of it is left undamaged). iNKT cells are critical in the induction of the liver damage (probably due to IFN- $\gamma$  secretion) [159]. Surprisingly, Con A pretreated mice develop tolerance against Con A rechallenge (by induction of Tregs). The induction of this tolerance however did not depend on iNKT cells [160]. This and other [137] observations show that iNKT cells are not absolutely required for establishment of peripheral tolerance. It was suggested that iNKT cells are important in the development of tolerance at low doses of antigen (by induction of Tregs) and deletion mechanisms dominate at high doses [137]. Importantly, however, if one assumes that iNKT cells act in peripheral tolerance by licensing the cross-presentation of an antigen, one should remember that this iNKT job (licensing) is only contributory, as cross-presentation can be licensed by traditional CD4<sup>+</sup> T cell mediated licensing (by TCR recognition of peptide-MHC-II complex expressed by APC). In fact MHC-II expression on DCs is upregulated in the absence of iNKT cells [139], possibly as a way to compensate for the loss of iNKT cell mediated licensing.

Lastly the described model fits well with the fact that liver is extremely vulnerable to viral infections (e.g. hepatitis virus infections) – because cross-presentation of viral antigens in the liver leads to the development of tolerance to viruses. One possible mechanism which was suggested to explain vulnerability of liver to viral

infections is immune subversion by virus-encoded proteins. However, it does not explain why viruses chose liver as their main target.

iNKT cells were shown to be protective in murine model of hepatitis B virus infection (but not in human HBV) [161]. iNKT may be beneficial in viral hepatitis by contributing to the generation of memory CTLs in the absence of cross-presented antigen. Clearly, two different responses (liver mediated tolerance and liver mediated protective immunity) fight each other. Differences in the ability of liver and LNs to cross-present may result in different types (tolerogenic vs inflammatory) of immune responses. One potential strategy to treat the hepatitis virus infections is to inhibit cross-presentation of viral antigen specifically in the liver, but increase its cross-presentation in the LNs and increase the number of OX40+ iNKT and OX40L+ pDCs in the liver.



**Figure 12. Suggested model of checkpoints regulating immune responses.**

Notably, it is unclear how liver is able to fight against pathogens if it always promotes tolerance to them. One possible explanation of this paradox is that short time pathogen presence in the liver does not result in cross-presentation of the antigens, and infection is resolved by regular protective immune response, while prolonged antigen availability favors cross-presentation and thus results in the development of tolerance. It was suggested that short-time antigen availability could result in cross-presentation by DCs favoring protective

immune response, while long term antigen availability results in cross-presentation by liver sinusoidal endothelial cells (LSECs) favoring tolerance[162].

## **2.6 Link between CD1d dependent immunity and lipid metabolism**

Since CD1-restricted lipid antigens are integral membrane components and CD1 proteins themselves are not able to extract lipids from membranes, researchers searched for proteins which transfer lipids onto CD1 proteins. Lipid transfer proteins characterized in the context of lipid metabolism were the first suspects for accomplishing this task and were confirmed to exert this function [163,164].

These include sphingolipid activator proteins (SAPs) and microsomal triglyceride transfer protein (MTP). SAPs and MTP (but not Apo E) are essential for NKT cell development [165]. SAPs, which comprise saposins A–D and GM2 activator protein, activate glycosidases which are required for sphingolipid degradation, contribute to the accessibility of endosomal/lysosomal membrane lipids to degrading enzymes [166] and promote lipid loading and unloading onto/from CD1 proteins in the endosomal/lysosomal compartments [163].

MTP, an endoplasmic reticulum resident protein involved in lipoprotein synthesis, loads endogenous lipids onto CD1 molecules in the endoplasmic reticulum, a process required for CD1 stabilization [167,168]. Impaired CD1-dependent immunity was recently described in abetalipoproteinemia (ABL) patients. ABL is a rare genetic disorder caused by mutations in the gene coding for MTP. DCs isolated from ABL patients are defective in lipid antigen presentation by all CD1 isoforms (to a lesser extent only for CD1b) [165]. Group 1 CD1 expression is considerably reduced in ABL presumably because the lack of CD1 stabilization results in their proteasomal degradation. Intriguingly, the cell surface expression of CD1d by MTP-deficient DCs was not affected in spite of the functional defect (inability to present exogenous lipid antigens, such as  $\alpha$ -GalCer). The authors speculated that MTP-dependent CD1d stabilization is required for endosomal loading of exogenous lipid antigen, but the lack of CD1d stabilization does not affect the vulnerability of CD1d to proteasomal degradation [165]. These findings highlight MTP as a unique regulator of human metabolic and immune pathways and reveal that ABL is not only a disorder of lipid metabolism but also an immune disease involving CD1.

Furthermore investigators asked how CD1-restricted lipid antigens are transported in the blood and how are they transferred inside the cell (as a part of lipoprotein particles, proteins or by themselves). ApoE, which favors lipoprotein clearance, was shown to be involved in the delivery of exogenous lipid antigen galactosyl-( $\alpha$ 1-2)-galactosyl ceramide (GGC, synthetic analog of  $\alpha$ -GalCer) for CD1d presentation via LDL receptor (LDLR) uptake [169]. This activity could be a part of the atheroprotective activities ascribed to ApoE [164]. Recent findings show that other lipoproteins and soluble proteins (e.g. fatty acid amide hydrolase (FAAH)) also play an important

role in lipid antigen transport and its delivery to APCs [170-172]. Apart from LDLR, scavenger receptors were also implicated in promoting lipid antigen delivery to APCs.

In conclusion, it is clear that CD1-dependent immunity and lipid metabolism merge via employment of the same lipid transfer proteins and lipoprotein particles. Thus, immunity and lipid metabolism were linked during evolution.

## **2.7 The CD1d-iNKT Cell Axis in Atherosclerosis**

(this chapter was modified (with critical changes and additions) from our paper[2])

A growing number of experimental observations suggest a deep connection between CD1d-dependent immunity and atherogenesis. CD1d-expressing cells (mainly APCs and vascular smooth muscle cells (VSMCs)) and NKT cells were detected in mouse and human atherosclerotic lesions [173,174]. As already discussed, CD1d-dependent immunity and lipid metabolism merge by using the same transport proteins and lipoprotein particles. Furthermore, several lysophospholipids and glycosphingolipids, known to accumulate in atherosclerotic plaques, are antigenic for NKT cell clones. Proatherogenic factors such as lysophosphatidic acid and oxidized LDL upregulate the expression of CD1d in DCs [175,176]. Lipid-related 'nature' of atherosclerosis itself suggests that there is a profound relation between CD1-dependent immunity and atherosclerosis.

Expression of CD1d by APCs is indirectly regulated by nuclear receptor peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) activation [175,176]. Intriguingly, group I CD1 family proteins are also controlled by PPAR $\gamma$  activation, but, in contrast to CD1d, their expression is downregulated upon PPAR $\gamma$  activation [175]. Of note, thioglycolate-elicited macrophages pulsed with oxidized LDL showed increased CD1d levels and induced NKT cells to produce IFN- $\gamma$ , a potentially proatherogenic Th1 cytokine [174].

The role of NKT cells in experimental atherosclerosis was addressed by two different approaches, either by manipulating NKT cell numbers (depletion or increase) or by activating the CD1d-NKT axis with NKT cell restricted lipid antigens (Table 2) [174,177-183].

### **2.7.1 NKT Cell Number Manipulation.**

The absence of CD1d is associated with impaired NKT cell development, and therefore CD1d $^{-/-}$  animals have been extensively used to characterize the role of NKT cells in atherosclerosis. Regardless of the proatherogenic background used, both LDLR $^{-/-}$  and ApoE $^{-/-}$  mice crossed with CD1d $^{-/-}$  mice resulted in double knockout animals with decreased atherosclerosis development (Table 2) [177-179]. Intriguingly, the proatherogenic effect appears to be more pronounced in the early stages of atherogenesis, but further studies are needed to address this issue[184]. Furthermore, selective iNKT cell deficiency (J $\alpha$ 18 $^{-/-}$ ) was also associated with a significant reduction in atherosclerotic lesions [180](in both genders). NKT cell adoptive transfer

experiments also confirmed the proatherogenic nature of NKT cells. Indeed, adoptive transfer of splenocytes from V $\alpha$ 14 transgenic mice (which have an increased number of NKT cells in the spleen) into LDLR $^{-/-}$ RAG $^{-/-}$  double knockout animals resulted in increased atherosclerosis in the aortic root compared to double knockout mice which received adoptive transfer of splenocytes from control mice or CD1d $^{-/-}$  mice [181]. Of note, when the effect of the adoptive transfer of specific NKT cell subsets was tested in thymectomized ApoE $^{-/-}$  mice, only the transfer of CD4 $^{+}$  NKT cells was associated with increased atherosclerosis, while that of double-negative NKT cells was not [182]. As CD4 $^{+}$  iNKT cells were shown to be biased toward a Th2 effector phenotype, these results are surprising, and a confirmation in a different experimental setting with functional thymus will be of help to fully understand the picture.

### 2.7.2 NKT Cell Stimulation by CD1d-Restricted Lipid Antigens.

iNKT cell stimulation by  $\alpha$ -GalCer in ApoE knockout mice is associated with increased atherosclerosis [174]. Furthermore, administering  $\alpha$ -GalCer to ApoE $^{-/-}$  mice with established lesions did not significantly increase the atherosclerotic lesion area, but it did decrease the collagen content [174], a feature associated with a less stable plaque. Also, the  $\alpha$ -GalCer analog OCH, which induces an anti-inflammatory Th2-biased NKT cell response [185], was reported to be proatherogenic [174]. The relevance of the proatherogenic effect of  $\alpha$ -GalCer-mediated iNKT cell induction was confirmed in ApoE $^{-/-}$ CD1d $^{-/-}$  double knockout animals in which  $\alpha$ -GalCer injection did not increase atherosclerosis [178].

In contrast with these findings, suggesting a proatherogenic role of NKT cell stimulation by CD1d-restricted lipid antigens [186,187], administration of  $\alpha$ -GalCer was associated with reduced intimal thickening and atherosclerosis in carotid arteries following perivascular injury in LDLR knockout animals but not in ApoE $^{-/-}$  mice [183]. However the approach used to induce atherosclerosis (carotid perivascular collar placement, also called carotid cuff placement) could be responsible for the observed difference. Clearly this model differs from the LDLR $^{-/-}$  (high fat diet) and ApoE $^{-/-}$  (chow or high fat diet) models. For example the absence of Rag-1, which results in the lack of T cells and B cells, is characterized by increased (4-5 fold) neointima formation in the carotid perivascular collar placement model [188]. On the contrary the lack of adaptive immunity (T and B cells) is atheroprotective in immunodeficient LDLR $^{-/-}$  and ApoE $^{-/-}$  models (in early and moderate atherosclerosis, under extreme hypercholesterolemic conditions the lack of T and B cells does not affect atherogenesis) [189]. To explain this result one could speculate that iNKT cells help to repair vascular injury by promoting the generation of appropriate memory T cells. And indeed iNKT cells were shown to promote the reparation of vascular injury [190]. On the other hand, in LDLR $^{-/-}$  (high fat diet) and ApoE $^{-/-}$  (chow or high fat diet) mice atherogenesis is associated with a loss of tolerance to lipoproteins (or minimally modified lipoproteins) [191].

Thus, in these models iNKT cells promote generation of autoimmune memory T cells leading to chronic inflammation instead of reparation of injury.

It was shown that prolonged hypercholesterolemia and high fat diet lead to decline in the ability of iNKT cells to be stimulated by  $\alpha$ -GalCer [192,193]. It is still an open question whether the decline in iNKT cell ability to be induced by  $\alpha$ -GalCer (e.g. in hypercholesterolemia) is due to inherent defect in iNKT cells themselves or due to the functional defect in APCs. Regardless of the mechanism, however, this functional loss may lead to the decline in the ability of iNKT cells to contain vascular injury during atherogenesis. Importantly it will also lead to the decline in the ability of iNKT to promote autoimmune responses. However the former property will dominate at some point. It is more important since atherogenesis can be maintained by oxidative degradation of low-density lipoprotein which activates macrophages even in the absence of T and B cells[189]. One could dampen the autoimmune responses in atherogenesis by promoting cross-presentation of autoantigens associated with atherosclerosis in the liver. But in extreme atherosclerosis downregulating autoimmune responses may not be enough. One also has to increase the egress of macrophages from the plaque [194] since, as noted before, macrophages promote vascular damage even in the absence of T cells. It is not Treg subset of T cells that promotes the reparation of vascular damage [195].

### *2.7.3 How Do These Findings Translate into Humans?*

One must be careful translating the data on iNKT cells obtained in mice to humans. The frequencies of iNKT cells in humans are lower (MAIT cells and/or NKT type II cells could substitute them). Moreover mice and humans differ in intracellular trafficking of CD1d [196].

There are very few reports on the role of CD1d-iNKT cell axis in human atherosclerosis. Circulating NKT cell levels are reduced in the peripheral blood of patients with previous cardiovascular events [173]. One interesting question is whether this reduction is a result of hyperlipidemia/atherogenesis or whether atherogenesis is the result of this reduction. It can be speculated that loss of iNKT in these patients and in aging leads to the loss of the ability to repair vascular injury and thus promote atherogenesis.

CD1d-positive cells were reported to be present mainly in neovascularized atherosclerotic lesions isolated from patients who had experienced cardiovascular events in the past (symptomatic patients), and not in the plaques from asymptomatic patients, where they are barely detected [173]. The earlier study (using different antibodies) however reported that CD1d is abundantly expressed in mice by vascular smooth muscle cells (VSMCs) surrounding the blood vessels in the intestine, colon and other organs[197]. It would be of value to confirm the absence of CD1d expression in VSMCs surrounding healthy aorta.

iNKT cells were detected in atherosclerotic plaques [173] where they constitute 1-3% of CD3+ T cells. The proportion of CD161+ T cells among the T cells in plaques has been reported to be in the similar range of 0.3–2% [198]. The role of iNKT cells in the atherosclerotic plaques, as well as mechanism of their enrichment in the plaque are unknown. One can speculate that they are recruited into neovascularized lesions in a similar way they are recruited to liver (as activated T cells). It was reported that the levels of ICAM-1 expression in the plaque correlate with the severity of atherosclerosis [199].

In vitro, a subset of CD4-positive cells expressing CD161 (mainly represented by NKT cells) was shown to promote VSMC apoptosis via FasL/Fas activation [200]; it was suggested that this mechanism could contribute to reduced plaque stability. Of note, these cells were shown to possess a Th1-biased IL-18 receptor-positive phenotype [200].

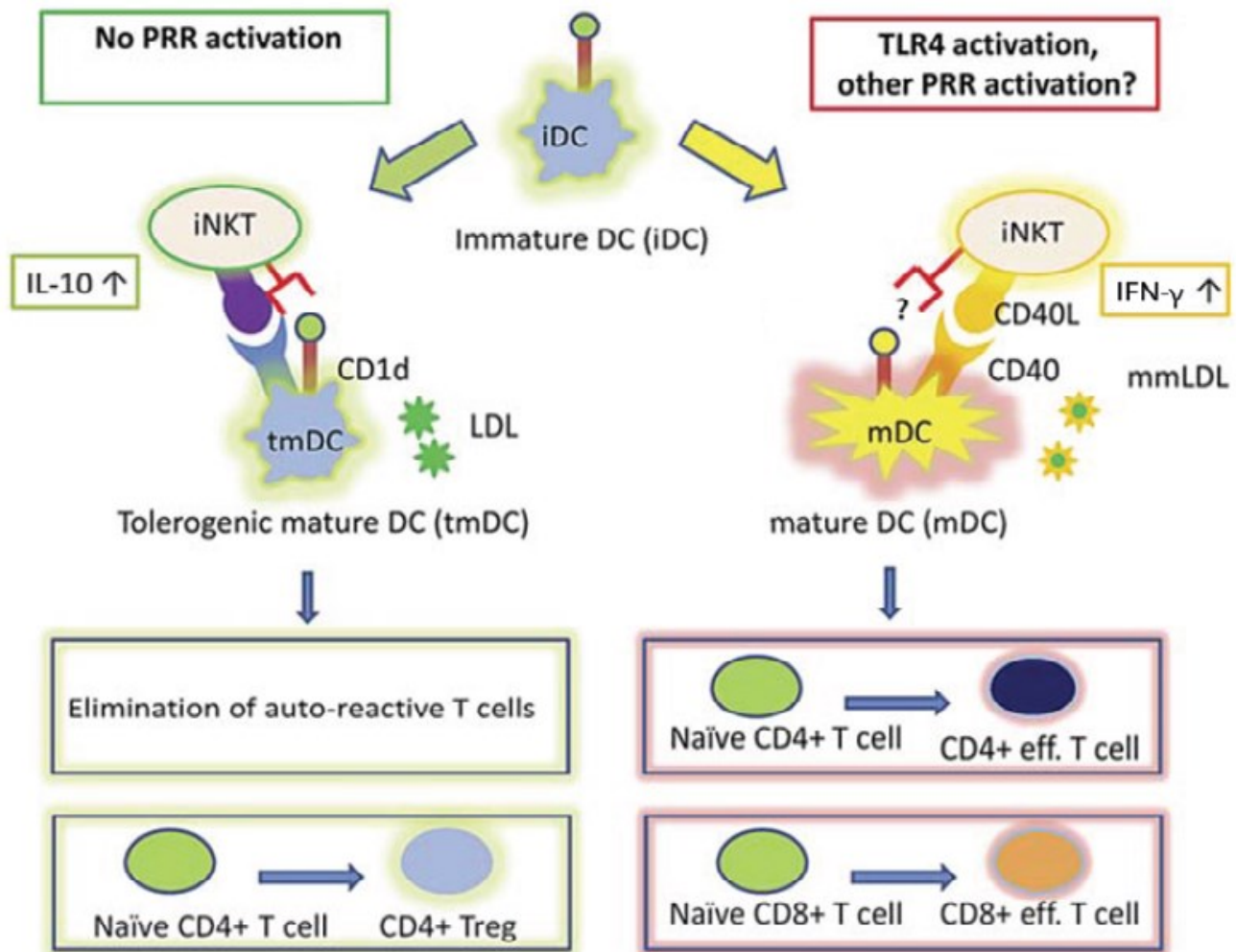
#### *2.7.4 Conclusion and Open Questions.*

Most of the available data suggest that NKT cells are proatherogenic in animal models, while data in humans on the pathophysiological role of these cells are less clear. Given the ability of iNKT cells to mature DCs depending on the presence of PRR stimulation[53], it is tempting to speculate that under physiological conditions, in the absence of pathogen recognition receptor stimulation, immature DCs could maintain the tolerance of the immune system to LDL by supporting the iNKT- mediated maturation of immature DCs into tolerogenic DCs (Figure 13 (below)). The presence of PRR activation (for instance TLR4 stimulation by bacterial products or by minimally modified LDL, which occurs during atherogenesis), followed by the increased CD1d-mediated presentation of stimulatory NKT cell ligands and increased IL-12/IL-18 levels, may polarize NKT cell responses, leading to the NKT cell-mediated maturation of immature DCs into inflammatory mature DCs (Figure 13 (below)). These inflammatory mature DCs can promote a cellular inflammatory response and can contribute to the disruption of tolerance to LDL [191].

In conclusion, several aspects have to be taken into account to fully address the role of NKT cells in atherosclerosis. Firstly, in all studies with  $\alpha$ -GalCer stimulation, soluble  $\alpha$ -GalCer was injected; this could result in iNKT cell anergy. The effect on atherosclerosis of DCs pulsed with  $\alpha$ -GalCer, which produce sustained iNKT cell expansion [201], should be addressed. Secondly, as chronic hyperlipidemia results in NKT cell anergy [192], the functional state of NKT cells in a particular experimental setting has to be analyzed in relation to the temporal window in which these cells become anergic. Thirdly, the possibility of turning the NKT cell response toward an atheroprotective one should be considered. In this context, the ability of DCs pulsed with  $\alpha$ -GalCer and LDL, and/or tolerized by IL-10, to limit the immunoinflammatory-related responses during atherosclerosis in the presence of TLR antagonists should be explored. Fourthly, the possibility of using the adjuvant properties



of  $\alpha$ -GalCer in DC-based vaccines designed to treat atherosclerosis should be explored [202]. Finally, characterizing the role of type II NKT cells in atherosclerosis will be of interest. Addressing all these aspects will improve our understanding of the biological role of NKT cells in atherosclerosis and may provide novel therapeutic targets for the immune-related aspects of atherosclerosis.



