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Review

Advanced omics techniques shed light on CD1d-mediated lipid antigen presentation to iNKT cells

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ABSTRACT

Invariant natural killer T cells (iNKT cells) can be activated through binding antigenic lipid/CD1d complexes to their TCR. Antigenic lipids are processed, loaded, and displayed in complex with CD1d by lipid antigen presenting cells (LAPCs). The mechanism of lipid antigen presentation via CD1d is highly conserved with recent work showing adipocytes are LAPCs that, besides having a role in lipid storage, can activate iNKT cells and play an important role in systemic metabolic disease. Recent studies shed light on parameters potentially dictating cytokine output and how obesity-associated metabolic disease may affect such parameters. By following a lipid antigen's journey, we identify five key areas which may dictate cytokine skew: co-stimulation, structural properties of the lipid antigen, stability of lipid antigen/CD1d complexes, intracellular and extracellular pH, and intracellular and extracellular lipid environment. Recent publications indicate that the combination of advanced omics-type approaches and machine learning may be a fruitful way to interconnect these 5 areas, with the ultimate goal to provide new insights for therapeutic exploration.

1. Introduction

CD1d-restricted iNKT cells constitute a unique subset of T lymphocytes that are reactive to lipid antigens presented via CD1d/beta-2-microglobulin β2m) complexes by Lipid antigen-presenting cells (LAPCs) [1] including macrophages, DCs and B cells [2,3]. iNKT cells play an essential role in local and global inflammation, acting as a bridge between the adaptive and innate immune system in an organ or tissue-specific manner. iNKT cells sense and respond to the local lipidome via CD1d/beta-2-microglobulin (β2M) complexes displayed to them by LAPC (Fig. 1A). iNKT cells have been found to be tissue resident, with cytokine secretion profiles distinct to the tissue of residence [4]. The interaction between the TCR on iNKT cells and the lipid-loaded CD1d on APC elicits a cytokine secretion response specific to the environment and the CD1d-presented lipid antigen, which plays an important role in modulating global inflammation [5–7]. Crosstalk is not only required to stimulate a cytokine response but is essential during thymic selection for iNKT frequency and function: without this, iNKT cell numbers are significantly reduced [8].

CD1d does not present peptides as MHC class-I and MHC class-II do, instead, CD1d/ß2M complexes present lipid antigens, that when recognized to CD1d restricted iNKT cells can promptly elicit an iNKT cell response that includes cytokine secretion. A recent paper eloquently displays the complexity of the iNKT cells cytokine profiles, highlighting

Abbreviations: α GalCer, alpha-Galactosylceramide; APC, Antigen Presenting Cell; AT, Adipose tissue; β2M, Beta 2 microglobulin; CERT, Ceramide transfer protein; CLIP, Class II associated invariant chain peptide; ER, Endoplasmic Reticulum; ERAP, Endoplasmic reticulum aminopeptidase; FFA, Free Fatty acids; GLIT, gamma-interferon-inducible lysosomal thiol reductase; GM2, A sialic acid containing GSL / Disialotetrahexosylganglioside; IFNγ, Interferon Gamma; iNKT cells, Invariant natural killer T cells; Li, Invariant chain; LAPC, Lipid Antigen Presenting Cell; MHC class-I, Major histocompatibility complex I; MHC class-II, Major histocompatibility complex II; MIIC, MHC class-II compartment; MTP, Microsomal triglyceride transfer protein; MTP-B, Microsomal triglyceride transfer protein B; NKT, Natural Killer T; NPC, Niemann–Pick C; PLC, Peptide loading complex; TAP, Transporter Associated with Antigen Processing; TCR, T-cell receptor; UGCG, UDP-glycose ceramide glucosyltransferase.

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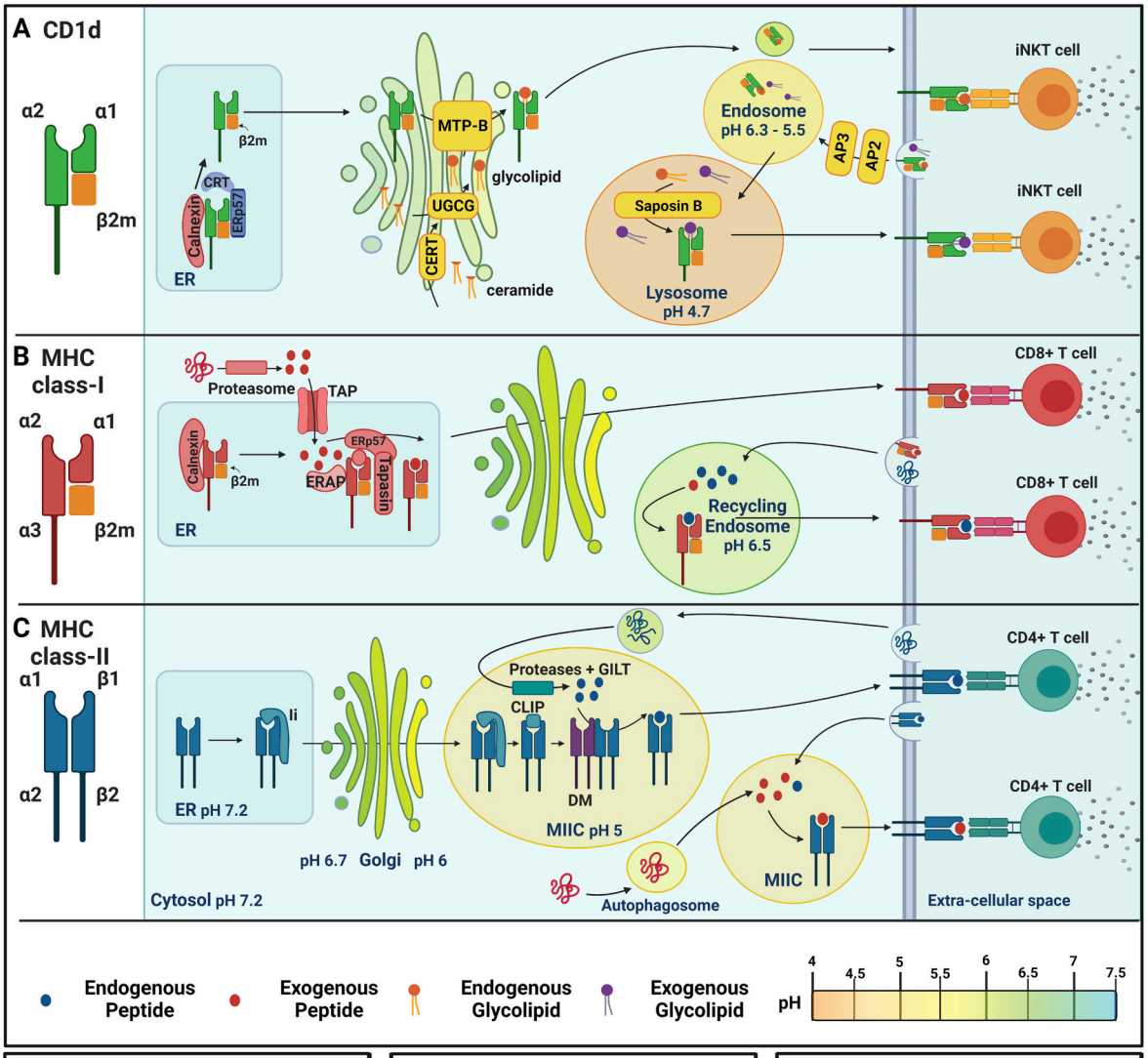

