Lipids in immunometabolism:

From lipid handling to lipid antigen presentation and back

Imogen Morris

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Lipiden in Immunometabolisme:

Van Lipidenverwerking tot Lipide-antigeenpresentatie en Terug

(met een samenvatting in het Nederlands)

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Imogen Morris

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Promotor:
Prof. dr. B.M.T. Burgering

Copromotor:
Dr. E. Kalkhoven

Beoordelingscommissie:
Prof. dr. C.R. Berkers (voorzitter)
Prof. dr. P.J. Coffer
Dr. N. Eijkelkamp
Prof. dr. K.F. van de Graaf
Dr. ir. Stienstra

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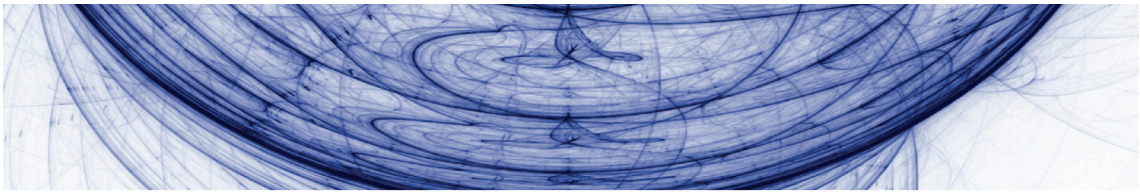
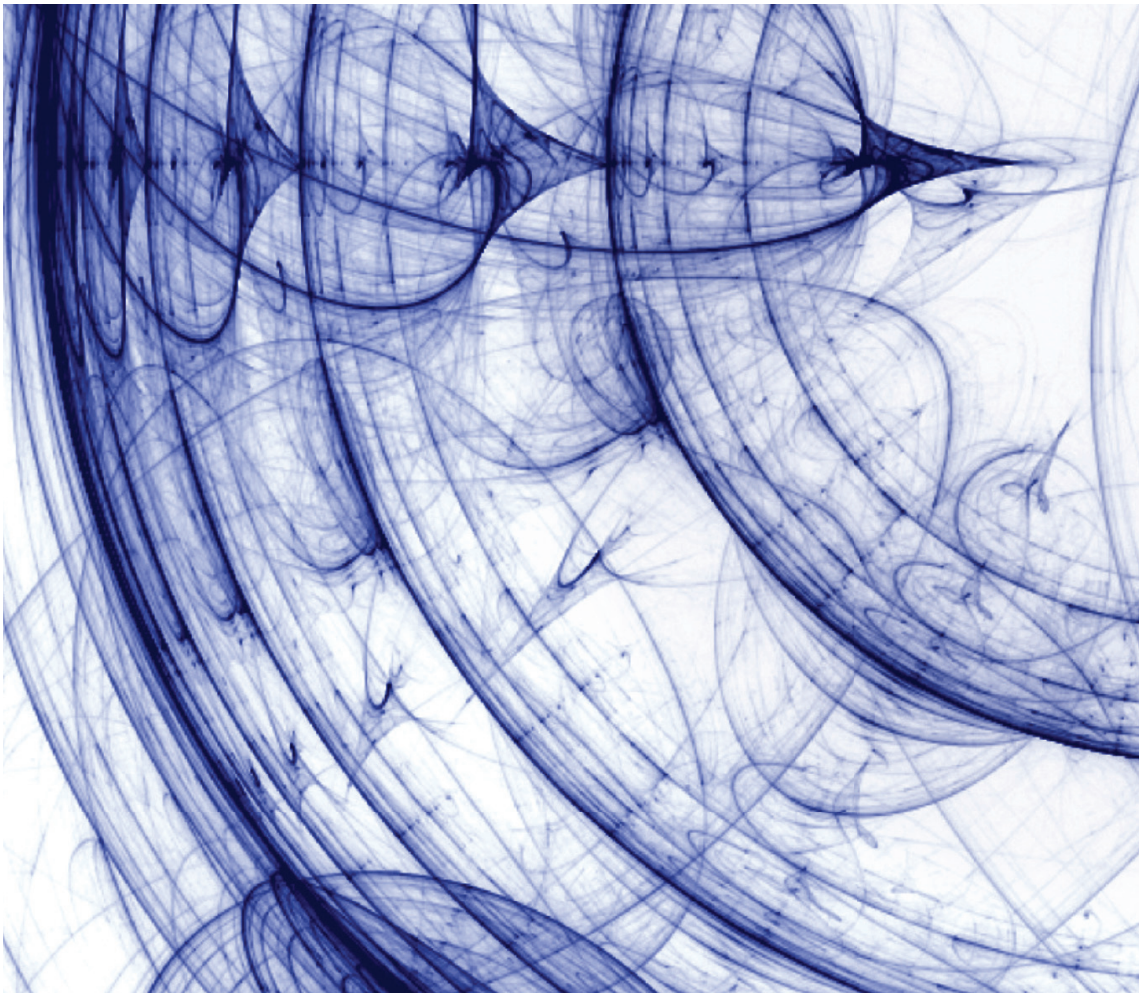
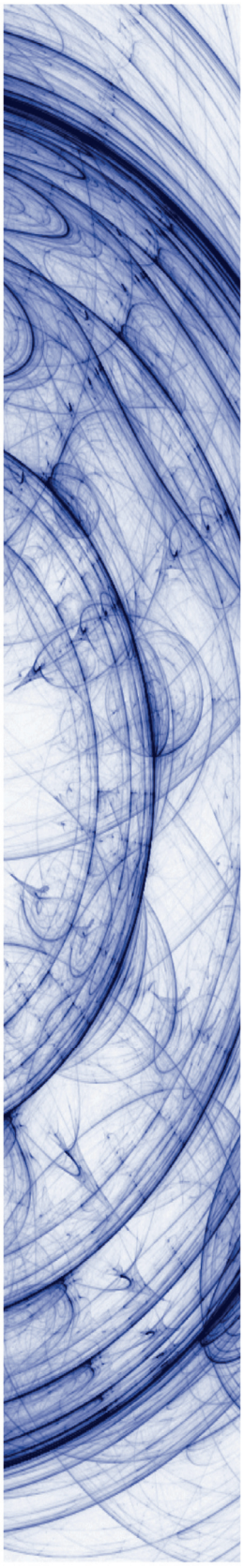