**Figure 13. The CD1d-NKT cell axis in atherosclerosis: emerging concepts.**

During atherosclerosis, in the absence of pathogen recognition receptor (PRR) stimulation (left side), immature DCs (iDC) could maintain the tolerance of the immune system to LDL by supporting the iNKT mediated maturation of immature DCs into tolerogenic mature DCs (tmDC). Tolerogenic DCs could then induce tolerance by elimination of a self-reactive T cell pool or by the polarization of naive CD4+ T cells into regulatory T cells. The presence of PRR activation [for instance TLR4 stimulation by bacterial products or by minimally modified LDL (mmLDL); right side] leads to an increase in the CD1d-mediated presentation of specific NKT-stimulatory lipids and to an increase in IL-12 expression by DCs (which is further enhanced by CD40-CD40L interaction). These immature DC-NKT cell interactions may promote the maturation of immature DCs into inflammatory mature DCs (mDC) and, thus, result in a cellular inflammatory response against LDL.

Treg = T regulatory.

**Table 2. Effect of modulation of NKT activity on lesion sizes in mice models.**

Approach	Mice model	Experimental design	Effect
Depletion or increase of NKT cell numbers	CD1d <sup>-/-</sup> vs. WT (female)	20w high-fat diet (HFD) starting at 10w of age [174].	59%↓ (AR)
	CD1d <sup>-/-</sup> LDLR <sup>-/-</sup> vs. LDLR <sup>-/-</sup> (both genders)	8 or 12w HFD starting at 5w of age [177].	= (TA)
	CD1d <sup>-/-</sup> LDLR <sup>-/-</sup> vs. LDLR <sup>-/-</sup> (both genders)	4 w HFD starting at 5w of age [177].	Male: 47↓ (TA)
			Female: = (TA)
	CD1d <sup>-/-</sup> ApoE <sup>-/-</sup> vs. ApoE <sup>-/-</sup> (female)	15w of age mice on chow diet [178].	26%↓ (AR)
	CD1d <sup>-/-</sup> ApoE <sup>-/-</sup> vs. ApoE <sup>-/-</sup> (male)	16w of age mice on chow diet [179].	68%↓ (AR)
	[CD1d <sup>-/-</sup> → LDLR <sup>-/-</sup> ] bone marrow chimeric mice (BMC) vs. [WT → LDLR <sup>-/-</sup> ] BMC mice (female)	4w after BM transplantation mice were placed on HFD for 5w [174].	39%↓ (AR)
	LDLR <sup>-/-</sup> RAG <sup>-/-</sup> recipient of CD1d <sup>-/-</sup> vs. WT B6 splenocytes (female)	After adoptive transfer of splenocytes 8-10w old recipient mice were placed on HFD for 12w [181].	= (AR) = (Thoracic aorta)
	ApoE <sup>-/-</sup> thymectomized on a third day of life (3dTx ApoE <sup>-/-</sup> ) vs. ApoE <sup>-/-</sup> subjected to sham operation (male)	8w HFD starting at 5-6w of age. In a separate experimental setup, just 1w before switching to HFD mice received (via IV injection in a tail vein) liver derived iNKT cells (CD4 <sup>+</sup> vs. DN) [182].	52%↓ (AR)
	Male 3dTx ApoE <sup>-/-</sup> recipient of CD4 <sup>+</sup> vs. DN iNKT (male)		229%↑ (AR)
	Jα18 <sup>-/-</sup> LDLR <sup>-/-</sup> vs. LDLR <sup>-/-</sup> (both genders)	8w HFD starting at 4w of age [180].	~20%↓ (AR) ~30%↓ (AA)
LDLR <sup>-/-</sup> RAG <sup>-/-</sup> recipient of Vα14tg vs. WT B6 splenocytes (female)	After adoptive transfer of splenocytes 8-10w old recipient mice were placed on HFD for 12w [181].	62%↑ (AR) = (thoracic aorta)	
NKT cell stimulation by CD1d restricted lipid antigens	ApoE <sup>-/-</sup> (female)	α-GalCer or OCH injections (IP) at 8, 10 and 12w of age, examination at 13w of age [174].	α-GalCer: 66%↑ (AR) OCH: 44%↑ (AR)
		α-GalCer injections (IP, ow) from 8w of age to 18w of age, examination 1w after last injection[174].	= (AR) = (TA)
			51%↓ (collagen)
	ApoE <sup>-/-</sup> (female)	α-GalCer injections (first IV followed by IP, tw) from 5 to 15w of age, examination 48 hours after last injection [178].	50%↑ (AR)
	ApoE <sup>-/-</sup> CD1d <sup>-/-</sup> (female)		= (AR)
	ApoE <sup>-/-</sup> (male)	α-GalCer injections (IP, tw) from 4 to 14w of age, examination 2w after the last injection[179].	100%↑ (AR)
		α-GalCer injections (IP, tw) from 4 to 14w of age, examination 10w after the last injection[179].	15%↑ (AR) 60%↑ (distal aorta)
	LDLR <sup>-/-</sup> (male)	Perivascular collars were place around both carotid arteries of 10-12w old mice. HFD was initiated 2w before the operation and continued after operation. 7w long α-GalCer injections (IV and IP (50%/50%), tw, starting after operation) [183].	84%↓ (carotid arteries)
ApoE <sup>-/-</sup> (male)	= (carotid arteries)		

↑ - increase in lesion areas; ↓ - decrease in lesion areas; = - no change in lesion areas. Abbreviations: w - week(s); tw – twice a week; ow – once a week; WT - wild type; HFD - high-fat diet; AR – aortic root; TA - total aorta; AA - aortic arch. Colors specify whether NKT cells exhibit atherogenic (red), atheroprotective (green) or neither (yellow) properties.

### 3 Specific Aims

To elucidate the role of iNKT cells in dyslipidemia I designed a project plan to meet the following aims of my PhD project:

1. Investigate whether the circulatory numbers of iNKT cell subtypes differ between patients with familial hypercholesterolemia (FH) and healthy individuals.
2. Investigate whether the function of iNKT cells, measured as the ability of iNKT cells to proliferate in peripheral blood mononuclear cells (PBMC) proliferation assay upon stimulation of with  $\alpha$ -GalCer, is impaired in FH patients compared to control individuals.
3. Analyze the expression of CD1 a,b,c,d by human PBMC.
4. Determine the effect of serum (bovine vs human) and lipoproteins on the expression of CD1 – a,b,c,d by immature human MDDCs.
5. Investigate the role of lipoproteins (LDL, VLDL, HDL) in the transport of synthetic lipid antigen  $\alpha$ -GalCer to APCs in an *in vitro* iNKT cell proliferation assay.
6. Conduct numerical and functional characterization of iNKT cells in different tissues (liver, adipose tissue, blood, spleen, lymph nodes) of mice with normal and altered lipoprotein levels (ApoE<sup>-/-</sup>, LDLR<sup>-/-</sup>, ApoA-I<sup>-/-</sup>, hApoA-I transgenic).

### 4 Materials and methods

#### 4.1 Materials

##### 4.1.1 Equipment

Beckman DU-640 Spectrophotometer  
Bio-Rad iMark Microplate absorbance reader  
BD FACSCalibur Flow Cytometry System  
ALC 4236 centrifuge  
Eppendorf centrifuge 5415 D  
Eppendorf centrifuge 5810 R  
Beckman ultracentrifuge L-60  
Beckman TL-100 ultracentrifuge  
Beckman Microfuge 18 Centrifuge  
Hermle Z400 centrifuge  
EuroClone SafeGrow 188 CO<sub>2</sub> incubator

Hanna instruments pH211 Microprocessor pH Meter

Applied Biosystems GeneAmp PCR System 9700

#### 4.1.2 Reagents

PBS-57-loaded mouse CD1d tetramers were a generous gift from the NIH Tetramer Core Facility (TCF) at Emory University (Atlanta, GA, USA).

Anti-mouse TCR $\beta$ -APC, CD19-FITC and anti-human CD4-APC, 6B11-PE, CD1d-PE antibodies were purchased from BD Biosciences. Anti-mouse CD4-APC and anti-human CD8-APC, CD3-APC, CD19-FITC, CD14-FITC, CD19-FITC were purchased from Immunotools. Anti-human CD1a, CD1b and CD1c antibodies were purchased from Ansell.

KRN 7000 ( $\alpha$ -GalCer) was purchased from Cabru (ARCORE (MB), Italy), catalog number: 11208.

The following buffers were used:

##### PBS:

Prepare 1 liter of 10X PBS by combining:

2 g KCl

80 g NaCl

2 g KH<sub>2</sub>PO<sub>4</sub>

10.488 g Na<sub>2</sub>HPO<sub>4</sub> • H<sub>2</sub>O (or 9.168 g Na<sub>2</sub>HPO<sub>4</sub> anhydrous, or 11.5 g Na<sub>2</sub>HPO<sub>4</sub> • 2H<sub>2</sub>O, or 17.3 g Na<sub>2</sub>HPO<sub>4</sub> • 7H<sub>2</sub>O).

Instead, to prepare 10X PBS, one can purchase PBS buffer (10X) powder (AppliChem, A0965,9010), weigh 95.5 g of the powder, add water up to 1 liter and mix.

Then dilute 10X PBS 10 times with water and adjust pH to 7.4

##### Staining buffer:

PBS (pH 7.4) containing 1% BSA and 0.04% Sodium Azide

##### MACS buffer:

PBS (pH 7.4) containing 2% FBS and 2mM EDTA.

##### ACK lysing buffer:

8.29 g NH<sub>4</sub>Cl (0.15 M)

1 g KHCO<sub>3</sub> (10.0 mM)

37.2 mg Na<sub>2</sub>EDTA (0.1 mM)

Add 800 ml of H<sub>2</sub>O and adjust pH to 7.2-7.4 with 1 N HCl

Add H<sub>2</sub>O to 1 liter total volume.

Filter sterilize through a 0.2- $\mu$ m filter and store at room temperature.

#### 4.1.3 *Mouse strains*

All experimental procedures were in accordance with the institutional guidelines for animal research. All mice (wild type, ApoE<sup>-/-</sup>, LDLR<sup>-/-</sup>, ApoA-I<sup>-/-</sup>, hTGApoA-I) were purchased from the Jackson Laboratories (Bar Harbour, Maine, USA). They all were on C57Bl/6J background. Additionally hTGApoA-I mice were on murine ApoA-I<sup>-/-</sup> background.

#### 4.1.4 *Patient cohorts*

Institutional Ethics Committees approved the study and informed written consent was obtained from all participating subjects. The study was conducted according to the standards of the Declaration of Helsinki and Good Clinical Practice. Blood samples from 166 subjects from two independent cohorts (FH patients and control subjects enrolled from a free-living population) were analyzed.

FH patients, attending the Lipid Clinic at the Bassini hospital, were selected based on the MEDPED score (make early diagnosis to prevent early deaths). All FH patients were under statin therapy getting close to therapeutic goals (with average value of total cholesterol 208 mg/dl, LDL cholesterol 129 mg/dl).

## 4.2 **Methods**

#### 4.2.1 *Isolation of human peripheral blood mononuclear cells (PBMC)*

1. Draw ≥ 15 ml of blood from median cubital vein of patient at the hospital and collect it into EDTA tubes for blood collection (violet caps). Mix to make sure that blood does not coagulate.
2. Transfer the blood into research laboratory. Equally distribute blood into 15 ml Falcon tubes (~ 10ml per tube) and centrifuge them for 12 minutes at 1510 g, break on (3000 rpm, swing bucket rotor ALC 5531, radius (at horizontal position) = 15 cm, ALC 4236 centrifuge).
3. Remove plasma and collect the white layer (buffy coat) into a new 15 ml tube (~2-4 ml).
4. Dilute buffy coat up to 13 ml total volume with PBS.
5. Transfer 3.5 ml of LSM (lymphocyte separation media) into a new 15 ml tube and overlay it with diluted buffy coat.
6. Centrifuge at 1600 rpm (break off) for 35 min.
7. Gently remove the top plasma layer and transfer PBMC ring into a new tube.
8. Add PBS (to improve yield use 2%FBS containing PBS for all washing steps if incubation in lipoprotein deficient conditions is not planned) up to 12 ml and centrifuge (1st wash) at 1200 rpm (break on high) for 12 min.
9. Remove supernatant. Resuspend cells in 2ml of PBS gently using 1ml pipetman (note: resuspension in small volume helps to avoid cell clumps).

10. Add PBS up to 12 ml, shake gently and centrifuge (2nd wash) again (same conditions).
11. Remove supernatant. Resuspend cells in 2ml of PBS gently using 1ml pipetman.
12. Add PBS up to 12 ml, shake gently and centrifuge (3rd wash) again, now at 1000 rpm (break on high) for 12 min (note: final washing step at lower speed helps to minimize platelet contamination).
13. Remove supernatant. Resuspend cells in 2ml of serum free RPMI-1640 or Aim V medium (1x pen/str, 1x glutamine, 1x Na pyruvate). Observe the cells under light microscope (40x). If platelets are still present at high levels perform additional washing step (note: failure to remove the platelets results in lower iNKT cell proliferation in PMBC proliferation assay).
14. Count cells with hemocytometer (HYCOR KOVA® GLASSTIC® SLIDE 10 WITH GRIDS). To count cells add 20  $\mu$ l of PBMC sample to the counting chamber, count cells in 10 random small squares at 40x magnification, determine the average number of cells per square, multiply by 90000 to obtain the cell concentration (number of cells/ml).

#### 4.2.2 *iNKT cell in vitro proliferation assay*

1. Divide PBMC samples into several 15 ml Falcon tubes if different serum conditions are used.
2. Dilute the PBMC samples appropriately (optimal final PBMC concentration for the assay is 0.5 million of PBMC per ml) in serum free RPMI-1640 or Aim V medium (1x pen/str, 1x glutamine, 1x Na pyruvate).
3. Plate cells into 24 well plate (1 ml per well).
4. Add serum to the wells if necessary.
5. Add 5ul (100U) of IL-2 (from 20U/ul stock) into all wells.
6. Add lipoproteins if necessary.
7. Make a 10x dilution of  $\alpha$ -GalCer stock solution (stock tube is 20  $\mu$ l of  $\alpha$ -GalCer solution of concentration 200 $\mu$ g/ml=200ng/ $\mu$ l in DMSO) with complete RPMI 1640 medium. 10 times diluted  $\alpha$ -GalCer solution will have concentration 20ng/ul. The stock solution (200 $\mu$ g/ml in DMSO) was prepared by heating up to 80°C and sonicating. Note: sonication has to be performed in an original glass tube (it does not work in epi tube).
8. Add 5  $\mu$ l of  $\alpha$ -GalCer (to a final concentration of 100 ng/ml) into appropriate wells.
9. Incubate plates at 37°C in CO<sub>2</sub> incubator for 10 days.
10. Continue with preparation of sample for flow cytometry analysis (described in the flow cytometry section 4.2.14).

#### 4.2.3 *Isolation of lipoproteins (LDL/VLDL/HDL) from human plasma*

We adopted a method for isolation of lipoproteins (VLDL, LDL and HDL) from human plasma described in 1955 by Havel R.J. *et al* [203]. It is based on the principle of ultracentrifugal flotation (Archimedes' principle

of buoyancy) – lipoproteins float up to the top by Archimedes' force created due to the centrifugation. Each type of lipoprotein is isolated from plasma by repeated centrifugations after progressively raising the non-protein solvent density of plasma from 1.006 g/ml to 1.019 g/ml (for VLDL isolation), to 1.063 g/ml (for LDL isolation) and finally to 1.21 g/ml (for HDL isolation) with potassium bromide (KBr). Below is the detailed protocol:

1. Draw ~30-70 ml of blood from median cubital vein of patient at the hospital and collect it into EDTA tubes for blood collection (violet caps). Mix to make sure that blood does not coagulate.
2. Transfer the blood into research laboratory. Equally distribute blood into 15 ml Falcon tubes (~ 10ml per tube) and centrifuge them for 12 minutes at 1510 g, break on (3000 rpm, swing bucket rotor ALC 5531, radius (at horizontal position) = 15 cm, ALC 4236 centrifuge).
3. Collect top plasma layer and transfer it into a single 50 ml Falcon tube (can be stored at 4°C for up to 2 days).
4. Bring the non-protein solvent density of the plasma from 1.006 g/ml to 1.019 g/ml by adding 0.03927 ml of KBr solution with density 1.35 g/ml per every milliliter of plasma. The formula for the calculation of this volume is:

$$X = \text{Volume of KBr solution of density } \rho(s) \text{ to add per 1 ml of plasma} = \frac{\rho(f) - \rho(i)}{\rho(s) - \rho(f)}$$

Where,

$\rho(f)$  – final non-protein density of the solvent

$\rho(i)$  – initial non-protein density of the solvent

$\rho(s)$  – density of added KBr solution (we use 1.35 g/ml density of KBr solution)

It is derived from conservation of mass equation:

$$\rho(i) * 1\text{ml} + \rho(s) * X = (1\text{ml} + X) * \rho(f)$$

5. Distribute plasma with corrected density into polycarbonate 13×51mm Beckman tubes (open-top, thickwall (for ultracentrifugation), Cat. Number: 349622). Add ~2-2.5 ml per tube, maximum 6 tubes per centrifugation.
6. Gently, using glass Pasteur pipette with latex bulb, overlay the mixture with small volume of KBr solution of the same density (~0.5-1cm in height). This process is called stratification.
7. Balance the tubes on the weighing machine.
8. Centrifuge tubes using TL-100 ultracentrifuge (Beckman rotor TLA 100.3) at 100000 rpm (540000g) per 2 hours.
9. Gently collect (using glass Pasteur pipette with latex bulb) and combine top VLDL layers into a single 15 ml Falcon tube (normally the total collected volume from 6 tubes is less than 2.5 ml).

10. Combine VLDL depleted solutions from 6 tubes into a single 50 ml Falcon tube and bring the density of the mixture to 1.063 g/ml by adding 0.1533ml of KBr solution (density 1.35 g/ml) per every milliliter of the mixture.
11. Distribute the mixture in to tubes, stratify it with KBr solution of the mixture density (1.063 g/ml) and balance the tubes by repeating steps 5 to 7.
12. Centrifuge as in step 8 (100000 rpm) but for 2 hours and 30 minutes.
13. Gently collect (using glass Pasteur pipette with latex bulb) and combine top LDL layers into a single 15 ml Falcon tube (normally the total collected volume from 6 tubes is less than 2.5 ml).
14. Combine VLDL/LDL depleted solutions from 6 tubes into a single 50 ml Falcon tube and bring the density of the mixture to 1.21 g/ml by adding 0.2335 g of solid KBr per every milliliter of the mixture.
15. Distribute the mixture in to tubes, stratify it with KBr solution of the mixture density (1.21 g/ml) and balance the tubes by repeating steps 5 to 7.
16. Centrifuge as in step 8 (100000 rpm) but for 4 hours.
17. Gently collect (using glass Pasteur pipette with latex bulb) and combine top HDL layers into a single 15 ml Falcon tube (normally the total collected volume from 6 tubes is less than 2.5 ml).
18. Prepare a desalting column (PD-10, Beckman, Cat. Number: 17-0851-01). If a new column is used - equilibrate it by filling it up to the top with PBS and letting the buffer to enter the column bed completely (by gravity), repeat this equilibration step 4 times. If a column was used before (washed, kept closed and filled with PBS), just let the buffer to enter the column.
19. After the buffer (PBS) entered the column bed apply VLVD sample (maximum 2.5 ml) to the column and let it enter the column bed by gravity.
20. Collect the desalted lipoprotein sample into a new 15 ml Falcon tube.
21. Wash the column 5 times with PBS by filling the column to the top with PBS and letting the buffer enter the column by gravity.
22. Add 100x EDTA solution (to 1x final concentration, 100x stock concentration is 50 mg/ml) to prevent oxidation of lipoproteins.
23. Filter the lipoprotein mixture into a sterile 1 ml Falcon tube through the 0.22  $\mu$ m sterilized filter (PES membrane).
24. Perform steps 19-23 for LDL and HDL samples.
25. Determine protein concentration of the lipoprotein samples by Lawry essay and store them at 4°C for maximum 1 week (better no longer than 2 days).