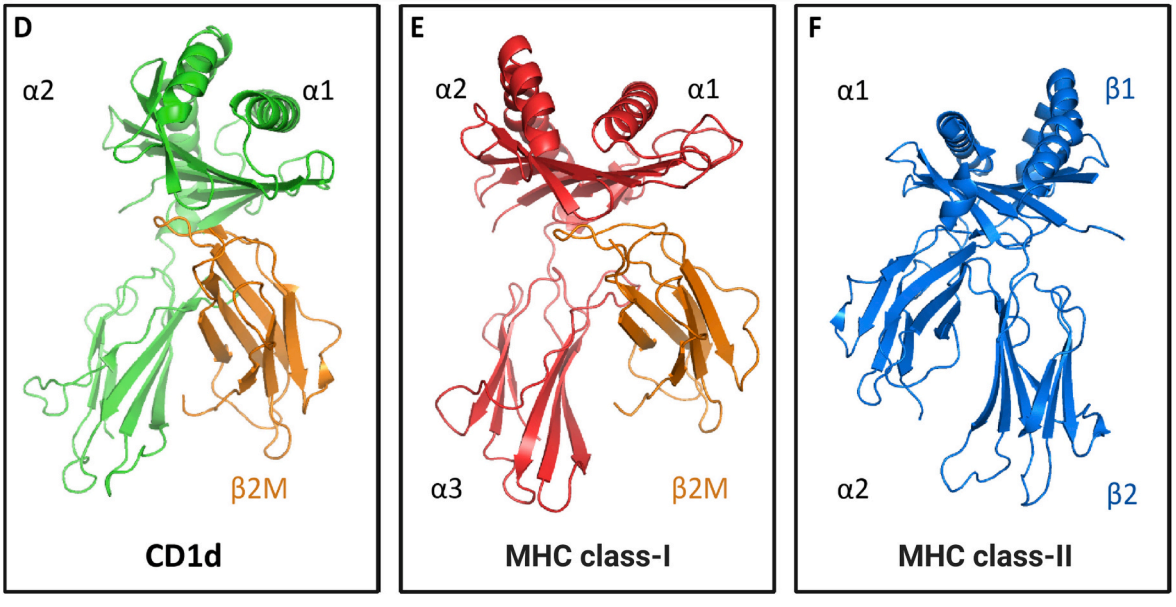

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Fig. 1. MHC class-I, MHC class-II, and CD1d loading and presentation pathways overview and comparison: A) CD1d is produced in the ER (pH 7.2), where Calnexin, CRT, and ERp57 enable b2M to stabilize the complex. CD1d then moves through the Golgi (pH 6.7 à pH6), ceramide transfer protein (CERT) transfers ceramides into the Golgi, where UDP-glycose ceramide glucosyltransferase (UGCG) converts them into loadable glycolipids via microsomal triglyceride transfer protein B (MTP-B). Through the endocytic pathway (pH 6.5), the loaded CD1d is presented at the membrane. CD1d is then recycled, via AP2 and AP3, into the endosome (pH 6.3 à pH 5.5) and lysosome (pH 4.7) and reloaded with exogenous lipid antigens via Saposin B to be presented at the membrane again. B) MHCI also uses Calnexin to load b2M in the ER, then it is transported to the peptide loading complex (PLC) within the ER. Transporter Associated with Antigen Processing (TAP) transports in peptides are trimmed by endoplasmic reticulum aminopeptidase- (ERAP-) 1 and ERAP2, and then loaded into MHCI by the chaperone Tapasin. The loaded MHCI is transported through the Golgi and presented at the cell membrane. MHCI can be recycled and represented through the recycling endosome (pH 6.5), presenting alternative peptides taken up during recycling. C) MHCII is initially folded with the chaperone protein invariant chain (Ii) in the ER, stabilizing the complex, and enabling transportation to the MHC class II compartment (MIIC). Here Ii is cleaved by lysosomal cysteine proteases (cathepsins), leaving a short peptide behind called class II associated invariant chain peptide (CLIP) bound to the peptide binding groove. Peptides that are destined to be presented must first be processed by lysosomal proteases and GLIT (pH5) D) Protein structure of CD1d E) Protein structure of MHCI F) Protein structure of MHCII.

and defining distinct populations within their tissue of residence [9] Recently, Adipose tissue (AT) -resident iNKT cells have taken center stage as key players that protect against the development of insulin resistance, [6,7,10–12] and removal of pro-inflammatory Fas-positive APCs by cytotoxic granule deposition [13,14]. In humans, AT-resident iNKT cell numbers correlate inversely with BMI and CD1d is particularly conserved between mouse (CD1d) and human (CD1a-e) [10,11,15–17]. Knock-out mice that through gene targeting specifically lack iNKT cells, spontaneously develop insulin resistance without being challenged by high-fat-diet (HFD) feeding [6,7,11]. Together, these findings raise the fascinating possibility that adipocytes through functioning as LAPCs can play a critical role in metabolic disease development. Indeed, adipocytes harbor an abundance of stored lipid antigens as potential CD1d ligands, which can directly stimulate local iNKT cells and maintain healthy adipose tissue. However, due to their low numbers in adipose tissues coupled with their rapid decline upon HFD intake, the careful study of the presumed iNKT cell anti-inflammatory function in adipose tissues using wet lab-based methods has been extremely challenging. Advanced high throughput omics-type methods are now coupled with machine learning, allowing for increasing resolution compared to wet lab techniques only. These omics-based techniques are now being used to explore the potential role of invariant natural killer T cells (iNKT cells) in systemic metabolic disease [18–21].