Chapter 1



General Introduction



Lipids and lipidomes

In contemporary biological research, exploring multifunctional molecules has become an area of great interest to the scientific community, offering a nuanced understanding of intricate cellular processes and organismal functions. Among these, lipids emerge as key players, showcasing their versatility as multifaceted molecules [1]. The significance of lipids extends beyond their traditional roles as mere components of cellular membranes, as emerging research unveils their involvement in diverse molecular and physiological processes[2]. Notably, lipids contribute not only importantly to cellular structures, but also to signaling pathways, and, critically, the regulation of energy storage and utilization, and even serve as antigens [3]. The uptake, storage and release of lipids, but obviously also their biosynthesis, interconversion and degradation, which all together can be referred to as 'lipid handling', are therefore all key elements in regulating the dynamic interplay between the different biological structures and processes in which lipids play key roles.

The term "lipidome" refers to the complete set of lipids present in a biological system, such as a cell, tissue, or organism, under specific physiological or pathological conditions. Lipidomes encompass a wide range of lipid species, including but not limited to fatty acids, phospholipids, glycolipids, and sterols [4]. The significance of the lipidome lies in the complexity and diversity of its constituent lipid species, the varied lipid species within the lipidome provide a nuanced toolkit that allows cells to tailor their functions to specific needs[1], [5], [6]. This adaptability is for example crucial for modulating membrane properties, ensuring the integrity of cellular structures, and fine-tuning the functions of distinct cellular compartments [3]. Moreover, the myriad lipid species serve as a reservoir of functional diversity, enabling cells to respond to dynamic environmental cues efficiently[1], [7], [8]. The complexity of the lipidome, therefore, emerges as a key factor in the orchestration of cellular processes [9].