#### 4.2.4 Preparation of lipoprotein deficient serum (LPDS) from human plasma

The method for preparation of LPDS was adapted from protocol published by Goldstein J.L. *et al* [204].

The detailed protocol is provided below.

1. Measure the volume of available plasma or serum.
2. Adjust the non-protein solvent density from 1.006g/ml to 1.25g/ml with KBr (0.3981 g KBr per every ml of plasma).
3. Distribute plasma with adjusted density into polycarbonate Beckman tube (with cap, capacity 26.3 ml, 25 x 89 mm, Cat. Number 355654). Make sure there is at least 20 ml of plasma per tube. Balance the tubes with caps.
4. Centrifuge for 72 hours at 40000 rpm (4°C) using 60L ultracentrifuge (rotor type 50.2 Ti).
5. Remove the floated lipoprotein layer.
6. Transfer LPDS with a glass pipette into Spectrum dialysis bag (Cat. Number 132650, MWCO: 6000-8000 daltons, flat width 23 mm, diameter 14.6 mm, volume/length 1.7 ml/cm, length 30m).
7. Close the bag, make sure there is enough air in the closed bag (since it will expand during dialysis).
8. Dialyze for 18 hours in 5 liters of dialysis buffer:  
EDTA: 0.001%  
NaCl: 0.9%  
Tris: 0.1M, pH 7.4
9. Add thrombin (20 Units/ml).
10. Remove the precipitate with glass rod.
11. Transfer LPDS into the tube of the same type and centrifuge (same rotor, same centrifuge) at 21000 rpm for 1 hour 30 minute (4°C).
12. Transfer LPDS to a 50 ml Falcon tube.
13. Measure the volume and bring it back to the original one by adding PBS.
14. Heat inactivate LPDS (to inactivate compliment) for 30 minutes at 56°C water bath.
15. Measure the protein concentration (described in chapter 4.2.6) and make sure there is no cholesterol in LPDS by determining its presence in cholesterol essay (described in chapter 4.2.5).
16. Sterilize by passing through 0.22 µm sterilized syringe filter into sterile 50 ml Falcon tube.
17. Store frozen at -20°C.

#### 4.2.5 Cholesterol essay

1. Add 0, 1, 2, 3, 4, 5, 10, 15, 20  $\mu\text{l}$  (in duplicates) of cholesterol standard solution (200mg/dl, Sclavo Diagnostics S.P.A.) to the wells of 96 well plate.
2. Add 2 $\mu\text{L}$  of plasma (or 20-40  $\mu\text{l}$  LPDS) to the wells (also in duplicates)
3. Add 200  $\mu\text{L}$  of Cholesterol CP reagent (ABX Pentra, Cat. Number: A11A01634).
4. Incubate at 37°C for 20/30 minutes.
5. Measure the optical density at 490/495 nm using iMark Microplate absorbance reader (Bio-Rad Laboratories Inc., Cat. Number 168-1130).

#### 4.2.6 Lowry method for protein concentration determination

We adapted a method described by Lowry O.H. *et al* [205] to determine the concentration of proteins in our samples. The detailed procedure is described below.

6. Prepare solutions A , B and Folin:

##### **Solution A :**

Sodium hydroxide 0.1 N (4 g/l)

Sodium bicarbonate 2% (20 g/l, anhydrous)

##### **Solution B :**

Copper(II) sulfate 0.5%

Potassium sodium tartrate 1%

Sodium hydroxide 0.1 N (4 g/l)

##### **Folin solution:**

Mix 1:1 H<sub>2</sub>O with Folin-Ciocalteu's phenol reagent (2N, Sigma Cat. Number F9252-500ml)

7. Prepare standards and sample by transferring 0, 5, 10, 20, 30 and 40  $\mu\text{l}$  of 1mg/ml BSA stock (prepared in distilled water, 1ml aliquots, kept frozen at -20°C) and adequate volumes of samples into borosilicate Pyrex glass tubes (13×100mm, Corning Cat. Number: 99445-13). Prepare duplicates of each sample and standard.
8. Bring the volume up to 200  $\mu\text{l}$  with distilled water.
9. Add 13  $\mu\text{l}$  of 10% SDS solution (prepared in water) to every tube.
10. Mix solutions A and B in a ratio 50:1. Call it a solution C.
11. Add 1ml of solution C to all of the tubes (containing samples or standards with SDS) and incubate the tubes for 10 minutes at 37°C water bath.
12. Add 100  $\mu\text{l}$  of Folin solution to every tube and incubate the tubes for additional 20 minutes at 37°C water bath.

13. Transfer the contents of the tubes to 1.5 ml cuvettes (Kartell S.P.A., Cat. Number: 1938, Semi Micro Family) and measure the optical density at 550 nm.

14. Calculate protein concentrations based on the standard curve and dilution factors (if sample was diluted).

#### 4.2.7 *Generation of human immature monocyte derived dendritic cells (MDDCs)*

Stock solutions:

GM-CSF: 200000 Units/ml in 50ul aliquots.

IL-4: 100000 Units/ml in 50ul aliquots.

Procedure:

1. Plate PBMC in 6 well plates at a density of  $8 \times 10^6$  cells per well in 3ml complete RPMI-1640 culture medium (10% FBS) and incubate at 37° C and 5% CO<sub>2</sub> for 1 h.
2. Carefully discard media with non-adherent fraction and gently wash cells with PBS 3-5 times. Using regular binocular light microscope check for the presence of lymphocytes after washes 3 to 5. Stop washing after no more lymphocyte contamination is observed.
3. Add warm complete medium to adherent cells containing GM-CSF 300 U/ml (1.5 ul of stock/ml) final concentration and IL-4 200 U/ml (2ul of stock/ml) final concentration.
4. Incubate for 5 days.

#### 4.2.8 *Isolation of murine hepatic mononuclear cells*

The protocol for preparation of hepatic mononuclear cells was modified from protocols suggested by Blom K.G. *et al* [206] and Watarai *et al* [207].

1. To prepare anesthetics (Avertine) dissolve 300mg of 2,2,2-Tribromoethanol in 300ul of tertiary amyl alcohol by energetic vortexing (better place it on a shaker for overnight shaking, note: cover the epi tube with aluminum foil to avoid exposure to light). Add the mix to 11.7 ml of H<sub>2</sub>O in 15 ml Falcon tube and mix energetically. Cover in aluminum foil and store at 4°C.
2. Prepare tubes filled with appropriate solutions for organ collection. Label them.
  - a) For liver: 50 ml Falcon tube filled with RPMI-1640 (4% FBS).
  - b) For blood: empty epi tube and empty 15 ml Falcon tube.
  - c) For spleen: 50 ml Falcon tube with 70 µm strainer on top. At least 50 ml of PBS with 0.1% BSA and 2mM EDTA per spleen should be prepared.
  - d) For adipose tissue: epi tube containing 1 ml of PBS with 5% BSA. At least 50 ml of PBS with 5% BSA per mouse should be prepared.
  - e) For lymph nodes: epi filled with 1 ml of RPMI-1460 medium with 2% FBS.

3. Prepare syringe pre-filled with 60 $\mu$ l of 0.05M EDTA (in water, 10% of blood volume).
4. Prepare staining buffer (1% BSA, 0.04% Na Azide in PBS).
5. Collect a mouse from animal facility.
6. Anesthetize a mouse with 250-300 $\mu$ l of Avertin solution.
7. Fix forelegs upward and hind legs downward with pins and swab a mouse with 70% (vol/vol) ethanol/H<sub>2</sub>O.
8. Open the abdominal and chest cavities by single incision using tweezers and scissors. At the shoulder joints and at tail area cut laterally.
9. Draw ~ 500-600 $\mu$ l of blood from the heart using 1 ml syringe prefilled with 60 $\mu$ l EDTA and transfer it into epi tube. Make sure that the blood is mixed with anticoagulant (continue with blood in chapter 4.2.12).
10. Cut the axillary vein and artery, exsanguinate whole blood before the removal of the liver.
11. Remove liver, let it bleed for at least 30 seconds.
12. Optional (recommended): insert a 25-gauge needle into the portal vein, attach a syringe containing 5 ml of ice-cold HBSS to the needle and very slowly inject 3-5 ml of HBSS to eliminate the blood.
13. Transfer liver into 50ml Falcon tube filled with 50ml of RPMI 1640 medium (4% FBS).
14. Collect spleen directly on the top of the strainer (continue with spleen in chapter 4.2.11)
15. Collect epididymal adipose tissue (AT) into epi tube containing 1 ml of PBS with 5% BSA (continue with AT in chapter 4.2.9).
16. Collect cardiac and inguinal LNs into different epi tubes containing 1 ml of RPMI-1460 medium with 2% FBS (continue with LNs in chapter 4.2.10).
17. Transfer the content of 50 ml Falcon tube (containing liver) onto 10ml petri dish. Mince the organ into small pieces with surgical scissors and force the pieces gently through a 200  $\mu$ m-gauge stainless steel mesh using a sterile syringe plunger. Transfer mechanically disrupted mix back into 50 ml Falcon tube.
18. Centrifuge the suspension at 620 rpm (60 g) with the off-brake setting for 1 min at room temperature (RT).
19. Transfer the resulting supernatant (45 ml) containing intrahepatic immune cells (IHIC) to a new tube and centrifuge at 1720 rpm (480 g) with the high-brake setting for 8 min at RT.
20. Resuspend the pellet in 10 ml of 37.5% Percoll solution (37.5% Percoll, 3.75% of 10x PBS and 58.75% of RPMI 1640 medium, all %s are by volume).
21. Centrifuge at 850 g (2270 rpm) with the off-brake setting for 30 min at RT.

22. Resuspend the pellet in 2 ml of ACK lysis buffer, incubate at RT for 3 min, then supplement with 1 ml FBS and with RPMI-1640 medium up to 10 ml total volume.
23. Centrifuge 480 g (1720 rpm) with the high-brake setting for 8 min at RT.
24. Remove supernatant and resuspend the pellet in ~ 300-600ul of staining buffer, continue with staining for flow cytometry analysis (described in chapter 4.2.14).

#### 4.2.9 Isolation of lymphocytes from murine epididymal adipose tissue

18. Cut epididymal AT from the mouse and collect into epi tube containing 1 ml of PBS with 5% BSA.
19. Transfer fat into the well of 6 well plate (or 35 mm petri dish). Add 1 ml of PBS with 5% BSA for a total volume of 2 ml.
20. Add 20 µl of Calcium Chloride stock (500mM, 100x, prepared in dH<sub>2</sub>O).
21. Add 20 µl of Collagenase II stock (200mg/ml, prepared in PBS).
22. Incubate for 20 minutes at 37°C with agitation.
23. Agitate by pipetting up and down, then pass through 70 µm strainer using plunger from 5ml syringe and collect in 50 ml Falcon tube by pouring PBS + 5% BSA through the strainer up to a total volume of 20 ml. Centrifuge at 1600 rpm for 5 minutes, break on.
24. ACK the pellet for 5 minutes with 2 ml of ACK.
25. Add up to 25 ml of PBS +5% BSA.
26. Centrifuge at 1600 rpm for 5 minutes, break on.
27. Resuspend the pellet in 200 µl of staining buffer for staining and flow cytometry analysis (described in chapter 4.2.14).

#### 4.2.10 Isolation of lymphocytes from murine lymph nodes

1. Collect lymph nodes (each type separately) into 1 ml of RPMI-1640 medium (2% FBS). Cut the LNs with small scissors.
2. Transfer LN mixture (1 ml) into the well of 6 well plate (or 35 mm petri dish). Add 1 additional ml of RPMI-1640 medium to a total volume of 2 ml.
3. Add 20 µl of Calcium Chloride stock (500mM, 100x, prepared in dH<sub>2</sub>O).
4. Add 20 µl of Collagenase II stock (200mg/ml, prepared in PBS).
5. Incubate for 20 minutes at 37°C with agitation, pipette to complete the digestion.
6. Collect into epi tube and spin at 1500 rpm in microcentrifuge for 5 minutes.
7. Resuspend in 1 ml of RPMI-1640 (20% FBS), transfer to a well of 24 well plate and incubate for 1 hour at 37°C, 5% CO<sub>2</sub>.

8. Collect cells into the epi tube. Centrifuge at 1500 rpm for 5 minutes.
9. Resuspend in 1 ml of staining buffer.
10. Count cells and stain ~ 3 million of cells in 100  $\mu$ l volume (staining and flow cytometry is discussed in chapter 4.2.14).

#### 4.2.11 Isolation of murine splenocytes

For isolation of splenocytes always use PBS containing 0.1% BSA and 2 mM EDTA. Preheat it to RT.

1. Place a cell strainer in the 50 ml Falcon tube. Transfer the spleen and 1 ml PBS directly into the 70 $\mu$ m cell strainer. Remove the plunger from a 5 ml syringe and use the black rubber end to mash the spleen. Release the splenocytes into the tube. Use grinding circular movements to homogenize the tissue. Rinse the filter at regular intervals with PBS. Continue to mash the spleen until all that remains is the white connective tissue of the outer membrane. Make up to the full volume of the tube with PBS (total 30 ml). Note: this has to be done immediately after removing the spleen from the mouse.
2. Centrifuge at 400  $\times$  g (1600rpm, 15cm radius, a swing bucket rotor, break off) for 10 minutes at room temperature and aspirate supernatant. The resulting cell pellet should be red in color. The supernatant may appear cloudy, due to smaller particles that cannot be centrifuged at this setting.
3. Resuspend the pellet in 5 ml of ACK Lysis Buffer. Incubate at room temperature for 5 minutes with occasional shaking. Stop the reaction by diluting the Lysis Buffer with 30 ml of PBS.
4. Spin the cells (400xg, 10 min, break off), carefully remove the supernatant and resuspend the pellet in 2 ml of the staining buffer (1% BSA, 0.04% Sodium Azide solution in PBS).
5. Count the cells using a hemacytometer.
6. Dilute with staining buffer to stain 1 mln of cells in 100ul.

#### 4.2.12 Isolation of murine PBMC

1. Transfer 450-700ul of blood into a fresh epi tube. Transfer 450  $\mu$ l of blood into 15 ml Falcon tube. Add 2.5 ml of ACK lysis buffer and incubate for 10 minutes at RT (the sample becomes transparent).
2. Stop the lysis by addition of 1 ml of FBS and RPMI 1640 medium up to total volume of 10 ml.
3. Centrifuge 8 min at 480g (1700 rpm on our centrifuge, radius of rotor is 15 cm), break on.
4. Remove the supernatant and resuspend the pellet in ~ 250  $\mu$ l of staining buffer (1% BSA, 0.04% Na Azide in PBS).
5. Divide the content of the tube into 2 epi tubes (100ul each). The sample is ready for staining and flow cytometry analysis (described at chapter 4.2.14).

#### 4.2.13 Experimental treatment of mice

All experimental procedures were in accordance with the institutional guidelines for animal research. Mice were fed chow diet and were sacrificed at 6 months of age with an injection of Avertin 2,5% (Sigma-Aldrich Co., USA). All  $\alpha$ -GalCer injections were administered intraperitoneally 3 days before the mice were sacrificed. Total 4 $\mu$ g of  $\alpha$ -GalCer was injected in a 200  $\mu$ l volume. To prepare  $\alpha$ -GalCer solution for injection  $\alpha$ -GalCer powder (250  $\mu$ g) in a glass tube was diluted with 1.25 ml of physiological solution (0.9% NaCl) containing 0.5% polysorbate (Tween 20) to a concentration 200  $\mu$ g/ml with heating up to 80-85°C and sonicating. The heating to 80-85°C should continue until the solution turns cloudy. This is ok. Bring it to RT and it will become clear quickly. Note: sonication is not necessary if solution is clear but if performed, sonication has to be performed in an original glass vial (it does not work in plastic epi tube!). This stock solution was aliquoted in 60  $\mu$ l aliquots. On a day of injection an aliquot was diluted in an epi tube 10 times with physiological solution (0.9% NaCl) containing 0.5% polysorbate (Tween 20). Incubate the epi at 55°C for 5 minutes. Solution (concentration of  $\alpha$ -GalCer - 20 $\mu$ g/ml) is ready for injection. Inject intraperitoneally using the “two-man” method[208].

#### 4.2.14 Flow cytometry

**Whole blood** from each subject was collected in EDTA anticoagulated vacutainer tube (with violet cap). Samples were stained and fixed within the day of collection. One channel (FITC) was used as a dump channel to exclude nonspecific staining (CD19-FITC (Immunotools), CD14-FITC (Immunotools)). Three separate stainings were performed for each sample to analyze CD3+ (APC, Immunotools), CD4+ (APC, BD) and CD8+ (APC, Immunotools) iNKT cells respectively. iNKT were identified by 6B11-PE antibodies (BD).

Same staining strategy was used to stain **PBMC**. The following procedure was used to prepare PBMC samples for staining:

1. After 8-10 days of incubation gently remove the cells from each well (24 well plate format) into 1.5 ml eppendorf tube by pipetting them up and down off the bottom of the well (they detach easily).
2. Centrifuge eppies at 1000 rpm for 10 minutes in microcentrifuge. Remove the supernatant.
3. Resuspend cells gently by pipetting up/down with P200 pipetman (25 times on day 8-10, 10 times on day 0) in 100  $\mu$ l of staining buffer (1% BSA, 0.04% Sodium Azide solution in PBS). Proper pipetting is used to disrupt iNKT colonies into single cells. Incubate 15 min at RT to allow proper blocking (by BSA).
4. Add antibodies. Incubate in the dark (RT) for 40 min. Add 600  $\mu$ l of BD lysis buffer (or MACS buffer) to each tube, transfer the contents from each tube into FACS tubes and analyze by flow cytometry.

For staining **murine mononuclear cells** the following staining strategy was used:

1. Distribute cells resuspended in staining buffer into epi tubes (100  $\mu$ l for each staining).
2. Block non-specific staining by adding FC-block (1  $\mu$ l) to 100  $\mu$ l of cells resuspended in staining buffer (4°C, 30 minutes).
3. Centrifuge loaded and unloaded NIH CD1d-tetramers (PE) briefly in a microcentrifuge. Add 0.8  $\mu$ l of CD1d tetramer to the sample and incubate for 30 min at 4°C.
4. Add 1  $\mu$ l of anti-CD19-FITC and 1  $\mu$ l of anti-TCRb-APC antibodies and incubate for 30 min at 4°C.
5. Add 600  $\mu$ l of BD lysis buffer (or MACS buffer) and analyze by flow cytometry. Note: all murine samples should be analyzed on the day of staining. Keep at 4°C before the analysis. Human samples, if fixed by BD lysing/fixing solution, can be analyzed at the 48 hour period.