So far, wet lab-based approaches did allow for the identification of a variety of lipid antigens of CD1d. Knowledge gained from these studies is now being utilized in machine learning and advanced omics approaches with considerable success. Notable developments include, but are not limited to, modulation of lipid synthesis genes [22–24], developing antibodies specific for CD1d lipid antigens and their related catabolic enzymes [24,25], fractionation of various lipid sources to narrow down candidates within the lipid class [26–28] (reviewed by [29,30]), TCR capture combined with advanced lipidomic approaches [27,31], plate-bound CD1d to load and assess potential lipid antigens [32], using desirable known features to synthesize lipid antigens [33], and more recently utilizing data from structural dynamics studies to predict potential candidates [34]. We hypothesize that collectively, these advances support the existence of an ever increasing repertoire of lipid antigens that is available for CD1d capture and display, and highlight the promiscuous nature of CD1d whose loading and presentation is regulated on many levels, influencing LAPCs ability to activate iNKT cells [9]. Considering CD1d's diverse antigenic ligands, we argue that “perfect fit with optimal stimulating capacity” might not be expected for all natural lipid antigen candidates identified thus far. Instead, CD1d can bind and present a wide array of lipid antigens to iNKT cells, resulting in a range of cytokine outputs, depending on the presence of adaptor molecules and other local circumstances in the CD1d antigen loading compartment (Fig. 1A). Supporting this, lipidomics-based profiling of organs in both health and disease now shows that the lipidomes of organs or disease types is impacted by genetics, environment, and diet [35].

In an attempt to clarify how LAPC-mediated CD1d presentation may contribute to obesity-associated metabolic diseases, here we review existing literature on the regulation of LAPCs-mediated lipid antigen/CD1d antigen presentation and iNKT cells cytokine responses. Using specific examples, we break down known mechanisms regulating iNKT cell cross-talk with adipocytes, according to the following five regulatory mechanisms in APCs:

1) Co-stimulation
2) Structural properties of the lipid antigen
3) Stability of lipid antigen/CD1d complexes
4) Intracellular and extracellular pH
5) Intracellular and extracellular lipid environment

2. CD1d, lipid antigen loading, presentation, and recycling

Structurally similar to MHC class-I molecules, CD1d is comprised of 2 α-helices (α1 and α2) and forms a heterodimer with beta 2 microglobulin (β2M). The α1 and α2 helices sit on the top of the β2M protein surrounding two highly hydrophobic pockets (A’ and F’), the β2M protein lies under the hydrophobic pockets stabilizing the whole heterodimeric complex (Fig. 1D). Two CD1d antigen loading strategies are considered dominant in LAPCs: the first occurs through the ER/Golgi following the synthesis of CD1d. The second involves the recycling of CD1d from the cell membrane replacing the original lipid antigen cargo via the more acidic environment of the lysosome (Fig. 1A). In the first pathway, endogenously produced lipid antigens are loaded via microsomal triglyceride transfer protein (MTP) [36,37] and presented at the cell membrane; this pathway, including biosynthetic enzymes and MTP (more specifically, the MTP-B isoform identified in adipocytes) [37,38]. The second pathway involves recycling CD1d from the cell membrane and replacing the lipid antigen inside the more acidic environment of the lysosome. Here MTP is also active, alongside saposins [39], GM2 (a sialic acid containing GSL) activator protein, Niemann–Pick C (NPC) 1 and 2 [40], and a-galactosidase(reviewed by [2]). Similar to the first pathway, the expression of these lipid loading machinery components has been shown in mature adipocytes [37]. For both pathways, once the lipid antigen is loaded into CD1d it is has been suggested that a conformational change occurs, which is essential for the lipid antigen-loaded CD1d to interact with the TCR expressed by iNKT cells [16,42] (Fig. 1A).

1) Co-stimulation during LAPC – iNKT cell interaction: resident iNKT cells population display local tissue-specific cytokine profiles

iNKT cells can secrete pro-inflammatory Th1 (i.e., IFNγ), anti-inflammatory, tissue repair Th2 cytokines (IL-4, IL-13, IL-10), and Th17 cytokines (IL-17A) upon stimulation, depending on their dominant cytokine profile are also referred to as NKT-1, NKT-2 and NKT-17 [9,19,43]. iNKT cell populations have been shown to have a cytokine profile signature associated with their tissue of residence [4,9] and stimulation type [44]. In addition to CD1d-TCR contact between LAPC that interact with iNKT cells, additional costimulatory interactions are required for and influence IL-4, IFNγ or IL-17-type cytokine secretion. CD28:CD80 cross-talk is essential for both IL-4 and IFNγ secretion, whereas CD40:CD154 interactions are needed for IFNγ secretion but

inhibit IL-4 secretion [45]. For IL-17 secreting iNKT cells, the transcription factor RORγt is required for NKT-17 specification [46] and IL-7 is required for proliferation and homeostasis [47]. Secreted cytokines such as IL-12 induce Stat4 phosphorylation and thus IFNγ secretion, leading to the upregulation of IL-12 receptors in a feed-forward loop [48]. Here we discuss the pro-inflammatory and anti-inflammatory cytokine skew of iNKT cells, for clarity and simplicity we focus on IFNγ for TH1 and IL-4 for TH2, however if should be noted that iNKT's secret many other cytokines which are essential for their function and inflammatory skew [9]. This multilevel cross-talk can explain the duality of iNKT cell cytokine secretion, enabling dynamic and flexible responses to its lipidome environment (reviewed by [41,49]).