Analytical chemistry approaches through which lipidomes are identified – predominantly NMR and LC- MS– are referred to as lipidomics, and together with computational tools, have enabled us to delve into the complex biology of lipidomes. Publications showing how circadian rhythms [10]–[12], genetics, diet, and sex influence lipidomes [1], even down to the level of site-specific lipid profiles (global to organelle) [13], have provided a wealth of knowledge and highlight the essential role an individual's lipidome has on health and disease [13]. Rapid advancements in lipidomics have also informed many fundamental scientific disciplines. These disciplines range from cell biology, as lipidome composition is essential for cell structure and membrane dynamics and integrity, to metabolism, centering around the central role of lipids in

energy homeostasis, to signal transduction and even immune responses. On a more translational level, changes in the lipidome can be used as biomarkers for disease and inform treatment type[5], [9].

Advancing biotechnology is beginning to combine our snapshots of lipidomes into functional data providing insights into therapeutic targets as well as allowing us to manipulate the lipidome itself. Understanding lipid metabolism with the potential to engineer lipid profiles can lead to a more holistic yet direct approach in many fields.

Lipidomes can be viewed in many ways and from many angles. For example lipidome composition informs many essential biological functions, nutrients and fuel, signaling factors, structure and conformation and storage. Recent large scale lipidomic studies have demonstrated how complex lipidomes can be [1], [7], [14]. Furthermore, organ- and organelle-specific lipidomes are affected differently by sex, genetics and environmental influences, leading to the development of interventions more suited to these factors. An example of common structures comprised of differing lipid species of the same lipid class depending on location is the mitochondria and its cardiolipins [15]–[17]. Cardiolipins are a class of lipids whose structural properties are essential for mitochondrial ultrastructure and so respiration, comprised from 4 fatty acids attached to a single glycerol head group, they are necessary for mitochondrial cristae conformation. In some cases organs have been shown to have a cardiolipin-specific fingerprint with defined fatty acid species though to be defined by metabolic requirements [15]–[18]. This specificity on the level of location and lipid species suggests that, although there is functional redundancy between similar lipid species in that they can perform the same function in a different location, suboptimal lipidomes could cause reduced resilience due to lack of lipid species diversity creating points of stress.

Adipocytes: specialized lipid handling cells

A cell type intimately linked to lipids is the adipocyte, a specialized cell type dedicated to lipid storage and release [19]. In mammals, different types of adipocytes have been specified, with the white and brown adipocyte being the best known [20]. While white adipocytes are dedicated to the storage of lipids and release in time of need, brown adipocytes can dissipate energy through uncoupled respiration and heat production (thermogenesis). Both white and brown adipocytes store lipids as triglycerides in lipid droplets (LDs), specialized cell organelles that will be discussed in more detail below. Mammalian white adipocytes contain a single large lipid droplet, and are therefore referred to as unilocular, and brown adipocytes contain multiple LDs per cell and are referred to as multilocular. Apart from storage and release, the process of

adipocyte differentiation as well as their functionality is regulated by lipids through the transcription factors PPARγ, exemplifying yet another layer of the intimate connection between lipids and adipocytes [21]. Interestingly, PPARγ also plays an important role in various immune cells [21], while LXR, another member of this transcription factor family that is activated by cholesterol-like ligands, is also functional in both adipocytes and immune cells. A further layer of lipid-driven activity in adipocytes is formed by the ability of adipocytes to interact directly with immune cells through lipid antigen presentation, which will be discussed in detail below. Together with the secretion of adipokines, this lipid antigen presentation pathway allows adipocytes to communicate with other cells and organs in the body, which puts them at the center of immunometabolism, the interface between the immune system and metabolism [PMID: 29328913]. Taken together, white adipocytes exhibit multiple lipid-driven functions and characteristics, including storage, signaling, and immune functions, through which they can adapt to specific physiological conditions [22]–[25].