#### *4.2.15 Statistical analysis*

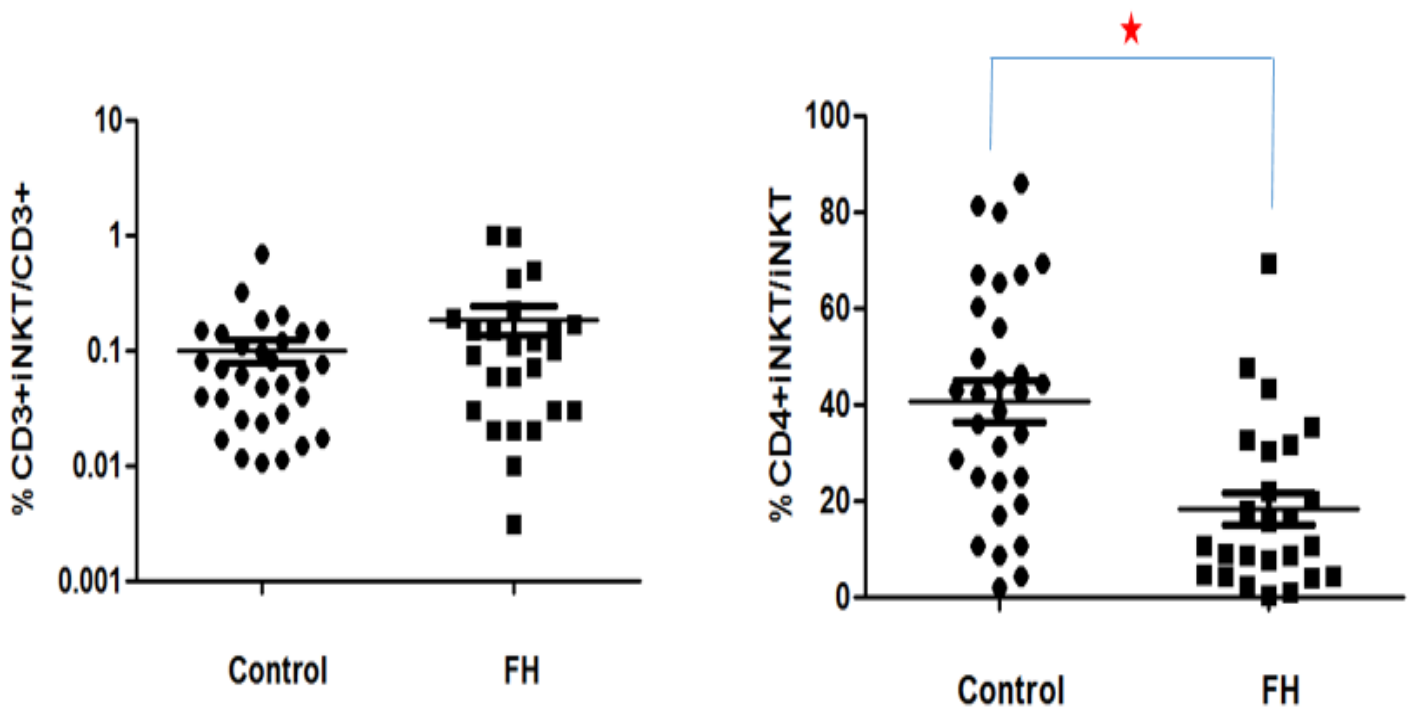
iNKT cell numbers and proliferation indices were compared between control and FH patients using Student's t-test or Mann-Whitney U-test. Correlation between the percentage of total iNKT cells and the CD4+iNKT/total iNKT ratio was determined by Pearson's correlation coefficient and Spearman correlation coefficient. As iNKT cell distribution is skewed, when necessary, the percentages of iNKT cells were log-transformed for the purpose of analysis. P value <0.05 was considered statistically significant. We performed statistical analyses using SPSS software (Released in 2011, IBM SPSS Statistics for Windows, version 20.0, IBM Corp., Armonk, NY, USA) and GraphPad Prism software (Released in 2007, GraphPad Prism version 5.00 for Windows, GraphPad Software Inc., San Diego, CA, USA).



## 5 Results

### 5.1 Numerical characterization of iNKT cell subsets in patients with Familial Hypercholesterolemia

Familial Hypercholesterolemia (FH) is a dominantly inherited genetic disorder characterized by high levels of low-density lipoprotein (LDL) and early cardiovascular disease (occurring with frequency 1 in 500 people in the case of heterozygous FH; and 1 in a million in the case of homozygous FH)[209]. FH patients, attending the Lipid Clinic at the Bassini hospital, were selected based on the MEDPED score (make early diagnosis to prevent early deaths, developed by the US MedPed Program, based on cut points for total cholesterol concentrations). Rare mutations of LDLR, ApoB or PCSK9 were detected in 60% of patients. All patients were under statin therapy getting close to therapeutic goals (with average value of total cholesterol 208 mg/dl, LDL cholesterol 129 mg/dl).



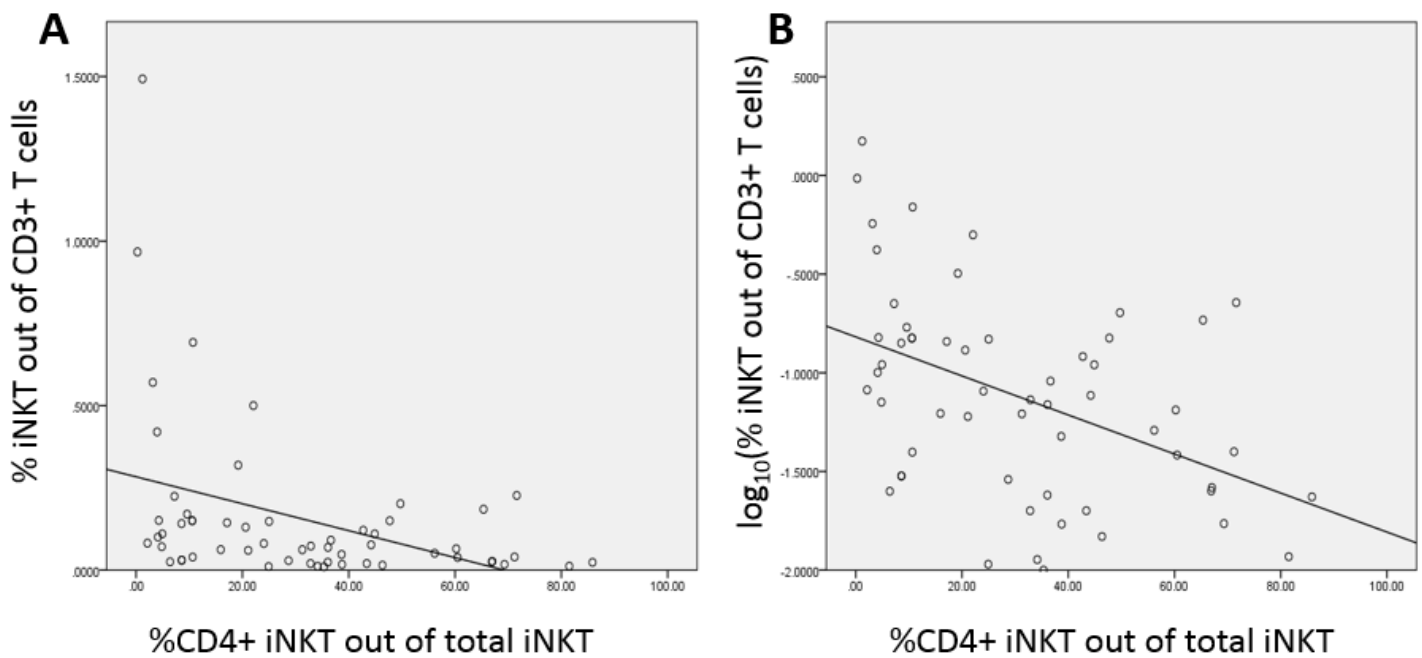
**Figure 14. Characterization of iNKT cells and CD4+ iNKT cell subset in patients with FH.**

FH patients exhibit a significant decrease (control patients:  $40.8 \pm 4.2$  ( $\pm$ SEM), N=31; FH patients:  $19.2 \pm 3.5$  ( $\pm$ SEM); N=26; \*P<0.01 (T test, statistical power=94.8%), P<0.001 (MW test)) of CD4 positive iNKT compared to age and sex matched controls, while the difference in total iNKT cell frequencies between the groups did not reach statistical significance (control patients:  $0.0984 \pm 0.0235$  ( $\pm$ SEM), N=31; FH patients:  $0.2261 \pm 0.0663$  ( $\pm$ SEM); N=26; P=0.079 (T test with Welch's correction, statistical power: 56.8%), P=0.074 (MW test)).

The percentages of circulating invariant NKT (iNKT) cells (out of CD3+ cells) were investigated in 26 FH patients and 31 age/sex matched control individuals by FACS analysis of the whole blood samples. iNKT cells

were detected by 6B11 (which mark the invariant chain of the iNKT TCR) and anti-CD3 antibodies. CD4+ iNKT and CD4- iNKT subsets were further analyzed. We report that FH patients present a significant decreased percentage of CD4 positive iNKT ( $40.8 \pm 4.2$  versus  $19.2 \pm 3.5$  ( $\pm$ SEM),  $P < 0.01$ , T test) compared to age and sex matched controls, while the total iNKT cell frequencies are similar between FH patients and controls (Figure 14 (above)).

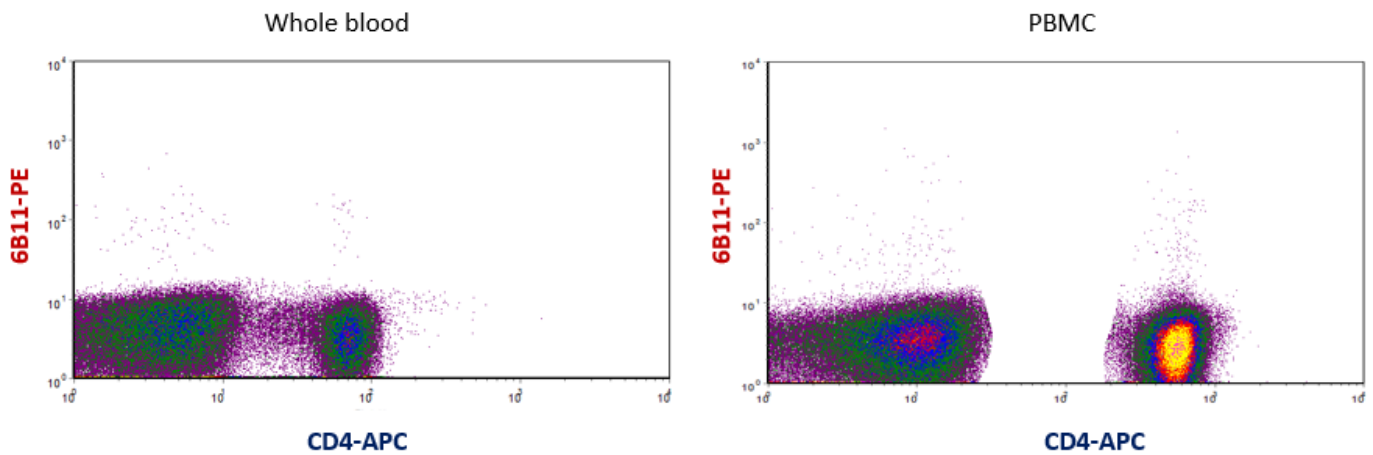
Another interesting observation obtained from this study is the negative correlation (Figure 15 (below)) between the percentage of total iNKT cells (out of lymphocytes) and the percentage of CD4+ iNKT cells out of iNKT cells, suggesting that high iNKT cells frequencies result from the expansion of double negative (DN) iNKT cells.



**Figure 15. Negative correlation between total iNKT and CD4+iNKT/total-iNKT ratio.**

**A:** Pearson correlation coefficient = - 0.38; P(2-tailed) = 0.004; Spearman correlation= -0.447; P(2-tailed) < 0.001; N=57; R<sup>2</sup>=0.144. **B:** Pearson correlation coefficient = - 0.453; P(2-tailed) < 0.001; Spearman correlation= -0.447; P(2-tailed) < 0.001; N=57; R<sup>2</sup>=0.205.

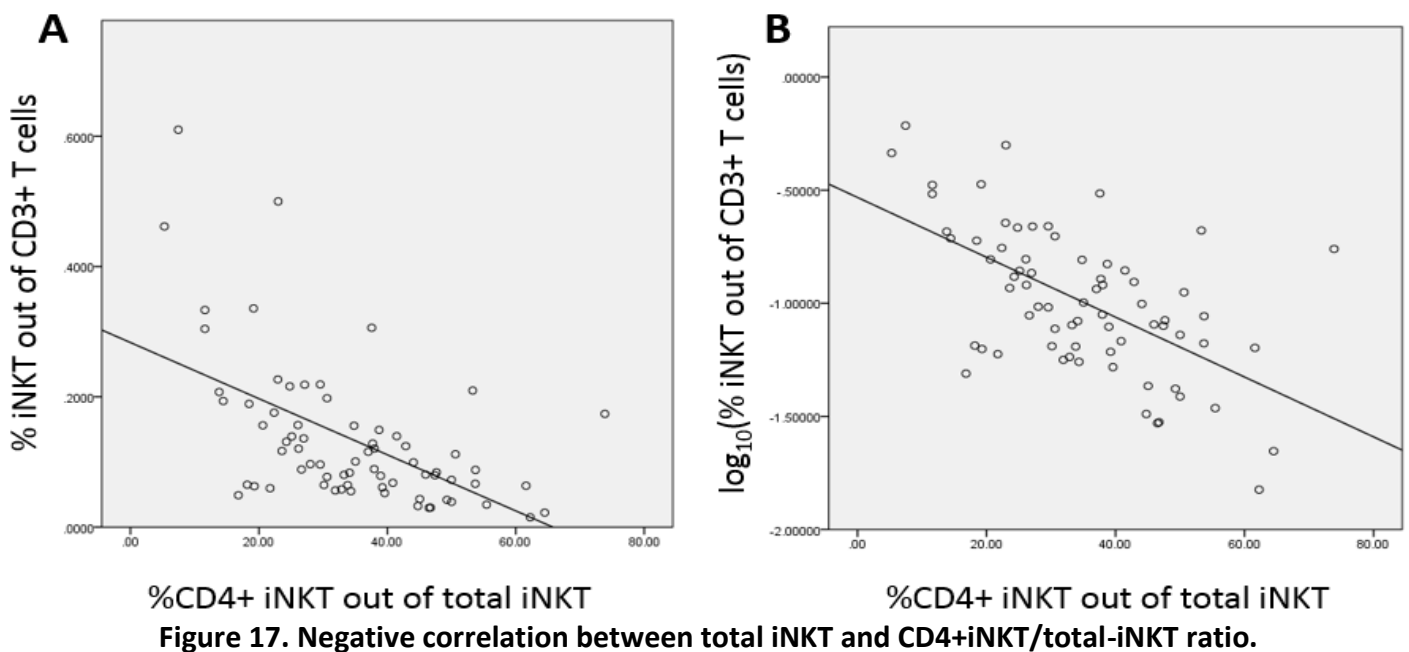
Of note, CD4+ iNKT cell numbers determined from the whole blood samples were more than two times lower than the ones determined from samples prepared from isolated PBMCs (0.038% vs 0.08%). It is likely that procedure of isolating PBMC results in an increased non-specific staining which is critical when performing flow cytometry with rare cell types (< 0.1% of T cells). Figure 16 (below) depicts color dot plot analyses of samples prepared from whole blood and PBMC. Clearly, there is less non-specific staining in the whole blood sample staining (there is more defined population of CD4+ iNKT cells in the whole blood staining).



**Figure 16. iNKT cell flow cytometry analyses from samples prepared from whole blood and from PBMC.**

Gated on lymphocytes from which 'sticky' cells were dumped.

Non-specific staining can be neglected when working with less rare cell frequencies ( $> 0.15\%$ ). The analysis of data obtained from PBMC samples also showed negative correlation between iNKT cell frequencies (this time out of all lymphocytes) and percentage of CD4+iNKT out of total iNKT (Figure 17) since the correlation mainly results from high iNKT frequency data points.



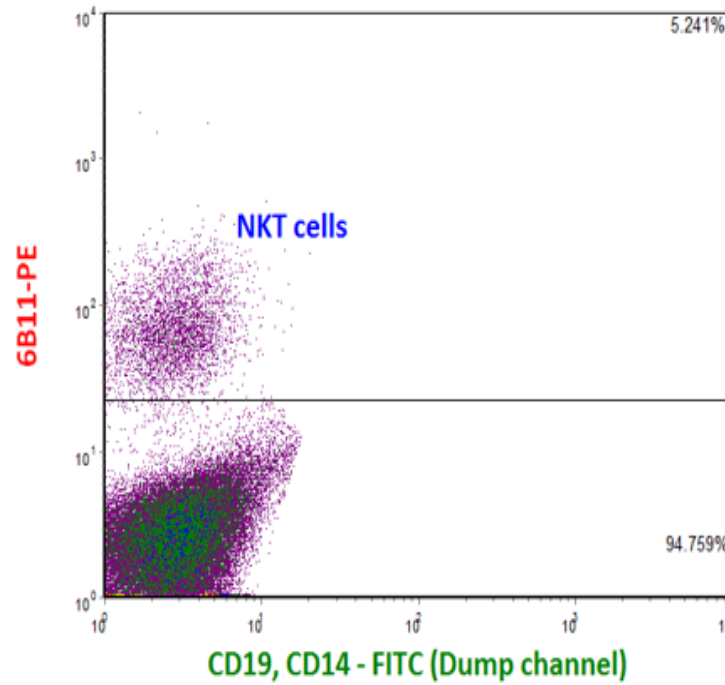
**A**

**B**

**Figure 17. Negative correlation between total iNKT and CD4+iNKT/total-iNKT ratio.**

**A:** Pearson correlation coefficient = - 0.55; P(2-tailed) < 0.001; Spearman correlation= -0.539; P(2-tailed) < 0.001; N=71;  $R^2=0.303$ . **B:** Pearson correlation coefficient = - 0.587; P(2-tailed) < 0.001; Spearman correlation= -0.539; P(2-tailed) < 0.001; N=71;  $R^2=0.345$ . Note: samples were prepared from PBMCs.

To conclude, we stress the major drawback of our studies - all our FH patients were treated with statins and their lipoprotein levels were normal. Importantly, the only patient who was not on statin treatment showed dramatically increased circulatory iNKT cell levels (Figure 18 (below)). Statins indeed are known to impair CD1d mediated presentation[210].



**Figure 18. iNKT cells frequency was extremely high in a single FH patients without statin therapy.**

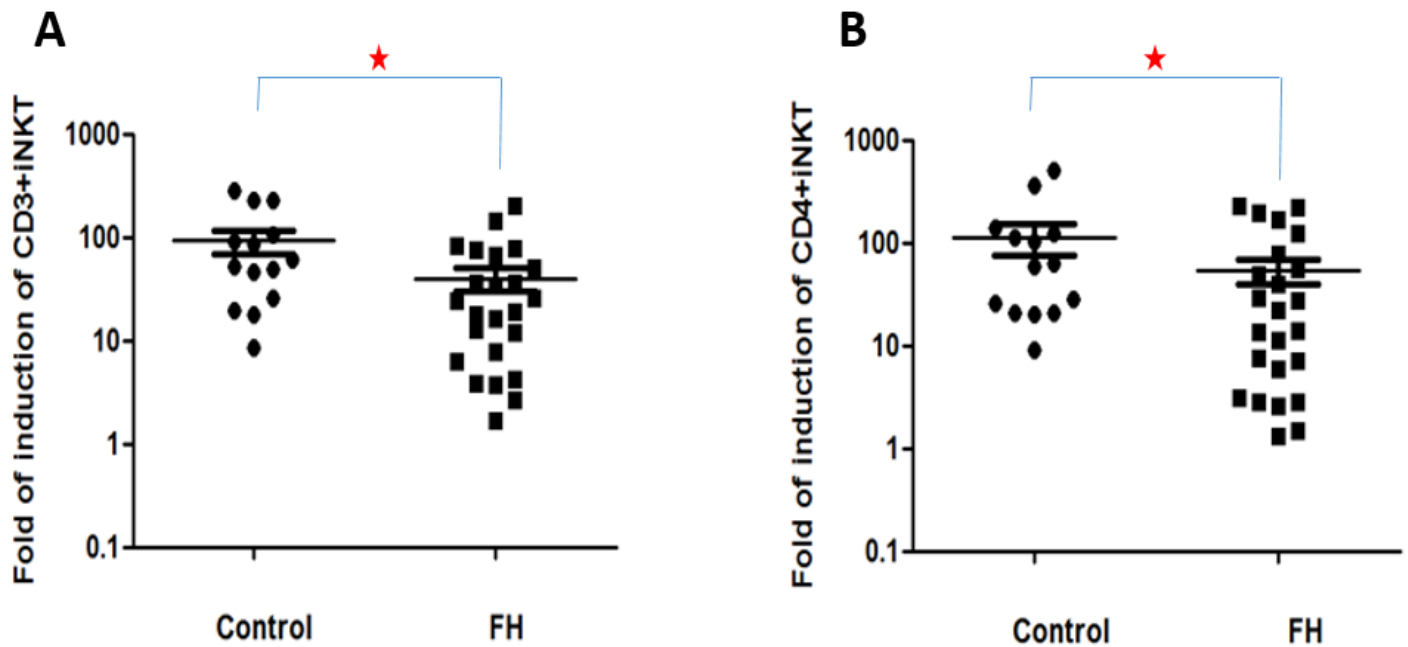
Gated on lymphocytes. FITC was used as a dump channel (CD19/14-FITC). Dumped cells are gated out.