2) Lipid antigen structural properties: diversity of identified lipid antigens underscores CD1d's promiscuity

Most lipid antigens identified so far exhibit a similar structure: a polar head group coupled to two alkyl chains which are then split and inserted into the A' and F′ pockets of CD1d, inducing a conformational change (Fig. 2A). An ever-growing array of lipids continue being elucidated that all fit into CD1d (Fig. 2C-F). One study into the structural properties of CD1d's lipid antigens eluted all lipids from secreted CD1d molecules, followed by mass spectroscopy characterization [31]. The authors defined 177 lipid species, mainly phospholipids, and some sphingolipids. The tail composition of the identified lipids showed considerable variety, with the *sn*-1 carbon chain length ranging from 12 to 22, containing up to 7 double bonds. The *sn*-2 chain ranges from 14 to 24 carbons long with 0 to 6 double bonds. The most common chain length for sn-1 and sn-2 was 18 with a single double bond. Of note, several tail and head combinations might be considered 'unconventional' CD1d antigens, such as cardiolipins identified here and elsewhere, despite their four tail structure [31,50]. Furthermore, this study identified a cluster of lipid antigens that would be considered weak stimulators, with only a few regarded as stable complex forming antigens, and many that would be considered unconventional species, highlighting the subtle regulation of CD1d binding. This massive diversity is probably just the tip of the iceberg because lipid species, as do iNKT cells, also have tissue-specific signatures [35], that moreover, alter during disease [51]. We refer to other recent reviews for providing comprehensive overviews and comparisons of lipid antigens antigenicity [29,52–54].

3) Stability of lipid antigen binding: from sea to CD1d

Alpha-Galactosylceramide (αGalCer), or KRN7000, is a CD1d lipid antigen with potent ability to activate iNKT cells, originally and perhaps unexpectedly purified from the sea sponge *Agelas mauritaniu* (reviewed by [55]). Despite the evolutionary distance between sea sponges and vertebrates, it has provided this field with its most useful tool to date due to its potent antigenicity. The strength of αGalCer's antigenicity is due to the composition of its tails which, in this case, confer stability when in complex with CD1d [28,56,57]. The stability of the interaction between CD1d and its lipid antigen provides a key hint about what is driving the Th1/Th2 cytokine secretion of iNKT cells following the antigen-specific contact with LAPCs [58]. Therefore, it may not just be how the lipid antigen induces a conformational change as CD1d becomes antigen-loaded, but also how stable the interaction is between the lipid antigen and CD1d. Studies of LAPC - iNKT cell cross-talk collectively demonstrated that transient CD1d – TCR interactions can results in the secretion of IL-4 (Th2), whereas a sustained interaction induces both IL-4 and IFNγ (Th1 and Th2) [59,60]. Oki et al. show that the composition of length and saturation of the lipid antigen tails informs the type of interaction with a longer tail resulting in a more sustained stable interaction [60]. Indeed, most known or synthesized lipid antigens induce a Th2 dominant cytokine secretion with few stimulating a Th1 dominant response [60–63]. With the recent advancements of immunometabolism research, the overall picture emerges that each tissue has a resident population of immune cells able to provide dedicated and appropriate responses to stimulation. iNKT cells are no exception, as when presented with the same lipid antigen, iNKT cells isolated from different tissues produce a tissue-specific cytokine signature [9,64,65].

3. Attenuation of activation

Borrelia burgdorferi, the bacterial species causing Lyme disease, produces 2 closely related lipids with the same head group but two tail variations. It is antigenic when the tails consist of palmitic acid (sn-1), oleic acid (sn-2), named BbGL-2c. On the other hand, the related lipid species BbGL-2f, where sn-1 is oleic acid and sn-2 is linoleic acid has the reverse orientation when loaded into CD1d and is non-antigenic. The slight difference in lipid antigen composition highlights the sensitivity of this mechanism and indicates that the conformational changes induced in CD1d by the tails of the loaded lipid antigen inform the antigenicity of the interaction [28] (Fig. 2C-F).

The ability of CD1d to be loaded with a variety of lipid antigens irrespective of whether they are antigenic or not underscores the highly context dependent cytokine output by iNKT cells following cross-talk. Previously, we discussed how different lipid antigen species modulate Th1/Th2 bias; however, the presence and abundance of another class of antigens also modulates the mechanism. Sphingomyelins, a class of non-antigenic lipid antigen species, are required for the initial thymic positive selection of iNKT cells [60,61,66]. Furthermore, using in vivo experiments, elevated levels of specific sphingomyelin species in lysosomes resulted in defective iNKT cell development [22]. The same sphingomyelin species, C24:1 and C16:0, were also used in a co-culture competition assay against αGalCer, both species of sphingomyelin were able to out compete αGalCer, reducing the IL-2 secretion from DN32. D3's in a dose dependent manner [61,67] (Fig. 2E). As such, this example illustrates the context-dependent roles of lipid species and provides clear evidence that disruption of lipid species ratios can have significant consequences on the development and functionality of iNKT cells. Putting the above findings into context are several studies that show that in obese adults, the same sphingomyelin species, C24:1 and C16:0, are significantly elevated and associated with many of obesities co-morbidities [68–70].

4) Intracellular and extracellular pH: pH regulation of CD1d pocket volume and lipid antigen loading

Using known lipid antigens extracted from various sources has enabled us to learn more about the lipid antigen antigenicity requirements. CD1d moves from a highly acidic environment in the endosome to the relatively neutral environment when displayed at the cell outer membrane and back through the recycling pathway of the acidic lysosome [32,71–73]. Therefore, the effects that pH plays on the affinity and stability of the lipid antigens contained by the hydrophobic pockets of CD1d must be considered.

The pH of any microenvironment is crucial for enzymatic activity, protein folding, and protein-protein interactions [74,75]. Across the endosomal pathway, the different compartments ranging from early endosomes to lysosomes have specific pH's, allowing dedicated enzymatic and chaperone protein functions. A CD1d-specific example of this is Saposins A-D, essential lipid antigen exchange proteins with the dominant species being Saposin B [76]. Using a modeling approach, Cuevas-Zuvira et al. have shown that the lysosomal pH 4.5 is required for Saposin A to undergo a conformational change, correctly orient itself over the CD1d portal and facilitate lipid transfer [34]. Instead, at pH 7, the lipid antigen exchange is not possible due to Saposin A's parallel orientation with CD1d [34]. In addition, at pH 4.5, CD1d residues His68 and Asp80, around the portal opening become protonated and thus electrostatically positive, which the authors postulate might facilitate