Lipid Droplets: where immune cells (again) resemble adipocytes

Lipid droplets (LDs) are dynamic organelles found in a variety of cell and species types, from archaea to animals, playing a crucial role in the storage and mobilization and homeostasis of lipids[26]. Initially considered inert nutrient storage organelles, recent research has unveiled their significance as essential modulators and regulators of local and global health [27], [28]. The discovery of LDs dates back to 1886 when Edmund Wilson observed "oil-drops" speculated to be passive components in cell functions [29]. Technological advancements in electron microscopy led to the identification of these conserved organelles in protozoic cells[30]. In the 1970s, Aubie Angel's pulse-chase experiment in rat adipocytes revealed the movement and storage of triglycerides in LDs [23]. The history of LDs is intertwined with the evolution of cell biology, with their functions now recognized to extend beyond lipid storage[31].

The evolution and diversification of LDs can be observed throughout the evolutionary tree, where they likely served primarily in energy storage gaining functions as complexity increased[26]. In plants, LDs play crucial roles in lipid synthesis and storage for seed germination and pollen tube growth, as eukaryotes diversified, LDs acquired more complex roles in different species and cell types. In animals, LDs have adapted to meet the specific needs of various cell types, regulating membrane composition, being hubs for signaling molecules, aiding in cellular stress tolerance and metabolic regulation. LDs exhibit diversity in morphology and composition, with each cellular context favoring specific lipid species storage. The adaptations of LDs in different

species have been driven by selection pressures, enabling them to perform site-specific functions [26], [30], [31].

Lipid droplets (LDs) are comprised if a mono layer phospholipid membrane encircling a core of neutral lipids, cholesterols and proteins, and are present in a broad variety of cell types including adipocytes and hepatocytes [32]. LDs come in many shapes and forms, from the large LDs in white adipocytes mentioned earlier that occupy almost the total cellular space, to the minute ones found in the Golgi and ER during their biogenesis [26], [33]. Next to their secretory phenotype and the ability to present peptide and lipid antigens, LDs present yet another level where adipocytes and immune cells share functional characteristics, as for example macrophages can also store lipids in LDs [32]. Lipid droplets have been shown to play a crucial role in immune cell functions by serving as dynamic storage depots, ensuring a readily available energy source during periods of increased metabolic demand. Lipid droplets serve as signaling platforms, actively participating in immune responses by modulating intracellular signaling pathways [32], [34]. The dynamic regulation of lipid droplets in immune cells underscores their importance in supporting the diverse and energy-intensive functions of immune cells, highlighting the intricate interplay between lipid metabolism and immune system activities.

The lipid species content of lipid droplets play many biological roles, with many linked to discrete nutrient sensing pathways [35], [36]. Therefore, the ability to modulate lipidomes holds significance as it offers a means to tune cellular functions, impacting crucial processes such as membrane structure, energy metabolism, and signaling pathways. Indeed, LD's are now being recognized as biomarkers with lipid profiles being associated with many phenotypes in both health and disease [33]. LD's therefore provide vital information about the nutrient and health status of each cell.

Adipose resident iNKT cells: an immunometabolism interface

While adipocytes and immune cells share functional characteristics, direct and indirect crosstalk between these cell types has also been identified on multiple levels, with the adipocyte-invariant natural killer T (iNKT) cell interaction providing an interesting example of direct crosstalk. Invariant natural killer T cells sense lipid antigens presented via CD1d via their semi-invariant TCR, and this crosstalk stimulates a cytokine secretion often linked to the species of lipid presented [8], **Figure 1**. iNKT cells have the capacity to secrete a complex cocktail of cytokines, including anti-inflammatory

cytokines like IL-10 and IL-4 and pro-inflammatory cytokines like IFNγ, defined by the environment of their residency [8], [37]–[40]. As many lipid types have been shown, and many more predicted, to be loaded and presented via CD1d the cross talk therefore depends on local lipidomes, allowing iNKT cells to act as nutrient sensors[41].