The observed correlation between total iNKT and CD4+ iNKT/total-iNKT ration suggests that that circulatory iNKT cell levels are actually increased in FH patients and one just has to collect more data to reach the statistical significance. Both the decrease in Th2-like iNKT (CD4+ iNKT) and the increase in Th1-like iNKT (DN iNKT) are atherogenic, since Th1 type immune responses are known to be atherogenic, while Th2 immunity is atheroprotective (depending on a stage of a lesion)[211]. As noted before, similar result (decrease in CD4+ iNKT and increase in DN iNKT) was observed in long-term type I diabetes patients. This similarity strengthens the known link between the diseases[212].

## **5.2 Functional characterization of iNKT cell subsets in patients with Familial Hypercholesterolemia**

To investigate functional properties of iNKT cells PBMC were isolated from 24 FH and 14 control patients, plated on plastic plates (1 million of cells per well, 24 well plate format), and incubated for 10 days in the 1 ml of RPMI1640 media (containing L-glutamine, Na-Pyruvate, pen/strep and 10% FBS) in the presence of IL-2 (100 U/ml) and  $\pm$   $\alpha$ -GalCer (100 ng/ml). Cells were further processed and analyzed by flow cytometry. Our data show

that both total iNKT and CD4+ iNKT cell abilities to proliferate following  $\alpha$ -GalCer stimulation are impaired in FH patients under statin treatment (Figure 19 (below)).



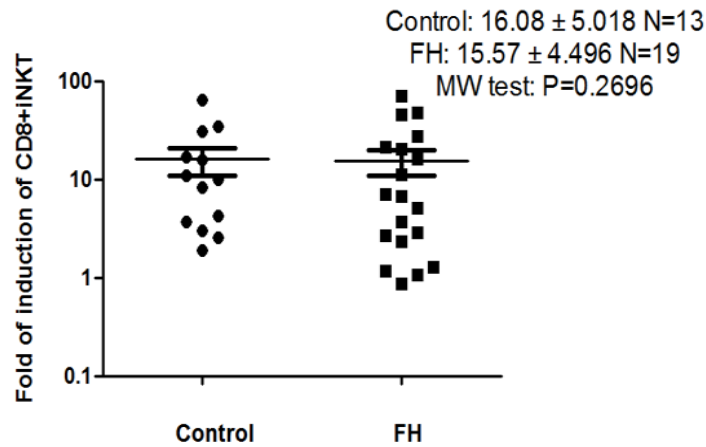
**Figure 19. Functional properties of total and CD4+ iNKT cell subsets.**

The fold of increase in total and CD4+ iNKT cells numbers after 10 day incubation of PBMCs (isolated from control and FH patients) in the presence of  $\alpha$ -GalCer and IL-2. **A:** Control:  $93.6 \pm 24$ ; FH:  $40.3 \pm 10$ ;  $P < 0.01$  (MW test); **B:** Control:  $114.4 \pm 39$ ; FH:  $54.5 \pm 15.1$ ;  $P < 0.05$  (MW test). Data format: average  $\pm$  SEM.

Data suggest that some of the FH patients (but not all) have a reduced ability to respond to  $\alpha$ -GalCer in PBMC proliferation assay. Of note, out of 24 FH patient (identified by MEDPED score) 11 were analyzed for the presence of mutations in LDLR, ApoB and PCSK9 genes. Mutation in one of the genes was confirmed for 6 of them. All these 6 patients had reduced iNKT cell proliferation (below 50 fold).

A rarer CD8+ iNKT cell subset showed a reduced ability to proliferate (compared to CD4+ and DN iNKT subsets). We observed no statistically significant difference between control and FH patients in CD8+ iNKT cell proliferation (Figure 20 (below)).

Certainly it would be more interesting to compare the ability of iNKT cells to proliferate in PBMC proliferation assay between normolipidemic and hyperlipidemic patients which did not receive statin therapy. Importantly, since (as discussed in the chapter 5.5) the ability of iNKT cells to proliferate in PBMC proliferation assay is very sensitive to the PBMC concentration, it would be of value to compare iNKT cell proliferation abilities in the assay between patients with low and high lymphocyte count and low/high red blood cell count.

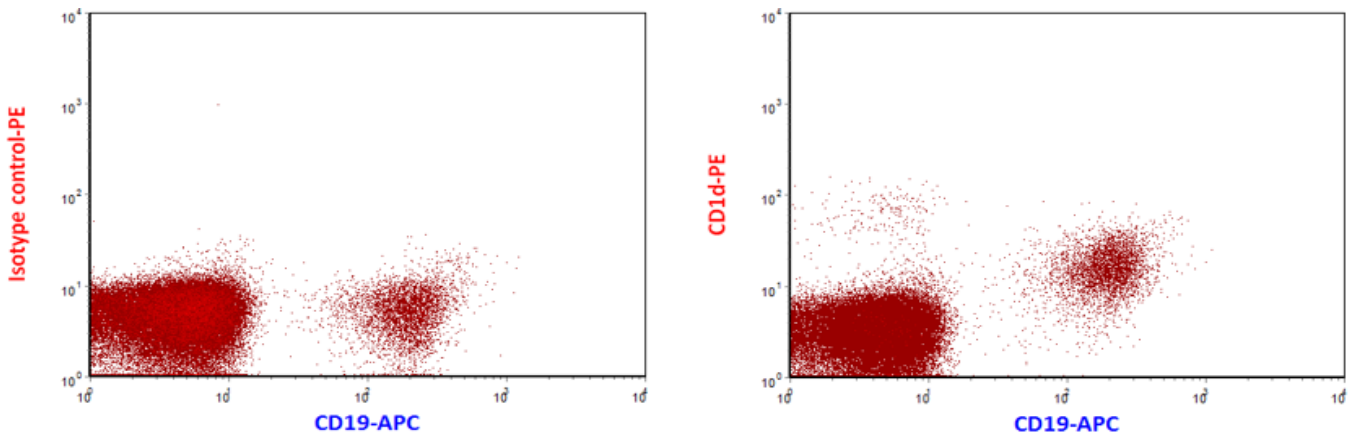


**Figure 20. Functional properties of CD8+ iNKT cell subset.**

The fold of increase in CD8+ iNKT cell numbers after 10 day incubation of PBMCs (isolated from control and FH patients) in the presence of  $\alpha$ -GalCer and IL-2.

### 5.3 Expression of CD1 a,b,c,d by human PBMC.

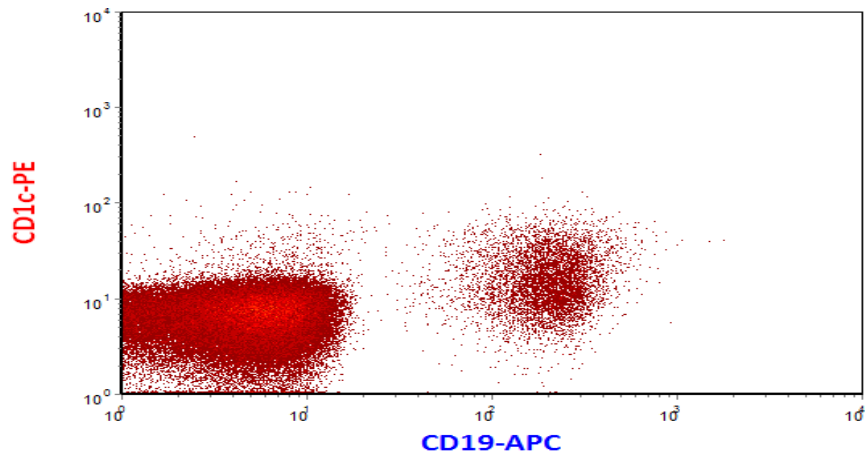
To determine which subtype of PBMCs expresses CD1d we stained them with anti-CD19, CD14+ and anti-CD1d (or anti-isotype control) antibodies. The major PBMC subtype expressing CD1d were CD19+ B cells (Figure 21 (below)). Much smaller unidentified PBMC subset (negative for CD19 and CD14) located between lymphocyte and monocyte gates on FSC/SSC plot expressed CD1d at the highest level.



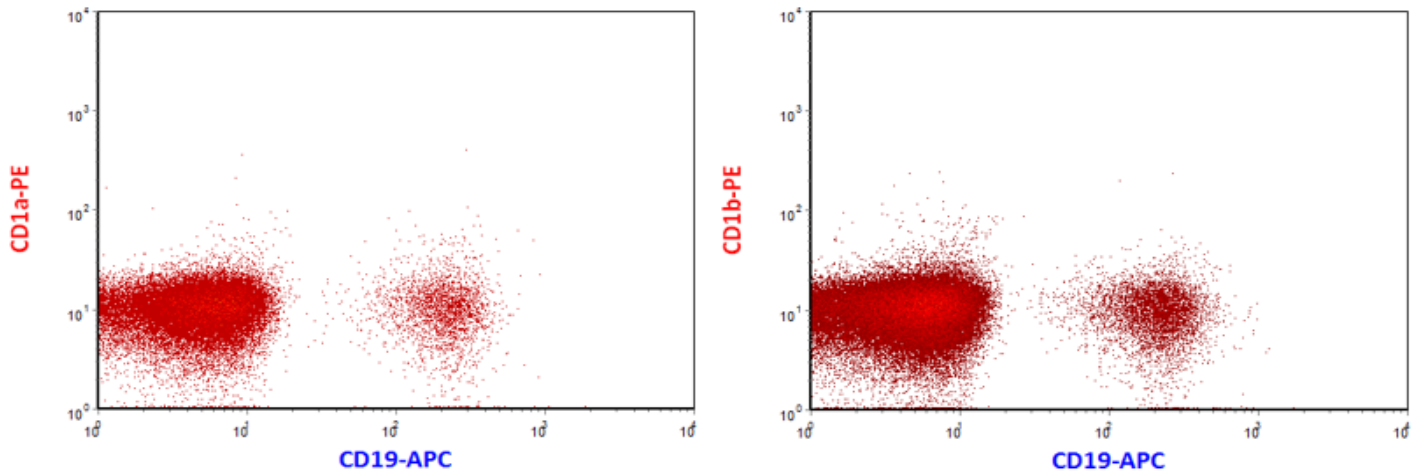
**Figure 21. CD1d expression by B cells.**

PBMC staining. Gated on lymphocytes plus the region between lymphocytes and monocytes. Note the presence of an unidentified CD1d expressing subset (CD19 negative, CD1d positive). Gated back on FSC/SSC plot this subset is located between lymphocytes and monocytes.

It has been reported that monocytes express CD1d at low levels [87]. However, we were unable to confirm the expression of CD1d on CD14+ monocytes. One has to reduce non-specific staining (e.g. by blocking Fc receptors with 'Human BD Fc block' reagent) to analyze protein expression at such a low level.



**Figure 22. CD1c is also expressed by B cells.**



**Figure 23. CD1a and CD1b are not expressed by B cells.**

Interestingly, although B cells are unable to present  $\alpha$ -GalCer and thus induce iNKT cell proliferation[213] their depletion results in the abrogation of iNKT cell proliferation in PBMC proliferation assay, while depletion on monocytes results only in a 1.5-2 fold reduction of PBMC proliferation [214]. It could be speculated that APCs lacking CD19 and CD14 identified in our analysis are the major iNKT cell inducers in PBMC proliferation assay. APCs, such as DCs are known to nibble (take small bites) other cells. One can further speculate that these unidentified APCs (probably DCs) obtain CD1d by nibbling B cells or by other type of transfer. It was shown recently that hepatic stellate cells (HSCs) in the liver are able to transfer MHC class I molecules to LSECs and these molecules were employed for LSEC cross-presentation to CD8+ CTLs[215]. This shows that MHC class I-like protein transfer from one cell type to another is possible.

Interestingly, CD19+ B cells from our assay also expressed CD1c (Figure 22), but not CD1a and CD1b (Figure 23). This is in line with the report that regulation of CD1c and CD1d expression in B cells differs from the one reported in DCs [216].

#### 5.4 Effect of serum (bovine vs human) and lipoproteins on the expression of CD1 – a,b,c,d by immature human MDDCs.

Monocytes were prepared from PBMC by plastic adherence. Immature MDDCs were derived from monocytes by 5 day incubation (RPMI-1640 medium, 37°C, 5% CO<sub>2</sub>, 10% fetal bovine serum (FBS) or autologous serum (AS)) in the presence of IL-4 and GM-CSF. 24 hour incubation of MDDCs (same conditions) in the presence or absence of LDL or oxLDL was followed by flow cytometry analysis for CD1a,b,c,d expression.

Clearly group I CD1 proteins were expressed at much higher level than CD1d. We report that both LDL and oxLDL reduced the expression of CD1a,b,c on MDDCs (Figure 24, Figure 25, Figure 26), while the expression of CD1d was slightly increased by the oxLDL (Figure 27, Figure 28).

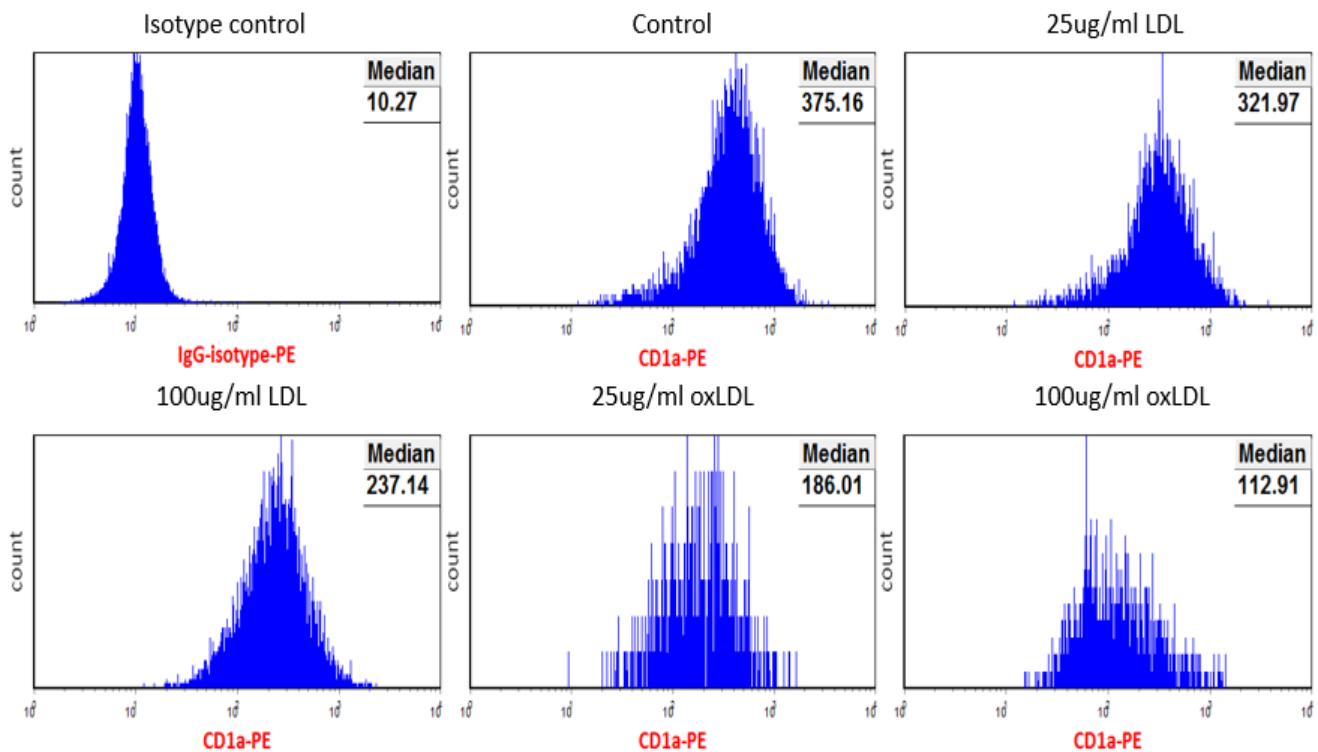


Figure 24. Expression of CD1a by MDDCs.

Importantly the expression of CD1a,b,c,d varied greatly between the individuals and the effect of LDL/oxLDL addition depended upon the purity of MDDC preparation (contamination with lymphocytes reduced or even reversed the observe effects, data not shown).

Interestingly, when we used human autologous serum (AS) instead of FBS in our MDDC assay, expression of CD1a,b,c by MDDCs drastically decreased (Figure 29). Human serum lipids (Lysophosphatidic acid and cardiolipin) were reported to induce inhibition of group I CD1 proteins, but upregulate the expression of CD1d [175].



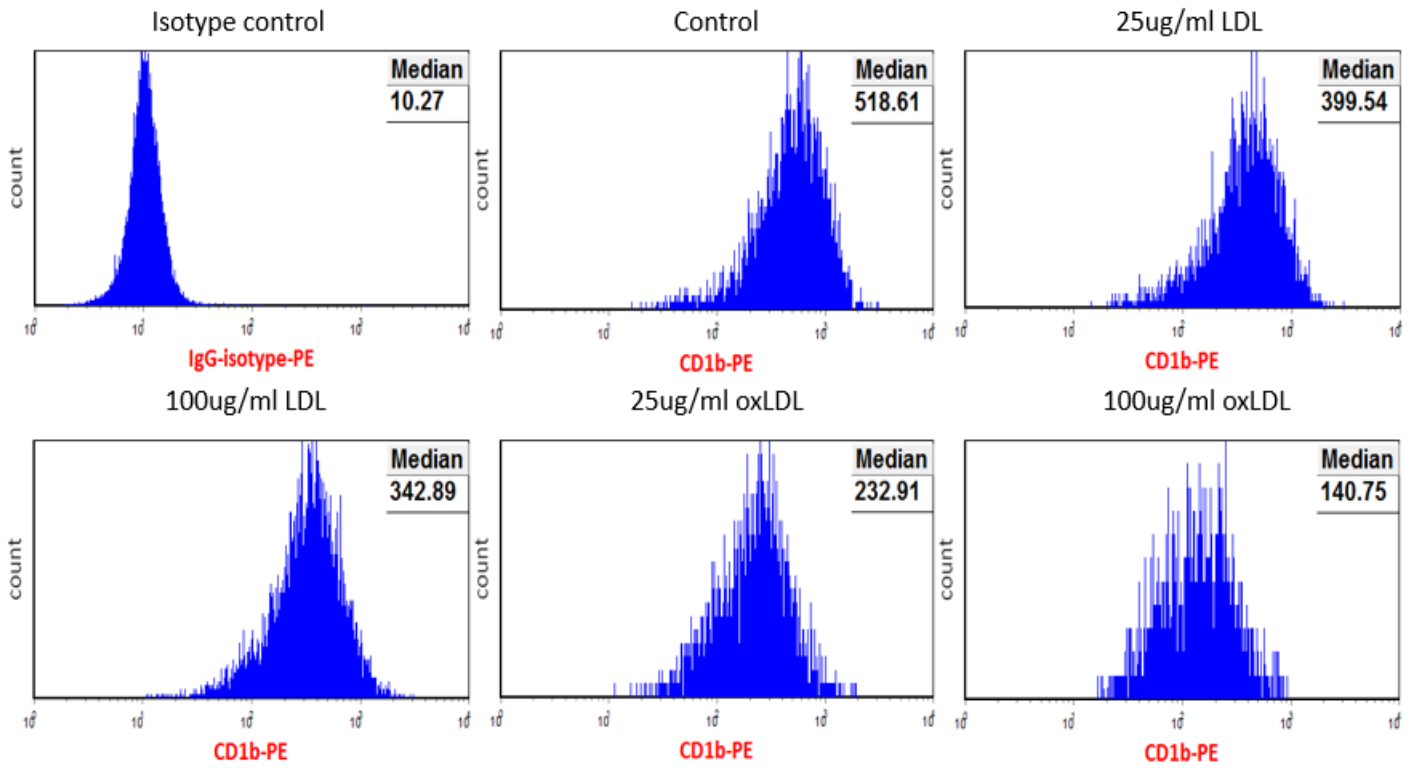


Figure 25. CD1b expression by MDDCs.