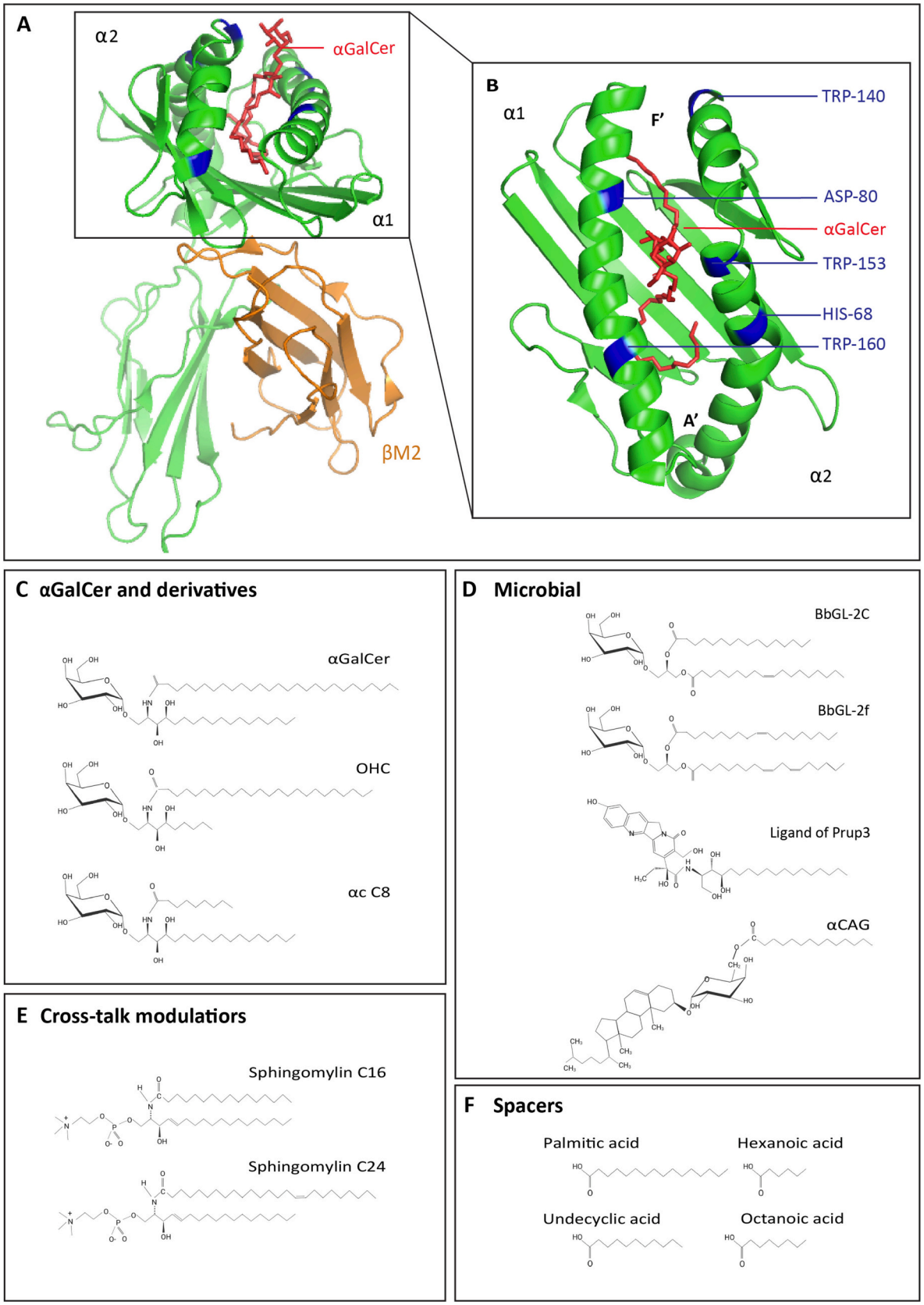


Fig. 2. Variations of CD1d antigens: A) Protein structure of CD1d loaded with aGalCer (red) B) Enlargement and top view of CD1d antigen portal pockets A' and F' loaded with aGalCer (red). Highlighted in blue are the residues, which play a role in the pH regulated loading of lipid antigens. Trp-140, Asp-80, Trp-153, His-68 and Trp-160. C) aGalCer and its derivatives OHC and ac:C8. D) CD1d ligands derived from microbial sources, BbGl-2C, BbGl-2f, Ligand of PruP3, and aCag. E) Cross-talk modulators Sphingomylin C16 and C24. F) Spacers, Palmitic acid, Hexanoic acid, Undecyclic acid, and Octanoic acid.

lipid antigen transfer even further, through stabilizing the lipid antigen head group once loaded [34](Fig. 2B). Complementing this work, another study shows that residues Trp153, Trp160, and Trp140 of CD1d induce conformational changes that cause an increase in volume of the hydrophobic A' and F′ pockets at pH 4.5 enabling the tails of the lipid antigen to be inserted, but not at pH 7 [42] (Fig. 2B).

4. pH-regulated loaded CD1d membrane distribution

The path that CD1d takes from protein synthesis and folding, lipid antigen loading, to display as lipid antigen/CD1d complexes at the cell surface, and back through recycling is highly regulated via vacuolar ATPase (V-ATPase). In a wet lab approach, H^{+}-ATPase inhibitors were used to neutralize lysosomal pH and subsequently assess the effect of αGalCer and a truncated version, (ac C8:0) on iNKT cell cytokine output [32]. Th1 (αGalCer) antigens were presented on the membrane grouped in cholesterol rich lipid rafts similarly to MHC class-II [77], whereas Th2 (ac C8:0) loaded antigens were more evenly distributed over the membrane [32]. This distinction acts as another layer of regulating Th1/Th2 cytokine output following cross-talk. Upon neutralization of lysosomal pH, membrane presentation of full-length αGalCer was drastically reduced, and no longer clustered in cholesterol rich lipid rafts, whereas the truncated version was observed with a two-fold increase at the membrane. To confirm that the stability and presentation of the lipid antigen are dependent on pH, the Th2-associated antigen (ac C8:0) was pre-stabilized in CD1d and transferred into a cell where endosomal pH had been neutralized. This complex could then be identified as being clustered in the cholesterol rich lipid raft areas instead of evenly spaced on the membrane [71]. This study together with others also show that the truncated αGalCer, ac C8:0, frequented the lysosome much more often than the full-length version, possibly due to the shorter version's receptor independent uptake [30,78]. Altogether, these data support that a combination of an increase of competitive lipids and acidic pH increases displacement in the lysosome during recycling. This intriguing mechanism leads us to speculate that an alteration of lipid species acquired from the environment during the CD1d recycling steps could lead to modulated cross-talk (Fig. 3) (reviewed by [30]).