The disappearance of adipose resident iNKT cells is viewed of as one of the canary warning signs that the adipose tissue has become dysfunctional due to excessive lipid storage [42], [43].Initial controversy on this could quickly be attributed to experimental differences [37]. Feeding B6 mice a high fat diet (60% western) results in many pathologies: after four weeks hyperglycemia and elevated fasting insulin levels develops, at 16 weeks the adipose tissue is drastically altered, with hyperplasia (increaser in adipocyte numbers) and hypertrophy (increase in lipid content per adipocyte) causing a 30% increase of adipose tissue, ectopic lipid storage, diabetes and hypertension [44]. After two weeks of high fat diet the adipose iNKT population already significantly decreased, and by ten weeks their population is negligible [42], [43]. A similar but less rapid and severe decline can be observed in other tissues such as the liver, spleen and blood, a phenotype reflected in obese humans [42]. It has been speculated that the obese adipose tissue creates an inhospitable environment for resident iNKT cells [45], [46]. Indeed, parabiosis experiments show that the adipose tissue is not repopulated by iNKT cells until after weight loss [42]. Further studies show that during the weight gain/iNKT decline phase of obesity, the iNKT cells change phenotype, skewing from anti-inflammatory to pro-inflammatory [38], [39], [47]. As this demise appears to be a critical turning point for the whole adipose tissues homeostasis as well as global inflammation we endeavored to develop several model systems which have allowed us to delve into the intricacies of specific areas of this demise. Understanding the mechanisms underlying the sensitivity of iNKT cells to lipid environments could provide valuable targets for therapeutic interventions to mitigate the impact of obesity-related diseases. Further research into the role of iNKT cells in adipose tissue and their interactions with other immune cells may offer promising avenues for future treatments.

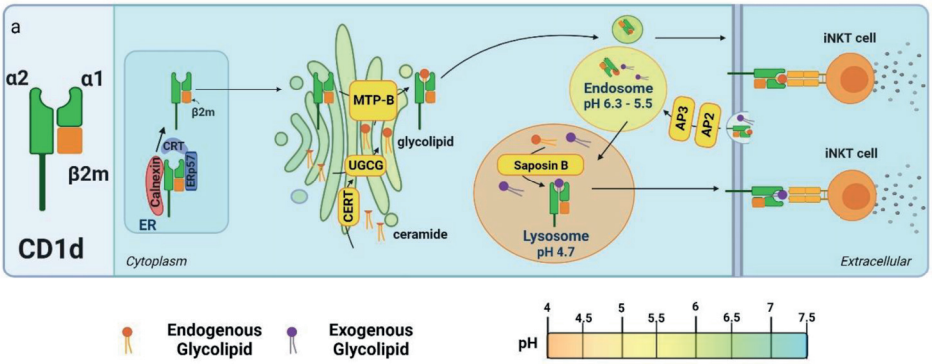


Figure 1. Schematic representation of adipocyte crosstalk with iNKT cells via adipocyte CD1d loaded with a lipid ligand and iNKT cell's TCR and the regulation of CD1d- mediated lipid antigen presentation to iNKT cells. Following crosstalk, the stimulated iNKT cell secretes various cytokines, which in turn contribute to the regulation of other immune cells as well as the adipocyte its self. This feed forward loop mechanism plays an essential role in the inflammatory balance, both on a local adipose tissue level as well as on a more global level. 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 - 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. **See chapter 6a for in depth review on lipid antigens.**

Pseudokinases: regulators of adipocyte and immune cell function

Next to cytokine/adipokine production, antigen presentation and LD formation being shared by adipocytes and immune cells, a yet understudied class of intracellular proteins called pseudokinases also seems to play an important role in both cell types. Pseudokinases are a class of highly conserved kinases which have lost their enzymatic function, instead acting as scaffolds, competitors, and anchors, making them important regulators or modulators of signal transduction pathway. Pseudokinases lack the residues required to interact with ATP and therefore cannot phosphorylate substrates [48]. Instead, they work as scaffolds, competitors, and modulators of protein-protein interactions (reviewed [49]). The loss of catalytic function through evolution is not limited to pseudokinases, but can also be found in pseudophosphotases [50] and pseudoproteases [51], via loss of canonical residues alternative confirmation of the catalytic site renders the kinase pseudo. In the case of the Tribbles family it is suspected that the co-evolution of amino acids distal from the now defunct active site facilitates non-catalytic roles [52], [53]. Computational protein-protein interaction analysis highlights the promiscuous pseudokinase family TRIBBLES (1-3) as having unique highly conserved conformations making them particularly relevant for drug development

[49], [54]–[56]. A previous study assessing the effects of standard vs intensive treatments for T2DM comparing Trib3 to SNP (c.251 A>G, Gln84Arg, rs2295490) found that this mutation should be taken into consideration for treatment of T2DM glycemic control because for some co-morbidities specific treatments significantly increased the detrimental outcome for the patient [57].