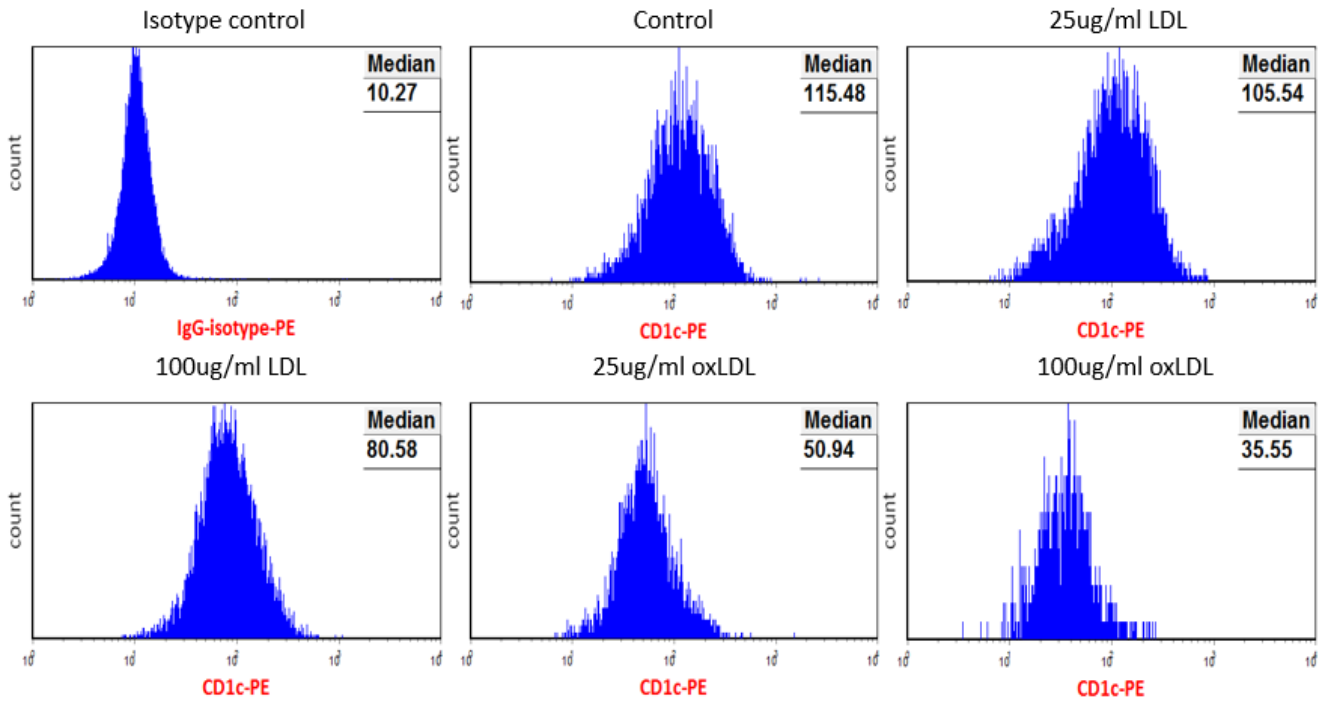


Figure 26. Expression of CD1c by MDDCs.

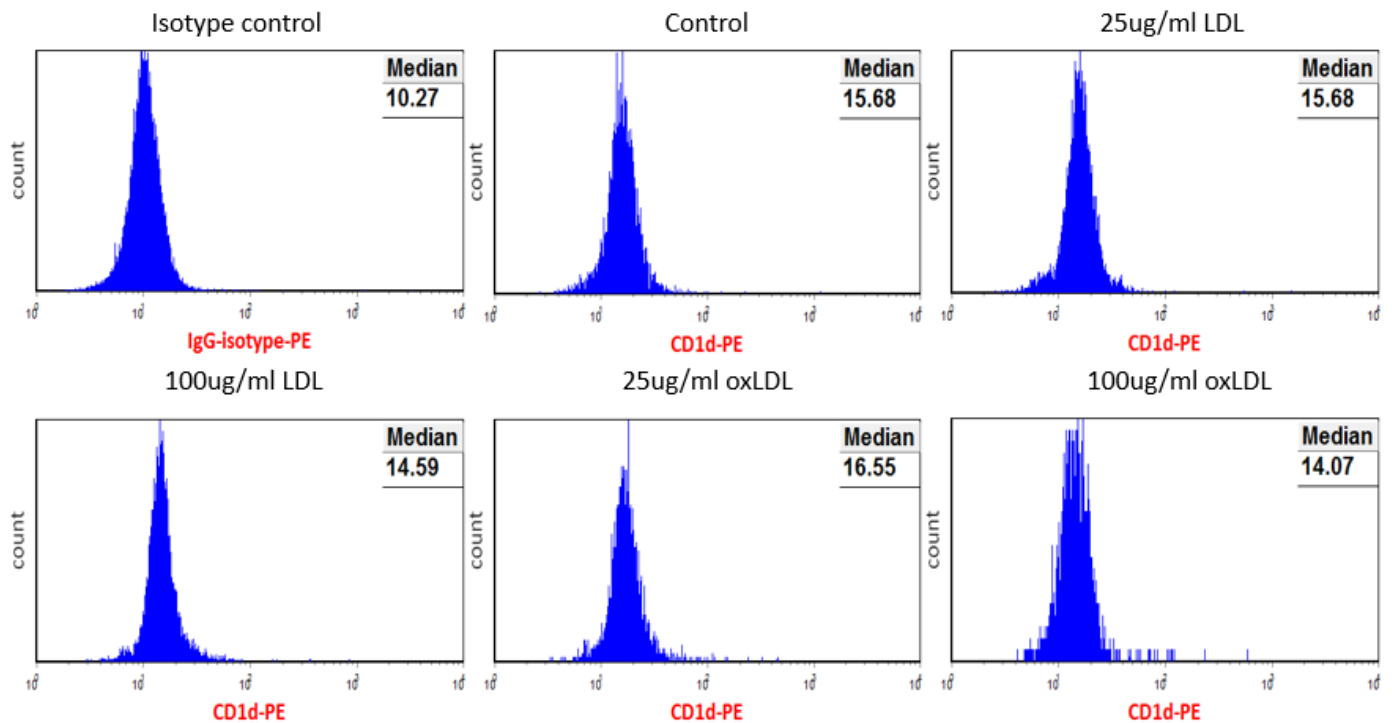


Figure 27. Expression of CD1d by MDDCs.

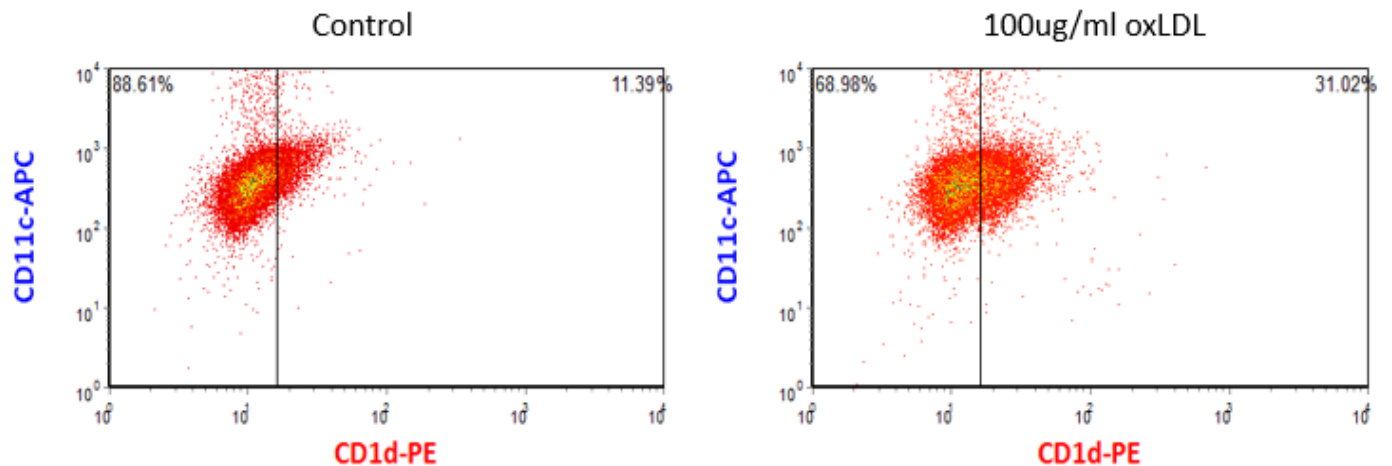
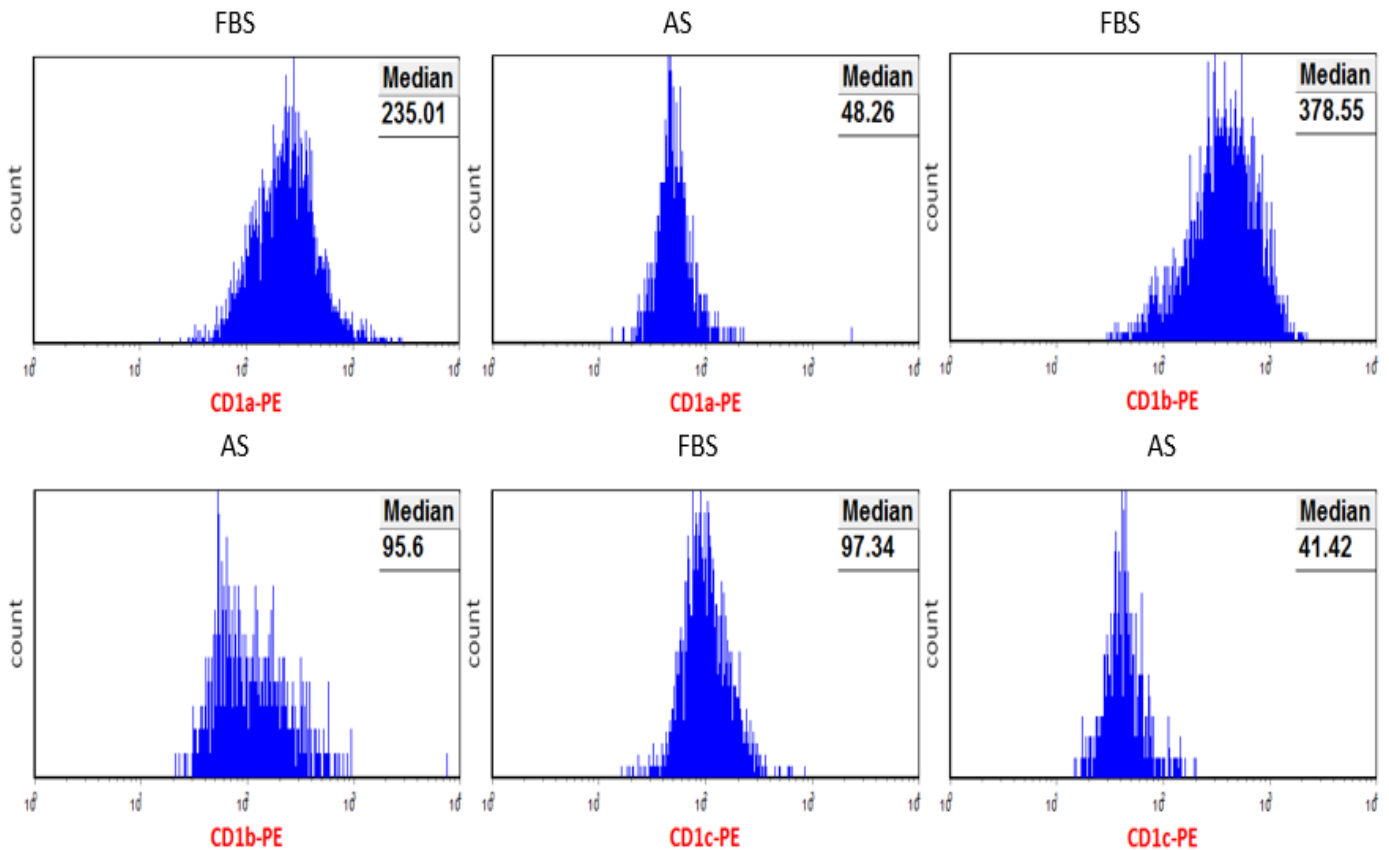


Figure 28. Expression of CD1d is elevated upon incubation of immature MDDCs in the presence of oxLDL.

Our data on CD1 expression by MDDCs are in line with published data generated under slightly different conditions [176,217,218]. To conclude, one cannot translate data obtained in the MDDC *in vitro* assay to what happens *in vivo*, because *in vivo* DCs differ greatly from MDDCs. Additionally one has to take into account that important factors (type of serum, contamination with lymphocytes, lipoprotein levels, serum IgG levels [219]) greatly affect the expression of CD1 proteins.

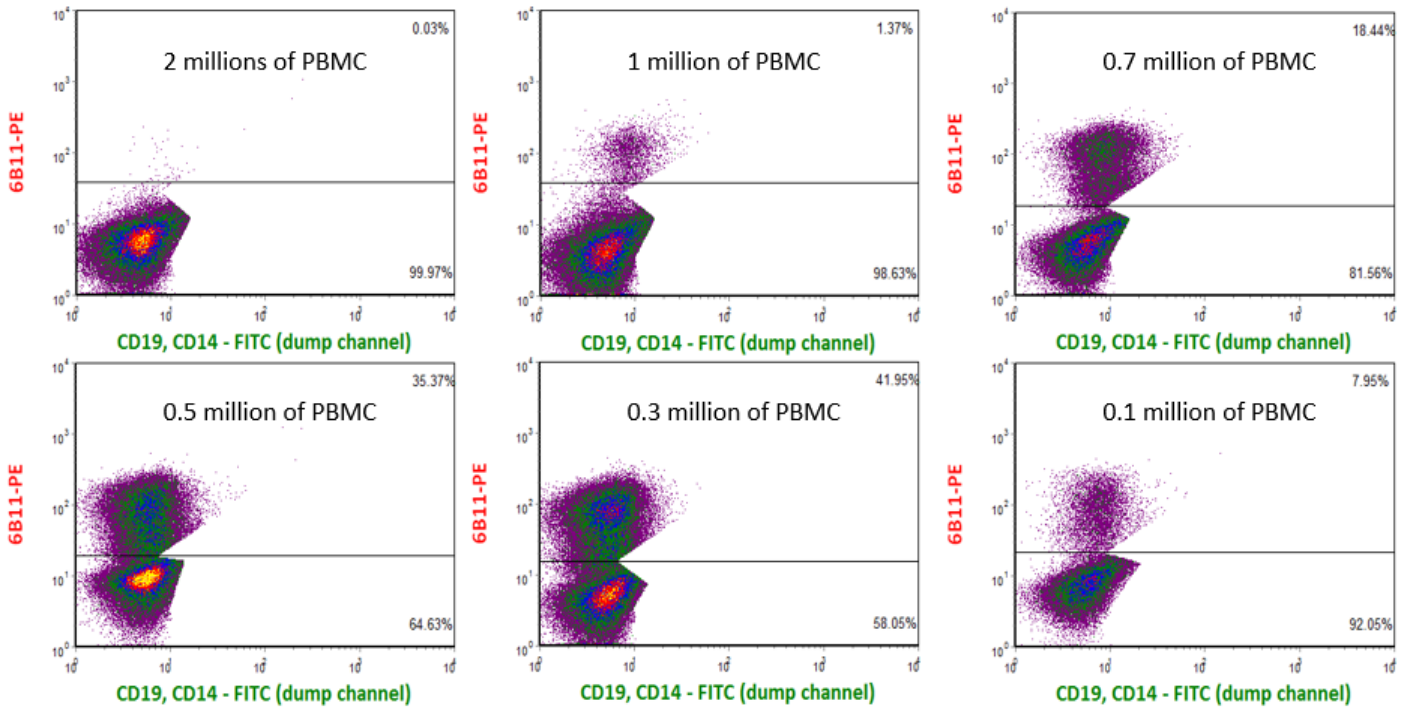


**Figure 29. Human serum downregulates expression of group I CD1 proteins by MDDCs.**

### 5.5 The role of lipoproteins in the transport of synthetic lipid antigen $\alpha$ -GalCer to antigen presenting cells (APCs) in an *in vitro* iNKT cell proliferation assay

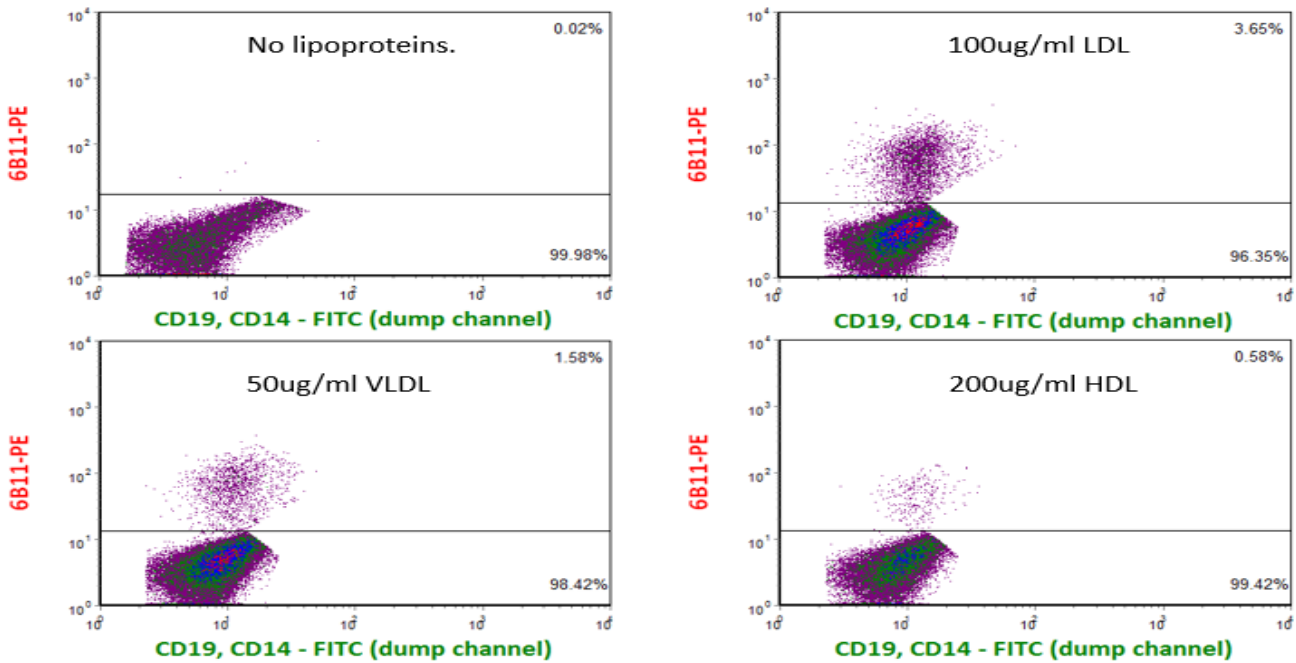
To investigate the role of lipoproteins (LDL, VLDL, HDL) in the transport of  $\alpha$ -GalCer to APCs in an *in vitro* iNKT cell proliferation assay, we conducted PBMC proliferation assay described in chapter 5.2 in the absence of serum (using Aim V media, which allows PBMC growth in the absence of serum) or in lipoprotein deficient serum (10% autologous LPDS) and added separately isolated autologous lipoproteins to the cell culture to find out if they can induce iNKT cell proliferation observed in the presence of serum (10% FBS). We observed no iNKT cell stimulation in the absence of lipoproteins and addition of lipoproteins did not affect the result (data not shown) under the conditions described in the previous experiment (1 million of cells per well). However, while performing iNKT cell proliferation assay, we noticed that iNKT cell stimulation is very sensitive to initial PBMC concentration (Figure 30).

Thus, we decided to repeat the experiment (using Aim V medium) in the absence of serum (with or without lipoprotein addition) or with addition of LPDS using 0.5 million of PBMCs per ml as an initial cell concentration. Remarkably, addition of lipoproteins drove iNKT cell proliferation (Figure 31).