Many studies and reviews have focused on finding highly antigenic lipid antigens; however, there are also lessons to be learned from many "unusual" lipid antigens. An example of this comes from *Helicobacter pylori,* which produces a Cholesteryl-glucoside lipid that despite its massive head group can be loaded into CD1d and for which CD1d-tetramers were made to successfully isolate a subset of iNKT cells [79,80]. Furthermore, this peculiar lipid antigen steered the iNKT cells towards Th1 polarization with IFNγ cytokine output [79]. A recent paper presents a conformational dynamics analysis approach; they assess the stability of the complex formed between various lipid antigens and CD1d under neutral (pH 7) and acidic (pH 4.5) conditions [34]. The

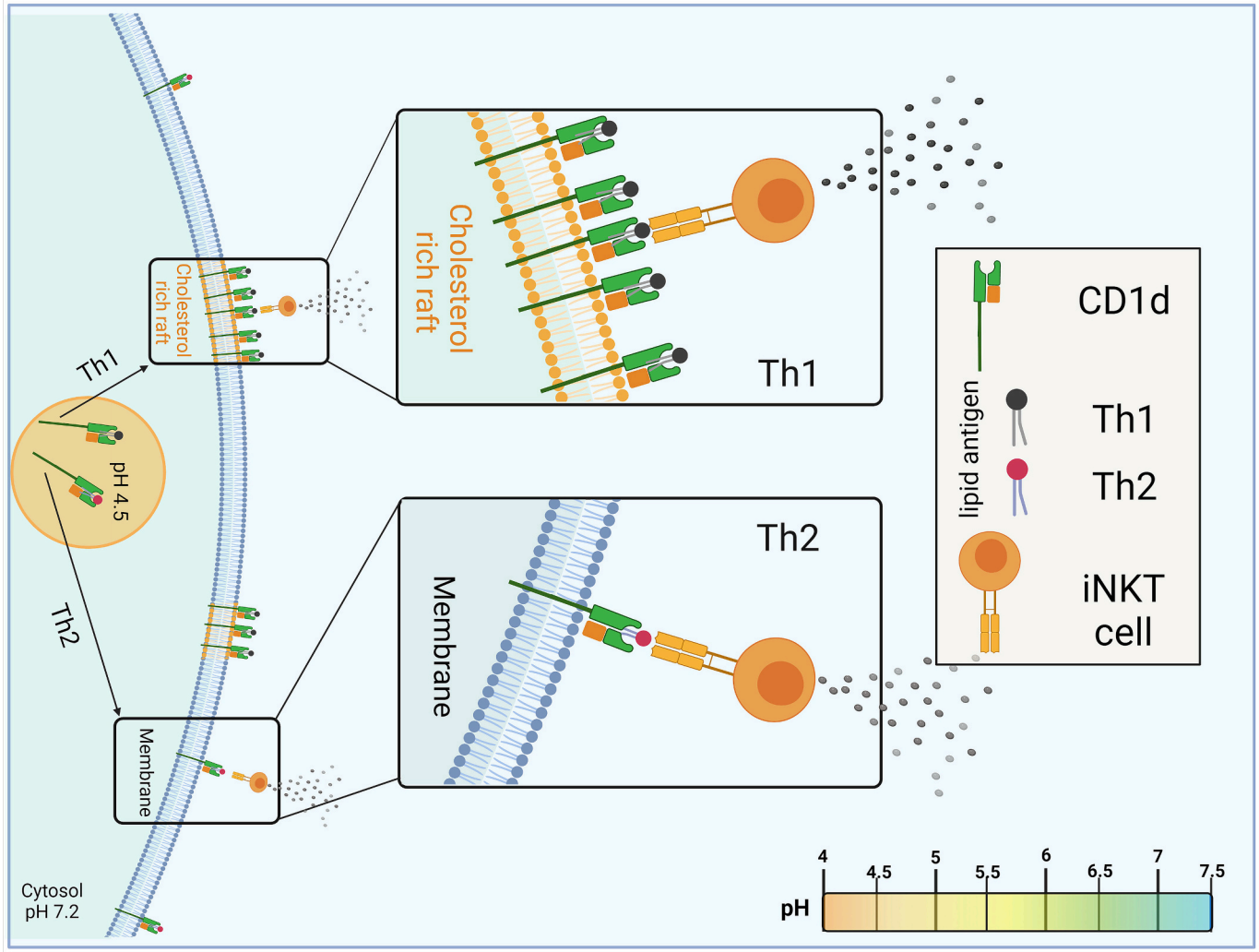


Fig. 3. pH regulated lipid antigen membrane distribution CD1d: The pH of the lysosome ensures that antigens associated with Th1 response following cross-talk with iNKT cells are transported to and presented at cholesterol rich membrane rafts clustering them together. Whereas antigens associated with Th2 cross-talk outcome are more evenly distributed throughout the cell membrane.

natural lipid antigen of Prup3, which has a similar cholesteryl ester head group and structure to the one extracted from *Helicobacter pylori*, was shown to also form a complex with CD1d and induce a robust iNKT cell cytokine response. In this work, the antigen tails appeared important for antigenic interaction rather than the massive head group. However, and most importantly, this study showed that while it is possible to load this lipid antigen, the interaction is highly unstable at any pH. Thus, using very high concentrations of lipid antigens during in vitro experiments or pre-loaded CD1d tetramer capture experiments allows us to understand the capacity of CD1d in its extremes. Whether this reflects in vivo conditions will require further research (Fig. 2C-F).

5) Intracellular and extracellular lipid environment: further expansion of CD1d's repertoire

There are several ways in which lipids can pass the cell membrane to be internalized. While we focus on the adipocyte here, it should be noted that many of the CD1d-antigen presentation mechanisms are likely to be conserved among a variety of cell types (reviewed by [15,81]). What has become clear is that long-chain fatty acids usually require a scavenger receptor to pass through the cell membrane, most notably CD36 [82,83]. CD36 has also been found in endosomes, with elevated expression levels associated with type 2 diabetes [84]. Short-chain fatty acids, on the other hand, can flip-flop across the membrane, and studies have shown that the shorter and more saturated the chain, the faster flip-flop occurs [85,86]. One consequence of FFA internalization is acidification of intracellular pH. This acidification was noted in several settings and appears to be easily reversible when BSA or albumin is added to the cell culture, except for dimerized FFA's, in which case acidic effects do not appear to be reversible [87]. Although the flip-flop mechanism has not yet been studied in obese adipose tissue, we speculate that increasing extracellular FFA's might play a role in CD1d loading and presentation of lipid antigens.