The family of Tribbles pseudokinases are highly conserved throughout the evolutionary tree of life, with Tribbles 2 being the earliest emerging and present in early metazoans and Tribbles 1 and 3 arriving much later in species that would derive the vertebrate linages. Tribbles 3 was the last to evolve and is mainly found in mammalian species where it has been found to have multiple roles. It is therefore speculated that the latter Tribbles played a role in the evolution of more complex interaction networks necessary for the evolution of the higher organisms. These context dependent roles mean Tribbles have been identified as regulators of many key cellular processes from balancing differentiation and proliferation to behaving as a nutrient sensor influencing metabolism and cellular stress responses and immune function, [58]. Tribbles 3 (Trib3) has a widespread expression (including liver, adipose, heart, kidney, small intestine, stomach, skin, and denervated skeletal muscle [59]) and has been implicated in wide variety of physiological and cellular processes in different cell types and organs [60], [61]. As a consequence, altered expression or activity of Trib3 has been associated with multiple diseases, including metabolic diseases [62](reviewed [63], [64]), **Figure 2**. Tribbles 3 had been noted to modulate the MAPK[65] and PI3K/Akt pathways in early research[66], [67], where it acts as a nutrient sensor for starvation, and has therefore been an interesting target for many disease progression studies. Tribbles 3, has been shown to play a diverse set of rolls, often linked to cellular stress due to lack of resources[68]–[70], and so has been implicated in many disease types ranging from type 2 Diabetes Mellitus to many forms of cancer[71]–[73]. In **chapter 4** we show how the many interaction partners of Tribbles 3 provides insights into the molecular mechanisms by which Tribbles 3 exerts its effects on so many levels of cellular function[74]. In 2016 Stevenson et al., showed that elevated TRIB3 induces macrophages to become foamy, speculating that this is due to TRIB3 reprioritizing macrophages roles, making lipid accumulation a higher priority than working as an inflammatory immune cell[75]. Gaining understanding on Tribbles 3 role in lipid handling and homeostasis will provide avenues into the underexplored influence of lipidomes on immunity. Manipulating Tribbles 3 emerges as a promising strategy, holding the potential to fine-tune immune responses and address associated disorders with precision and specificity.