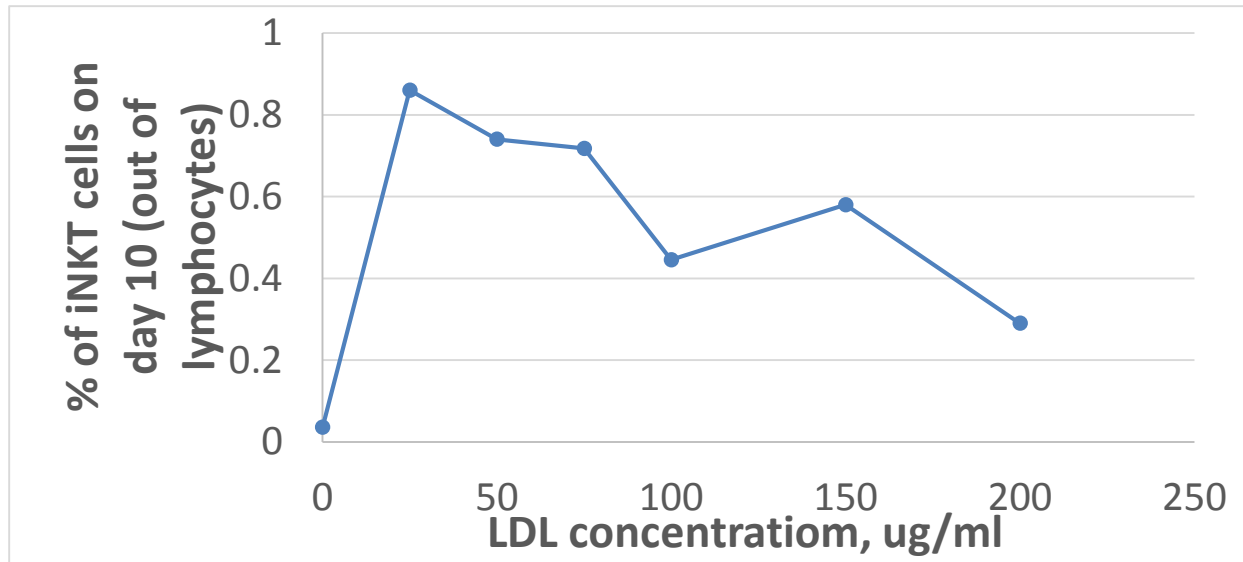
**Figure 30. iNKT cell ability to proliferate in PBMC proliferation assay is very sensitive to initial PBMC concentration.**

The ability of iNKT cells to proliferate in an in vitro iNKT cell proliferation assay is optimal at PMBC concentration of 0.5 million of cells per ml. Experimental settings are described in chapter 5.2. Only initial PBMC concentration is changed. Gated on lymphocytes (dumped 'sticky' cells are gated out).



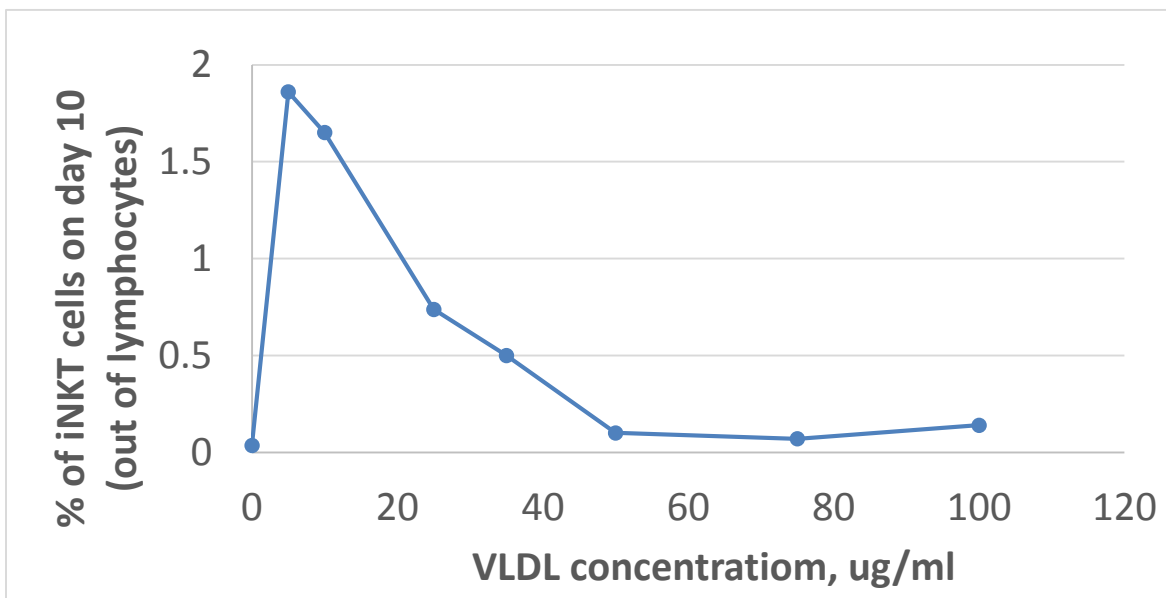
**Figure 31. Lipoproteins transport lipid antigens inside APCs to drive iNKT cell proliferation.**

Since we have chosen arbitrary concentrations (close to physiological) of lipoproteins in our essay (Figure 31), we then asked how the ability of iNKT cells to proliferate in the essay depends on the lipoprotein concentration. We report that all testes lipoproteins (LDL, VLDL, HDL) were able to induce iNKT when 100ng/ml of  $\alpha$ -GalCer was used in the essay. Importantly we observed that there is a concentration of lipoprotein at which maximum iNKT cell induction is reached (Figure 32, Figure 33, Figure 34).

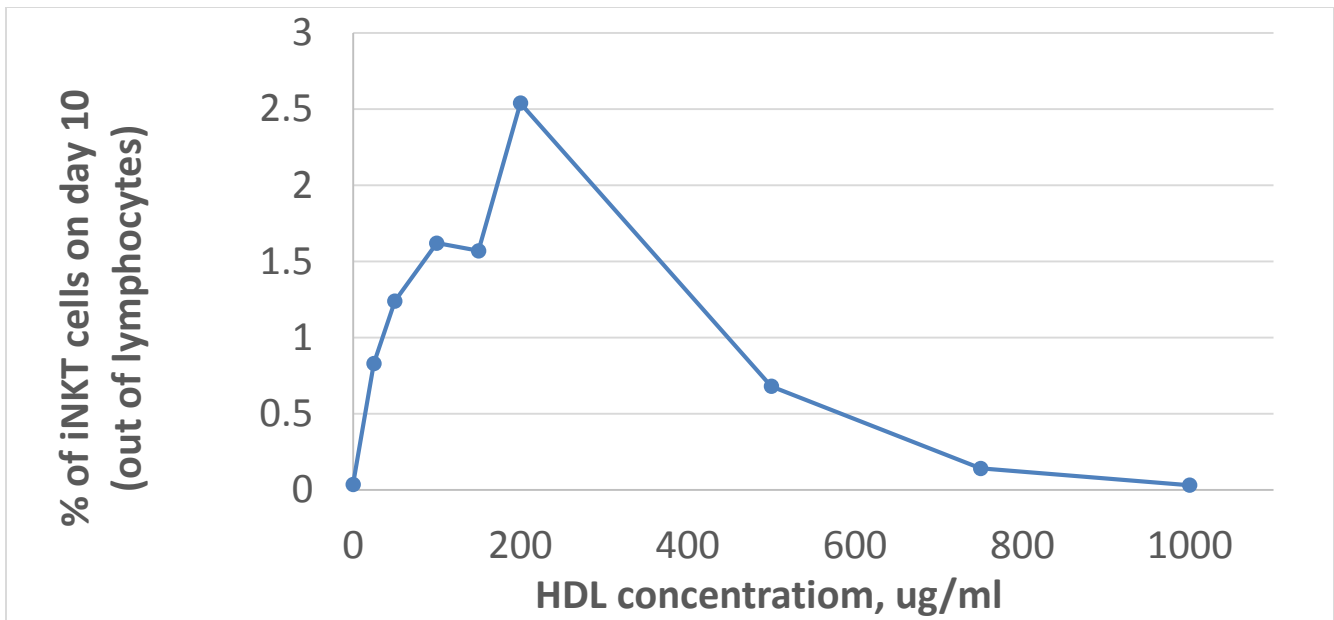


**Figure 32. iNKT cell proliferation essay in the serum free conditions in the presence of different concentrations of LDL.**

Note: Unlike in the case of VLDL and HDL, LDL is very toxic for cells at the concentrations above 100ug/ml.



**Figure 33. iNKT cell proliferation essay in the serum free conditions in the presence of different concentrations of VLDL.**



**Figure 34. iNKT cell proliferation assay in the serum free conditions in the presence of different concentrations of HDL.**

Normally in the iNKT cell proliferation assays we use 100 ng/ml concentration of  $\alpha$ -GalCer. But since in the last experiments all three lipoproteins (LDL/VLDL/HDL) showed the ability to induce iNKT, I decided to find out who is the best iNKT inducer by lowering down the concentration of  $\alpha$ -GalCer 10 times (to 10ng/ml). Only HDL was able to induce NKT cells at such a low concentration of  $\alpha$ -GalCer and the induction was almost at the same level as when 100 ng/ml  $\alpha$ -GalCer concentration was used. I conclude that HDL is the major type of lipoprotein that helps to deliver physiological  $\alpha$ -GalCer to APCs for further CD1d mediated presentation to iNKT cells.

Similarly, a group from Scripps Research Institute (La Jolla, USA) showed that  $\Delta EC_{50}$  value of  $\alpha$ -GalCer is lowest for **murine** HDL [172]. Thus HDL is the major transport particle that delivers  $\alpha$ -GalCer to APCs in both mice and humans.

As already mentioned, it was reported that soluble serum proteins (e.g. FAAH) also participate in the transport of lipid antigens to APCs [171], meaning that iNKT cells should also proliferate in our PBMC proliferation assay (Aim V media, 0.5 million of PBMC/ml) in the presence of LPDS, which is depleted of lipoproteins but not of soluble proteins. Indeed our preliminary data show that iNKT cells are induced at such conditions (data not shown). More data have to be collected to confirm the fact.

## 5.6 Numerical and functional characterization of iNKT cells in different tissues (liver, adipose tissue, blood, spleen, lymph nodes) of mice with altered lipoprotein metabolism

To investigate whether lipoprotein levels affect iNKT cell numbers and functions *in vivo* we assessed the numbers and functions of iNKT cells in different tissues (liver, adipose tissue, blood, spleen, lymph nodes) of mice with normal and altered lipoprotein levels (ApoE<sup>-/-</sup>, LDLR<sup>-/-</sup>, ApoA-I<sup>-/-</sup>, hTGApoA-I (human ApoA-I transgenic on murine ApoA-I<sup>-/-</sup> background)). Our preliminary data demonstrate the key impact of lipoprotein levels on murine iNKT cell numbers and functions.

### 5.6.1 iNKT cell numbers are decreased in the liver of LDLR<sup>-/-</sup>, ApoE<sup>-/-</sup> and ApoA-I<sup>-/-</sup> mice.

We showed that iNKT cell numbers are decreased in the liver of LDLR<sup>-/-</sup>, ApoE<sup>-/-</sup> and ApoA-I<sup>-/-</sup> mice (compared to WT mice). Transgenic expression of human ApoA-I on mApoA-I<sup>-/-</sup> background normalizes hepatic iNKT cell levels (Figure 35). Since only a few LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice were available for my experiment I did not collect enough data to confirm the significance of the observed difference in the case of these genotypes (LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup>). But the trend is clearly seen and the decline in hepatic iNKT cells (after 16 weeks of age) in LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> murine models was already reported [172,179]. Of note, hepatic iNKT cells were also reported to decrease in numbers upon switching to atherogenic diet [174].

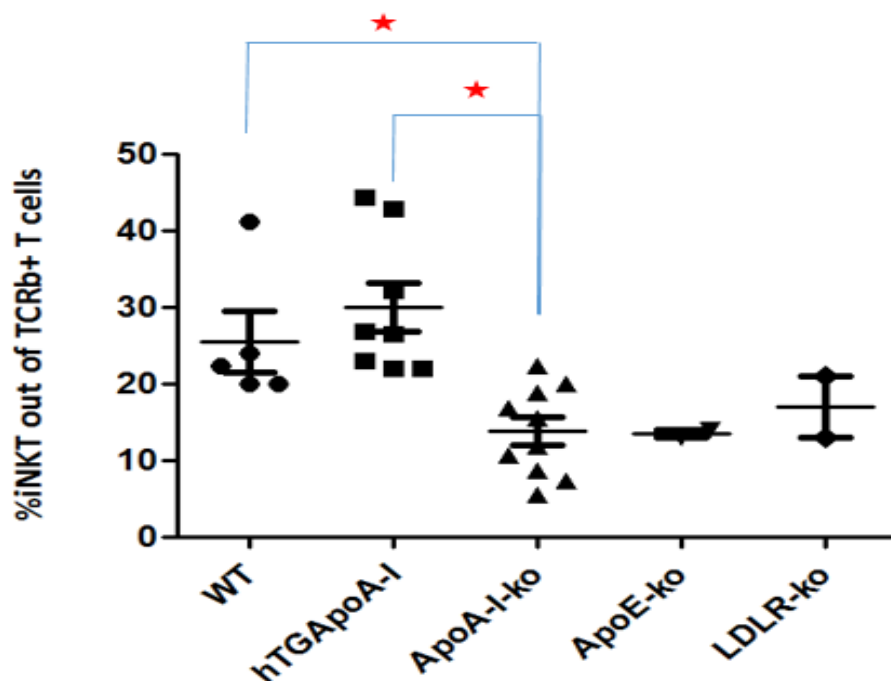


Figure 35. Hepatic iNKT cell frequencies in different murine genotypes.

The P values for the indicated differences are <0.05 (MW test).

### 5.6.2 iNKT cell numbers are increased in the blood of LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice.

Interestingly, iNKT cell numbers in the blood of LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice were increased compared to WT mice. No change in blood iNKT cell levels was observed in hTGApoA-I and ApoA-I<sup>-/-</sup> mice (Figure 36). Due to the mentioned unavailability of appropriate numbers of LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> only few data were obtained. More data has to be generated in the future to confirm the observed difference, which is statistically significant when data for LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> genotypes are combined (Figure 36, on the right).

### 5.6.3 iNKT cells are enriched in cardiac lymph nodes.

We report that cardiac LNs contain an increased number of iNKT cells compared to other types of LNs (inguinal, mesenteric, and axillary) (2-25% vs 0.1-0.3% of T cells) (Figure 37). Given the ability of iNKT cells to boost immune responses, it can be speculated that iNKT cells boost protective immune responses and autoimmune responses in the atherosclerotic plaques located in the vascular walls of the aortic arch which likely drain lymph into cardiac lymph nodes. It was shown recently that lymphatic vasculature plays important role in HDL-mediated reverse cholesterol transport (RCT), suggesting that HDL-mediated delivery of lipid antigens into APCs can occur not only in the plaque, but also in the cardiac lymph nodes since HDL travels where from the plaques [220,221].

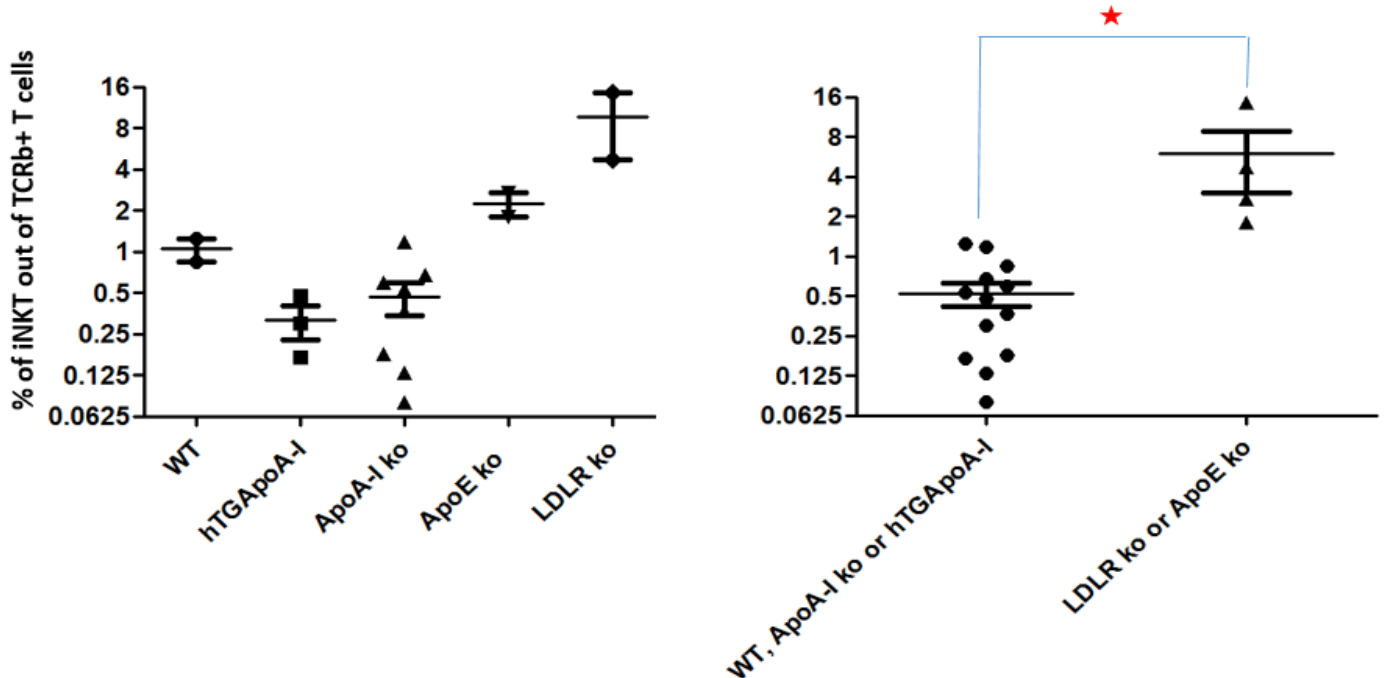


Figure 36. iNKT cell numbers are increased in the blood of hyperlipidemic mice.

Note: log2 scale was used for vertical axes. P<0.05 (MW test).



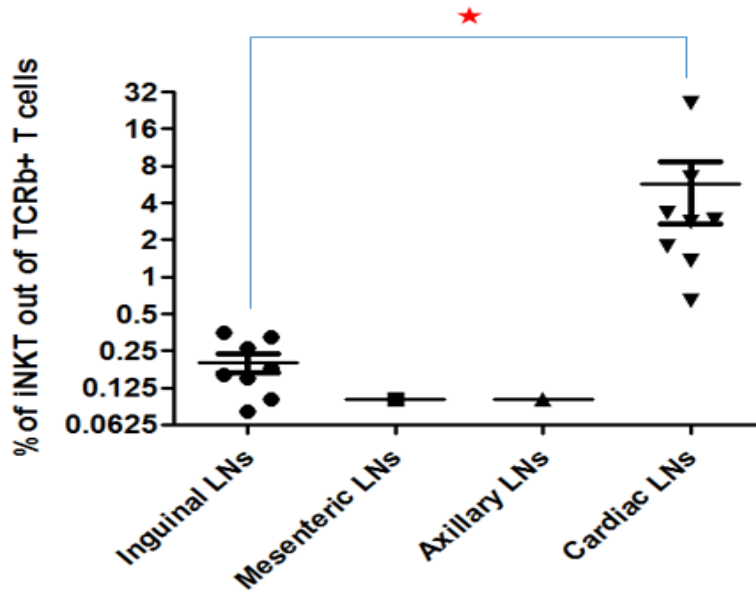


Figure 37. iNKT cells are enriched in cardiac LNs.

#### 5.6.4 iNKT cells are enriched in adipose tissue.

As already mentioned, it was reported that iNKT cells are enriched in murine epididymal adipose tissue [70,71,105] (up to ~10-20% of T cells), but one report states the opposite – that there are no iNKT cells in adipose tissue [222] (<0.1%). We report that iNKT cells are indeed enriched in adipose tissue, but constitute on average 1-4% of T cells (not 10-20%) (Figure 38). One possible explanation for the results obtained by other groups is that they did not use a dump channel to remove sticky cells which (as we observed (Figure 39)) are abundant in adipose tissue.