The precise lipid antigen content buried in CD1d pockets has proved elusive, but in some cases provided insight into functionality. Initially, it was assumed that lipid antigens acted independently; however, on closer inspection of crystallized CD1d, in native refolding conditions with short-chain α-galactosylceramide, a small short chain lipid was detected deep in the A' pocket. It was assumed that this small lipid was a contamination and remained overlooked for many years, but it is now suspected that this lipid, along with other FFA's, might act as spacer. How spacers are inserted into the A' pocket remains to be elucidated, however, we speculate it is likely to involve hydrophobic interactions. Due to the requirements of lipid antigen stability once loaded into CD1d (as discussed above), a spacer might make up for the short tail or even lack of one tail enabling previously non-antigenic lipids to become antigenic by filling one of the hydrophobic pockets, and thereby facilitating the essential conformational change upon binding [32,34,88–92] (Fig. 2F). During CD1d recycling, spacers pass through the lysosome, which has a lower pH than the initial endosome and contains many extracellular milieu-derived lipids acquired via scavenger receptors, low-density lipoprotein-receptors, or flip-flop (Fig. 1A) [32]. These additional extracellular lipids may re-tune the focus of CD1d from exposing endogenous lipids to exposing lipids captured from the extracellular environment. Furthermore, these extracellular exogenous lipid antigens have the potential to outcompete the initial lipid antigen if they have a higher CD1d affinity. As such, iNKT cells are exposed to antigenic lipids not only from the LAPC but also the local lipid environment. This dual sourcing of lipid antigens highlights the dynamic and flexible nature of iNKT cells response.

5. Phenotype of AT-resident iNKT cells

The various adipose tissue depots have come into the spotlight due to our increasing understanding of adipose tissue and its local tissue-resident immune cell populations [18,93]. CD1d is expressed by LAPCs, which has expanded for adipocytes their function as we understand it to include lipid antigen presentation besides lipid storage and adipokine secretion [41]. iNKT cells express the transcription factor promyelocytic leukemia zinc finger (PLZF) which is essential for their rapid response following stimulation [94,95]. In perinatal mice adipose tissue-resident iNKT cells express PLZF, during maturation PLZF is downregulated [9]. Adipose resident iNKT cells instead express E4BP4, induced by uptake of FFA's, which drives IL-10 production upon cross-talk stimulation [96]. A recent publication underscores the possibility that FFA uptake by splenic iNKT cells, in particular palmitic acid, induces downregulation of PLZF and induction of E4BP4 [9]. Adipose tissue-resident iNKT cells are the predominant source of secreted IL-4 in healthy tissue and work to maintain the inflammatory balance. On the other hand, iNKT cells can also secrete IFNγ and IL-17-family cytokines [9]. Thus, the plasticity of iNKT cells influence on inflammation balance is supportive to rapidly modulate the inflammatory environment depending on their stimuli. Whether AT-resident iNKT cells are predominantly Th1 or Th2 in the context of obesity has provided some controversy over the years, this could be due to differences in diet composition, microbiota, mouse breeding background or control group [6,7,10,11,97]. Overall, most studies agree that AT-resident iNKT cells are the primary source of circulating IL-4 in lean adipose tissue, putting them in the anti-inflammatory Th2 branch (reviewed in context [41,98–102]). However, the intrinsic ability of iNKT cells to secrete cytokines of Th1, Th2 and Th17 polarity at the same time suggests that internal and/or external stimuli can potentially direct the cytokine output, thereby contributing to the local inflammatory environment observed in AT of obese individuals [9,64,100].

6. The impact of obesity on adipose iNKT's

Obesity affects many aspects of the body, including the intestinal microbiota composition which plays a large role in lipid metabolism and absorption [103] dysbiosis, obesity and T2D (reviewed [104,105]). Bacterial species can both enhance (*Lactobacillus brevis and Streptococcus thermophilus*) and reduce (*Lactobacillus rhamnosus*) the availability of neutral sphingomyelin lipid antigens present in the gut [106], and have been found to contain sphingolipids which act as modulators of iNKT proliferation during pregnancy (*Bacteroides fragilis*) [107]. Bacteroides are an abundant component of a healthy microbiome, with sphingolipids making up a large portion of their membranes. Their increased abundance has been correlated with inflammatory bowel disease and inflammation [108] and its elevation in the gut being associated with accelerated obesity [109,110]. The gut microbiome undergoes significant changes during obesity, which in turn alters the lipid profile of both the intestines as well as systemically [111,112] aggravating inflammation [113,114] and likely affecting the lipid antigen repertoire available for CD1d-mediated presentation to the intestinal resident iNKT cells [115,116]. Obesity [117–119] and hyperglycemia [120] have also been shown to decrease barrier function increase the permeability of the intestines allowing the microbiota to pass through and colonize tissues and organs [121]. Anhê et al. demonstrated that morbidly obese individuals with T2D have significantly reduced diversity in their tissue microbial profile when compared to morbidly obese without T2D [121]. Furthermore this study shows preferential colonization of liver, subcutaneous and visceral adipose tissue as well as finding a significant increase of *Bacteroides* abundance in mesenteric adipose tissue in obese individuals with T2D [121], which as discusses above are associated with elevated inflammation.

The display of lipid antigen/CD1d complexes at the adipocyte plasma membrane drives iNKT cell activation in adipose tissue [6,7]. Adipocyte-specific CD1d knockout studies consistently deplete the local iNKT population, leading to a pro-inflammatory glucose intolerant environment irrespective of dietary intervention [6,122,123]. It was assumed that obesity leads to a reduction of CD1d expression on adipocytes, thereby causing the decimation of the adipose tissue-resident

iNKT cell population. However, while there is indeed less CD1d expressed and presented following a high fat diet, it is not completely abolished [122]. Studying adipose tissue-resident iNKT cells during the course of obesity development has been challenging due to their modest cell numbers, which rapidly decreases further during the initial stages of obesity onset [6,7,18,124]. To address this challenge, we recently developed a *semi*-ex-vivo assay to assess adipose tissue-iNKT cell cross-talk, our data demonstrates that co-culturing ex-vivo AT-iNKT cells with lipid loaded insulin resistant adipocytes results in a decrease of IL-4 and an increase of IFNγ secretion [64]. It has also been noted that tissues and organs have site-specific lipid profiles, highlighting the different metabolic needs of various tissues [125]. During obesity, the abundance and ratios of stored and circulating lipid antigens and lipids, which could act as spacers, co-stimulators or signaling molecules, among other functions, increases and alters [126,127]. We hypothesize that with recent research showing obesity driven global pH changes [128], it follows that the abundance of lipid antigen species available also influences the likelihood that a stable complex will be formed and presented. Furthermore, the dysregulation in acidic homeostasis seen in many diseases may impact the ratio of which lipid antigens can be loaded [129–133]. This mechanism might not only take place in adipocytes, but all APC's expressing CD1d, and therefore might be relevant for NKT cell activation by vaccination, infection or even cancer [57,134–137]. Therefore, whilst the mechanism of LAPC - iNKT cell cross-talk is consistent, the content of CD1d is most probably dictated by the lipid antigen cargo of the cell that expresses it.

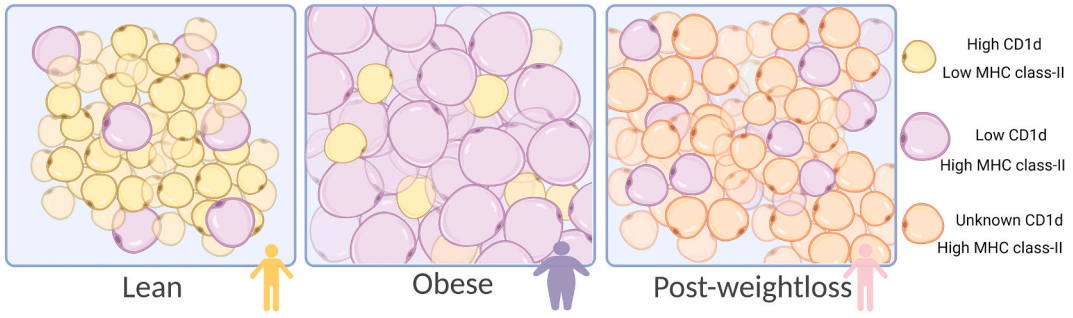
7. Concluding remarks

While many mechanisms of lipid antigen/CD1d antigen presentation appear conserved to all LAPCs, we described that CD1d-mediated antigen presentation can be modulated depending on the demands of the tissue location. We provided an overview of mechanisms that partake in the dysregulation observed in LAPC - iNKT cells during obesity. As we have seen, Th2 output requires a much shorter cross-talk interaction with the iNKT cells TCR, whereas Th1 output requires a more sustained interaction. We discussed that CD1d lipids linked to a Th1 response localize to cholesterol rich lipid rafts in the membrane, whereas Th2 associated loaded lipid antigens are excluded from these areas, in a pH-regulated manner. Finally, it is becoming more apparent that even if the antigen is not a snug fit for CD1d, if enough is available and in the right conditions, e.g. pH, spacers and abundance, then it could potentially be loaded and induce iNKT cell activation [138–140]. Taken together, we speculate that regulation of adipocyte pH, both intra- and extra-cellular, could provide an interesting avenue of research. We propose that there is more to CD1d-iNKT cell cross-talk than individual lipid antigens; instead it involves a reactive mechanism that acts as a local environmental sensor of lipids. By combining lessons from lipid antigens considered as weak stimulators and novel omics-type techniques, we may gain insight into the regulation of this mechanism. This could hold the key to identifying the lipid antigens of CD1d with the potential to accurately predict targetable lipid modulating biomarkers or beneficial CD1d lipid antigen cocktails.

8. Future perspectives

We are currently seeing a concerning increase in obesity globally, while efforts to develop a weight loss intervention that is long lasting has been elusive. One of the key reasons for this seems to be that obesity elicits a permanent change in adipocyte function (reviewed [141]), e.g.

Box 1 (Fig. 4)



Adipocyte MHC class-II presentation in obesity

While not being part of the hematopoietic system, adipocytes share several characteristics with professional APCs [141], including the ability to present peptide and lipid antigens. In comparison to MHC class-I/II peptide binding, the interaction between CD1d and its lipid antigens is usually less stable and more transient at the cell surface (Fig. 1A-C) [146]. Peptide antigen presentation by MHC class-I and MHC class-II molecules has been studied in many cell types (reviewed by [147]). Both MHC class-I and MHC class-II are expressed by adipocytes and have been linked to adipocyte dysfunction, particularly during obesity [148] (Box 1). While initial studies on the function of CD1d focused on traditional APC of both myeloid (monocytes, macrophages, dendritic cells) and lymphoid lineage (B lymphocytes, thymocytes but not mature T cells) [3], functional CD1d is also expressed by other cell types including adipocytes (reviewed by [150,151]). CD1d, a molecule that is dedicated to presenting lipid antigens, has long been deemed "MHC class-I like" due to the commonalities in structure and processing ([152] reviewed by [7]) (Fig. 1A,B). However, we can also see many similarities with MHC class-II (reviewed by [153,154]) (Fig. 1A,C). Adipocyte communication with immune cells is not limited to CD1d, adipocyte MHC class-II expression plays a curious role in obesity. Several studies have shown that MHC class-II adipocyte expression and presentation are consistently elevated and sustained during obesity [149]. However, it appears that it is the overall size of adipocytes, irrespective of diet, that modulates MHC class-II expression [155]. Adipocyte MHC II induces IFNγ secretion by $CD4^+$ T cells, which feeds back to the adipocyte, increasing adipocyte MHC class-II expression. Additionally, it has been suggested that free fatty acid (FFA) secretion by obese adipocytes can also stimulate MHC class-II expression in a JNK-STAT1 manner as well as inhibiting V-ATPase function [155]. Of note, following weight loss, it has clearly been demonstrated that many of obesities comorbidities are alleviated [156]. Nevertheless, some alterations persist, e.g., elevated MHC class-II adipocyte expression, showing a permanent change to the adipose tissue, and thus its resident immune population.

MHC class-II expression, see Box 1 (Fig. 4). Weight loss does shrink the size of obese adipocytes, but it does not reduce their increased number, who in turn secrete reduced adipokines and leptin when compared to weight matched counterparts [142,143]. In a recent publication, researchers found lipid metabolism is subject to circadian rhythm and speculate that this could help us understand better the link between consumption time, diet, obesity, and its comorbidities [140,144,145]. Combined, this research provides early insights at the potential to predict lipidomic profiles containing beneficial lipid antigens that could skew the inflammatory balance advantageously, enabling iNKT modulation in both health and disease.

Declaration of competing interest

The authors report no conflict of interest.

Data availability

No data was used for the research described in the article.

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