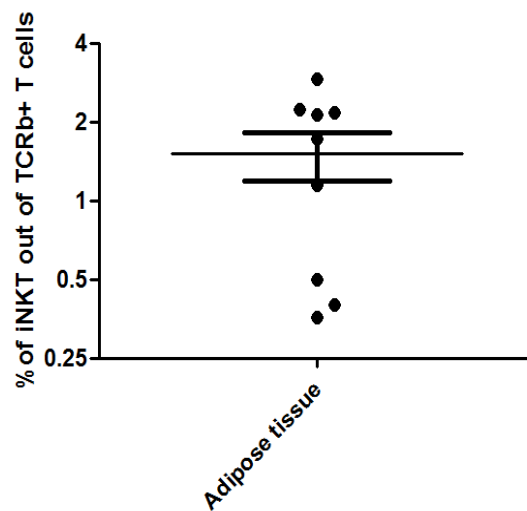
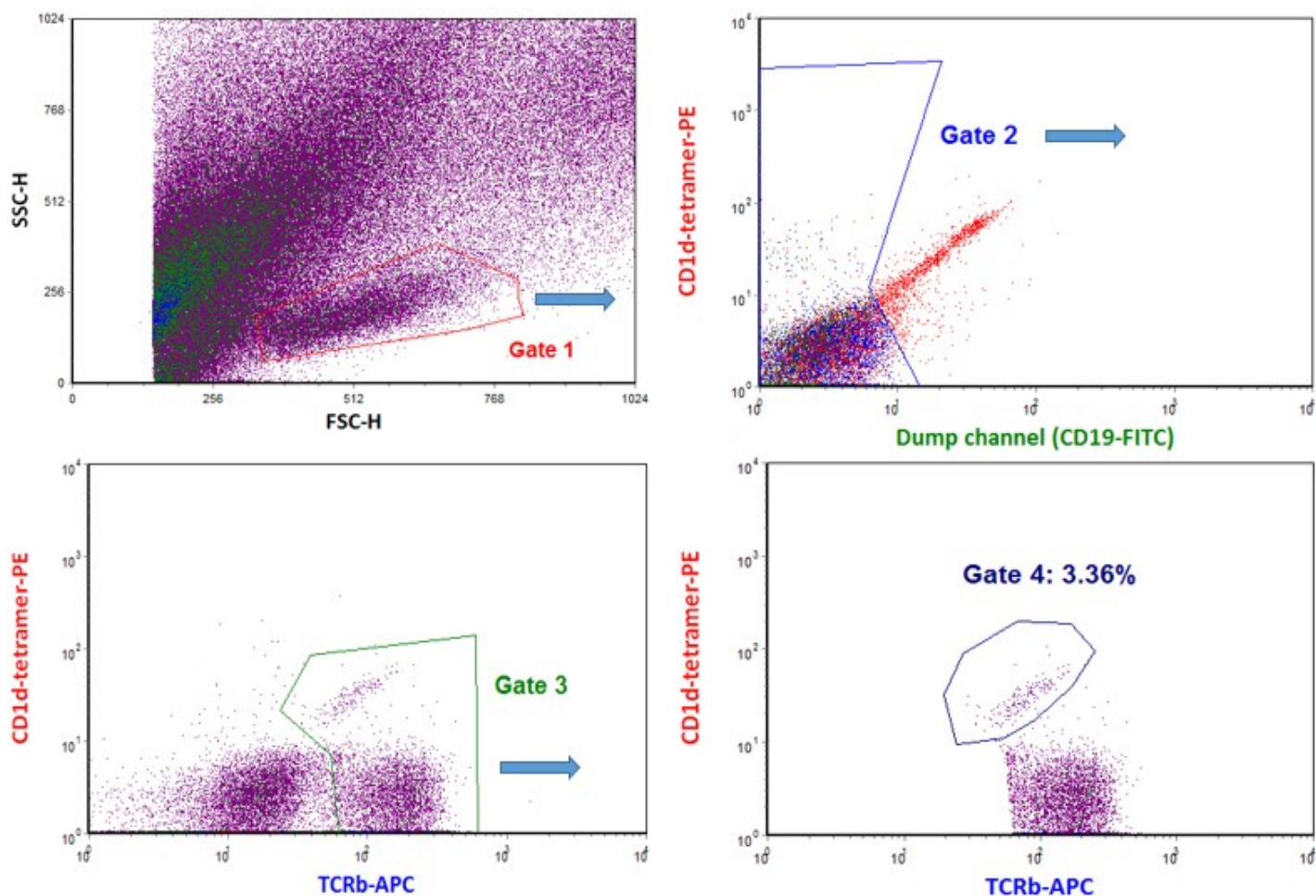


Figure 38. iNKT cells are enriched in murine adipose tissue.

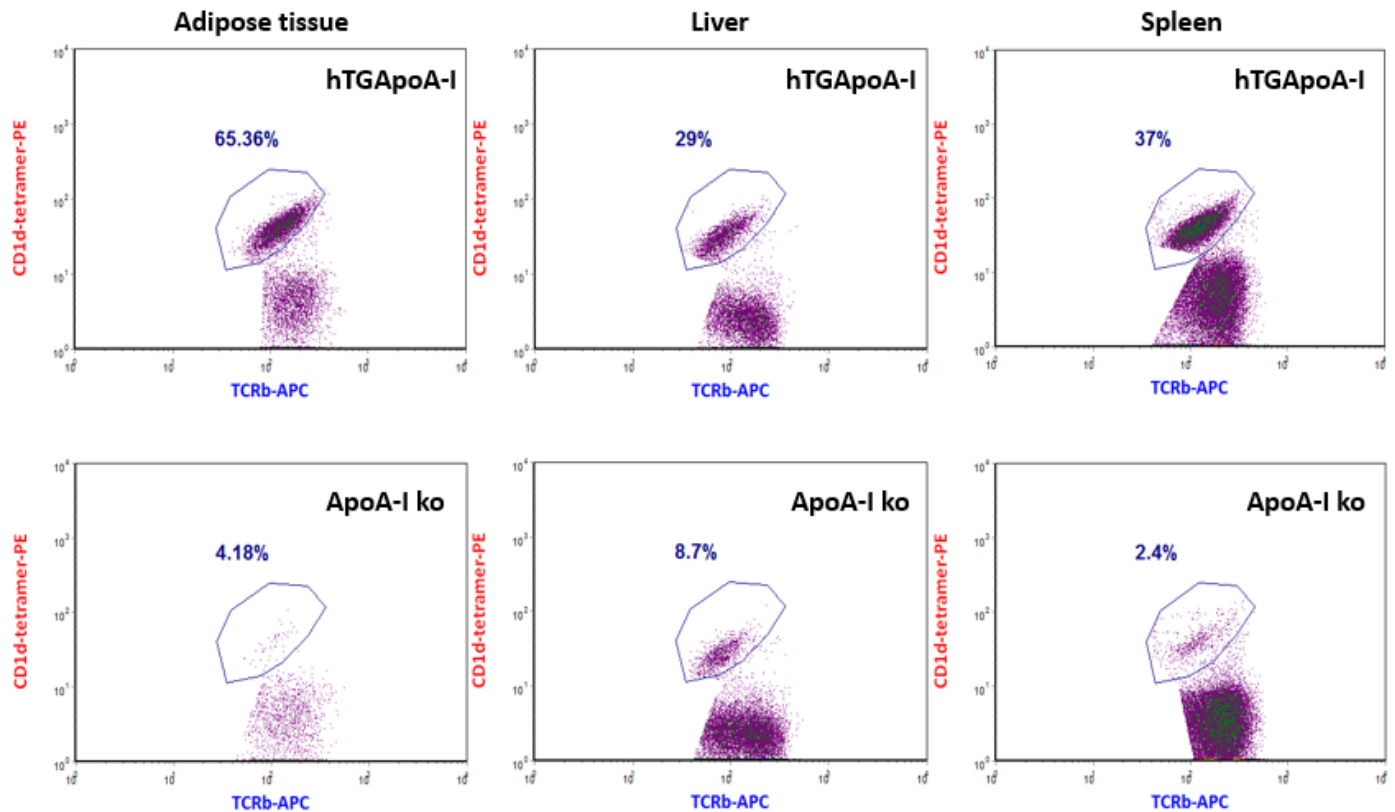


**Figure 39. Gating strategy for iNKT cell numerical analysis of adipose tissue lymphocytes.**

Note: if dump channel is not used, iNKT cell frequency would be 19.3%. Non-specific staining estimated with help of unloaded CD1-tetramer is only 0.3% (16% if sticky cells are not dumped).

### 5.6.5 Functional properties of iNKT cells in *hTGApoA-I* and *ApoA-I<sup>-/-</sup>* mice (preliminary data).

To test functional status of iNKT cells *in vivo* we IP injected mice with 4ug of  $\alpha$ -GalCer and estimated iNKT cell frequencies in different murine tissues 3 days after injection (at this time point iNKT cells were reported to reach maximum numbers). We expected liver to remain an organ with highest iNKT cell frequency. Surprisingly, in 50% of cases iNKT cell frequencies in adipose tissue were increased (from 1-4%) to extremely high values, which is 60-65% of T cells, while iNKT frequencies in the liver were only slightly increased from regular 25-30% to 30-40% (Figure 40). Of note, we observed a high degree of variability in our data. Later in the 'caveats' section I suggest the approach to reduce the observed variability. Our preliminary data suggest that iNKT in *hTGApoA-I* mice proliferate at the similar rate upon  $\alpha$ -GalCer IP injection as iNKT cells in WT and *ApoA-I<sup>-/-</sup>* mice. More data have to be collected to draw the conclusions.



**Figure 40. *In vivo* iNKT cells stimulation by IP injection of  $\alpha$ -GalCer.**

iNKT cell frequencies were determined 3 days after IP injection of 4ug of  $\alpha$ -GalCer.

## 6 Discussion

### 6.1 Suggested model for the role of iNKT cells in the context of atherosclerosis.

Based on our data, we suggest a following model of iNKT cell functioning in the context of atherosclerosis. The model is based on the newly developing concept that the loss of tolerance to lipoproteins is a possible cause of atherosclerosis development [191]. We suggest that high levels of iNKT cells in the liver (30% of T cells) maintain tolerance to LDL (or minimally modified LDL) probably via their ability to license hepatic APCs for cross-presentation [120]. Inability of liver to enforce tolerance to neoantigens (e.g. modified LDL) generated in the atherosclerotic plaques results in autoimmune responses which contribute to the plaque growth and destabilization. Based on our data showing that high levels of lipoproteins reduce iNKT cell functional properties (chapter 5.5), we suggest that hyperlipidemia with associated dysfunctional iNKT cells results in the reduced ability of liver to enforce tolerance to antigens that are cross-presented in the liver. The break of tolerance is further amplified by high numbers of iNKT cells in cardiac lymph nodes and overall high numbers of iNKT cells in the circulation (which is probably a result of high IL-15 levels in the blood). Of note, when neoantigens are not cross-presented in the liver, iNKT cells in the liver actually drive the generation of

autoimmune responses in the plaque by promoting memory T cell generation in concert with pDCs, which would explain recently reported atherogenic function of pDCs [223].

Importantly this model provides clues for understanding the etiology of not only atherosclerosis, but of any kind of autoimmune disease.

The observed decrease in hepatic iNKT cell numbers in LDLR<sup>-/-</sup>, ApoE<sup>-/-</sup> and ApoA-I<sup>-/-</sup> murine models could further reduce the ability of liver to induce tolerance to neoantigens. However, this result could be artificial. It is known that hyperlipidemia results in the increased number of activated T cells in the circulation [224]. Similar increase in the activated T cell frequency could happen in ApoA-I<sup>-/-</sup> model since HDL, of which ApoA-I is a major component, is known to affect the activation state of lymphocytes by modulating cholesterol content in lipid rafts [225]. But activated T cells are known to have an increased ability to retain in the liver. Thus, after we remove the liver from the mouse and let it bleed (or even perfuse it with 3-5 ml of Hank's Balanced Salt Solution (HBSS)), less lymphocytes come out of the liver in the case of LDLR<sup>-/-</sup>, ApoE<sup>-/-</sup> and ApoA-I<sup>-/-</sup> murine models, since they have more activated T cells.

I hypothesize that APCs sense the level of extracellular lipids (in the context of lipoproteins) and after prolonged exposure to high lipoprotein levels APCs stop performing their regular jobs (they are no longer able to activate iNKT cells) and switch to other jobs resulting in the loss of important immune functions performed by the liver.

## 6.2 Caveats

### 6.2.1 *Effect of perfusion on the frequency of isolated hepatic iNKT cells*

Hepatic iNKT cells are located literally in the blood and can travel in the direction opposite to the blood flow when they patrol liver sinusoids [226]. As in the case of other activated T cells, iNKT cells are retained in the liver as a result of constitutive Ca<sup>2+</sup>-dependent ICAM-1/LFA-1 interactions [227]. It was shown that liver resident T cells can be removed from the liver by simple perfusion at physiological flow rate with Ca<sup>2+</sup>-free HBSS containing 10mM EDTA [228]. In fact we observed that almost all hepatic iNKT cells are removed when liver is cut once (just to make a hole) and either perfused with 10 ml of Ca<sup>2+</sup>-free PBS (through the heart) or just immersed into 50ml of RPMI-1640 media for 15 minutes.

The established protocol for isolation of hepatic mononuclear cells recommends to bleed the liver and then perfuse it slowly with 3-5ml of HBSS [207]. Most of the blood leaves the liver during the bleeding. Many investigators avoid perfusion (as we did). However the differences in pressure applied to liver (when it is touched) may result in differential loss of lymphocytes from the liver during the bleeding.

Additionally, as already mentioned, differential loss of lymphocytes from the liver during bleeding/perfusion can also result from the differences in the frequency of circulating activated T cells.

Thus, to understand whether iNKT cells numbers in the liver are really reduced in hyperlipidemic conditions, I suggest to estimate iNKT cells numbers under two conditions: with recommended 3-5ml perfusion and without it (but, importantly, also without bleeding).

#### *6.2.2 IP vs IV immunizations*

It is known that in 25% of cases IP injection fails to deliver the injected material into intraperitoneal cavity [229,230]. It is possible that high degree of the variability in iNKT cell numbers on day 3 after  $\alpha$ -GalCer IP injection results from this issue. We are currently adopting a new method of IP injection (material is injected into an empty space created by folding the skin)[208]. To avoid mistakes associated with this issue I suggest to switch to IV injections, since both are meant for systemic delivery (parenteral).

#### *6.2.3 A need for a second positive maker for unambiguous iNKT cell identification in human PBMC*

As already mentioned, the detection of rare cell types (< 0.1% of T cells) necessitates the use of a second positive maker (e.g. using anti-V $\alpha$ 24 antibodies with 6B11 antibodies), as recommended in the recently described protocol [92]. Additionally, it would be useful to dump dead cells in the dump channel or a separate channel. Identification of CD4+ iNKT requires for 5 (6) color flow cytometer (2 'positive' channels, 1 'dump' channel, anti-CD3, anti CD4, plus a channel for dead cells (if not dumped)).

#### *6.2.4 Effect of age, microbiota and circadian clockwork on murine iNKT cell numbers*

It was shown recently that microbial exposure in neonatal life affects iNKT cell frequencies and functions later in the adulthood [78,231]. Thus, microbiome differences between laboratories could lead to contradictory results. Additionally, one should take into account that WT mice and knockout mice may differ in their microbiome, thus, keeping WT mice with knockout mice in the same cage may affect the outcome of the experiments since mice infect each other with microbes. Fat animals are known to possess a less diverse microbiome. It was shown that keeping a fat mouse with a lean mouse in the same cage makes a fat mouse lean[232].

iNKT cell numbers are low in early life, increase at 8-16 weeks of life, and decrease at the old age (after one year of life). In our experiments we chose to work with 6 month old mice. The practice should be kept. Finally there is well-established link between circadian clockwork and immunity [233]. The time of day the tissues are collected and  $\alpha$ -GalCer is injected should be kept the same.

### **6.3 Summary and Future Directions**

To summarize, I report the following findings:

a) While the total iNKT cell numbers did not differ between FH and control patients, the ratio of CD4+ iNKT to DN iNKT was reduced in FH patients under statin therapy compared to the control healthy individuals. We also report a negative correlation between total iNKT cell numbers and CD4+ iNKT /DN iNKT ratio.

b) The method used for estimation iNKT cell function (PBMC proliferation assay) is very sensitive to PBMC concentration and the optimal PBMC concentration for the method is 0.5 million of cells per milliliter. Importantly, at such concentration it is possible to analyze the effect of lipoproteins on lipid antigen delivery to APCs in serum/protein free conditions.

c) Out of PBMC cell subsets only B cells and unidentified rare population express CD1d at detectable levels. CD1d is expressed at very low levels by MDDCs.

d) HDL is the major transporter that transports lipid antigens to APCs for CD1d mediated presentation to iNKT. While lipoproteins are required to deliver lipid antigens to APCs, both high concentration of lipoproteins and high cellular concentration abrogate this delivery.

e) iNKT cells are enriched in cardiac lymph nodes compared to other types of lymph nodes. iNKT represent ~ 1-4% of lymphocytes in adipose tissues.

f) iNKT cells levels are decreased (2 fold) in the liver of LDLR<sup>-/-</sup>, ApoE<sup>-/-</sup> and ApoA-I<sup>-/-</sup> mice, but increased in the blood of LDLR<sup>-/-</sup> and ApoE<sup>-/-</sup> mice (compared to WT mice).

In the end, I suggest to pursue the following directions in the research.

a) Numerical and functional characterization of iNKT cells in the blood, liver, adipose tissue and cardiac LNs of normolipidemic WT (chow diet) and genetically modified hyperlipidemic mice (LDLR<sup>-/-</sup>, ApoE<sup>-/-</sup> mice on high fat diet). It will be of value to perform perfusion of liver with 3 ml of HBSS and to determine if perfusion removes different amount of lymphocytes from the liver of WT and hyperlipidemic mice.

b) Testing if there is a correlation between IL-15 levels and iNKT cell frequencies in human blood and murine tissues.

c) Identifying the effect of lipoprotein levels in human blood on iNKT cell frequencies and functions (as the abilities to proliferate and to express cytokines in PBMC proliferation assay).

d) Testing if there is a difference in the ability of APCs isolated from hyperlipidemic and normolipidemic patients to stimulate iNKT cells.

e) Identification of Scavenger Receptors which promote lipoprotein mediated delivery of  $\alpha$ -GalCer to APC.

Finally, it would be interesting to design experiments to put the suggested model of checkpoints regulating immune responses under the test.

## 7 Abbreviations

ABL – abetalipoproteinemia

ACK lysis buffer – Ammonium-Chloride-Potassium lysis buffer

APC – antigen presenting cell

ApoA-I – apolipoprotein A-I

ApoB – apolipoprotein B

ApoE – apolipoprotein E

AS – autologous serum

aTreg – adaptive regulatory T cell

Con A – concanavalin A

CTL – cytotoxic T cell

DC – dendritic cell

DMSO – dimethyl sulfoxide

DN – double negative

FAAH – fatty acid amide hydrolase

FBS – fetal bovine serum

FH – familial hypercholesterolemia

HBSS – Hank's Balanced Salt Solution

HDL – high density lipoprotein

HSC – hepatic stellate cell

hTGApoA-I – human ApoA-I transgenic on murine ApoA-I<sup>-/-</sup> background

ICAM-I – Intercellular Adhesion Molecule I

iDC – immature dendritic cell

iMATE – intrahepatic myeloid-cell aggregates for T cell population expansion

iNKT – invariant natural killer T cell

IP – intraperitoneal

iPS cell – induced pluripotent stem cell

iTreg – induced regulatory T cell

IV – intravenous

LDL – low density lipoprotein

LDLR – low density lipoprotein receptor

LFA-I – Lymphocyte function-associated antigen I  
LN – lymph node  
LPDS – lipoprotein deficient serum  
LSEC – liver sinusoidal endothelial cell  
MACS buffer – magnetic cell sorting buffer  
mDC – mature dendritic cell  
MDDC – monocyte derived dendritic cell  
MEDPED – make early diagnosis to prevent early deaths  
mmLDL – minimally modified low density lipoprotein  
MTP – microsomal triglyceride transfer protein  
MWCO – molecular weight cut-off  
NKT – natural killer T cell  
nTreg – natural regulatory T cell  
OCH – (2*S*, 3*S*, 4*R*)-1-*O*-( $\alpha$ -D-galactopyranosyl)-*N*-tetracosanoyl-2-amino nonane-1,3,4-triol  
oxLDL – oxidized low density lipoprotein  
PBMC – peripheral blood mononuclear cells  
PBS – phosphate buffered saline buffer  
PCSK9 – proprotein convertase subtilisin/kexin type 9  
pDC – plasmacytoid dendritic cell  
PPAR $\gamma$  – nuclear receptor peroxisome proliferator activated receptor  $\gamma$   
PRR – pathogen recognition receptor  
SAP – sphingolipid activator proteins  
tmDC – tolerogenic mature dendritic cell  
Treg – regulatory T cell  
VLDL – very low density lipoprotein  
VSMC – vascular smooth muscle cell  
 $\alpha$ -GalCer –  $\alpha$ -galactosylceramide

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