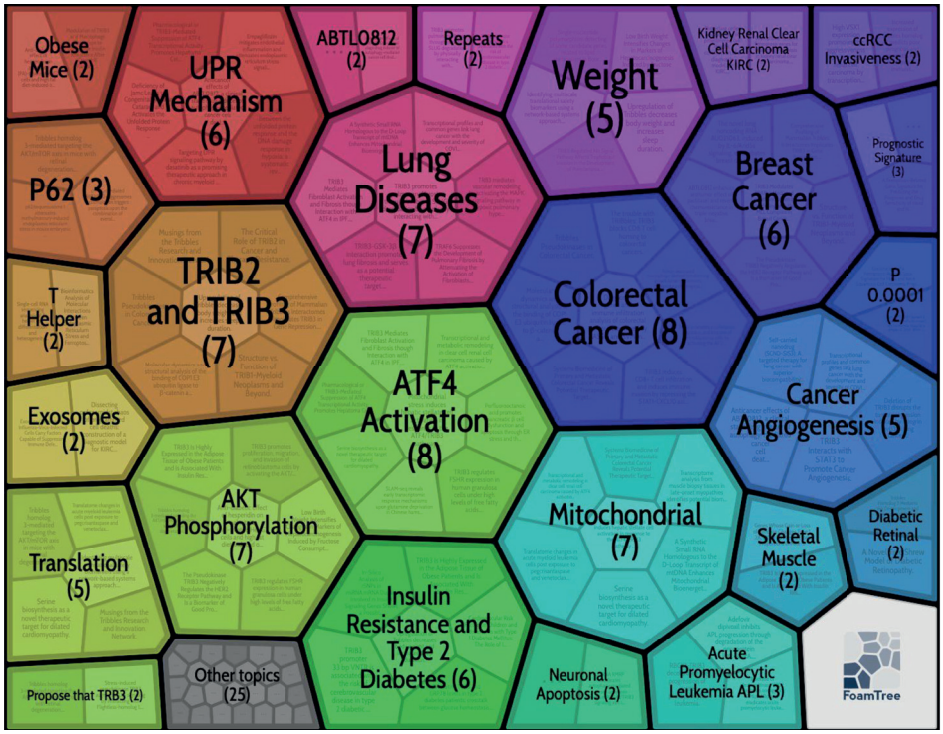


Figure 2. Carrot² is an open source search results clustering engine it organizes your search results into topics. Search term: Trib3. Associated publications in brackets next to cluster term Carrot2 Osiński, S., Weiss, D. (2005). Carrot2: Design of a Flexible and Efficient Web Information Retrieval Framework. In: Szczepaniak, P.S., Kacprzyk, J., Niewiadomski, A. (eds) Advances in Web Intelligence. https://doi.org/10.1007/11495772_68

Highly Conserved Mechanisms in Organisms: Lipidomes and Pseudokinases provide optimal choices

The lipidome represents a complex and dynamic network of lipid species interacting with each other to provide nutrients, discrete cellular structures as well as acting as signaling molecules and antigens. the evolution of the lipidome species enabled diversification and specification of cellular structures enabling more complex and higher functions connecting cellular and global functions. The advancement seen in mass spectroscopy resolution and mass accuracy has allowed us to delve in to the lipid fingerprint of organs, tissues, cells and even organelles, highlighting the diversity of structural properties of distinct lipid species [76], [77]. The adipocyte is located at the center of lipid metabolism in mammals and the various adipose tissue lipidomes [14], [24], [78] define both its structure and function which in turn is associated with the metabolic health of the adipocyte. A clear example of this is the abundance of

mitochondrial cardiolipins, which is an indicator as to how metabolically active the adipocyte is, ranging from low in white adipose tissue too high in brown adipose [79], [80]. Another example is the membrane lipid composition of white adipocytes [81] which has been linked to obesity, inflammation [82] and ageing [25], [83], [84]. A third example is the interaction and subsequent cytokine response between the lipid sensing iNKT and adipocyte CD1d is also be defined by its local lipidome [8], [38], [85], [86]. Finally, pseudokinases like Tribbles 3, have emerged as multifaceted players in metabolism, including lipid metabolism, but their global cellular activities are poorly understood. Taken together, understanding the mechanisms governing lipidome composition– including the role of pseudokinases–, lipidome function and lipidome conservation or diversification across different cell types as well as the global organism could provide valuable insights into fundamental cellular processes as well as unveil their dysregulation in various diseases. Furthermore, the interaction between lipidomes and pseudokinases emerges as a fascinating area of research, potentially uncovering novel regulatory mechanisms and therapeutic targets.

Thesis outline

While both the intracellular lipidome of adipocytes as well as the extracellular lipid environment have been implicated in optimal adipocyte function, little is known on the effects on adipocyte-iNKT cell interaction. Furthermore, the pseudokinase Tribbles 3 may play a role in regulating the intracellular lipidome of adipocytes, but the underlying molecular mechanisms are undefined. Both research areas will contribute to a better understanding of optimal adipocyte function, including interplay with immune cells (iNKT cells) which may have diagnostic or ultimately even therapeutic value for human diseases like type 2 diabetes.

We therefore aimed to:

1. Investigate how the lipid environment impacts the adipocyte-iNKT cell interaction, both from an adipocyte and an iNKT cell angle.
2. Investigate if and how the pseudokinase Tribbles 3 regulates the adipocyte lipidome.

To address these objectives, we utilized and developed several model systems allowing us to strategically address the different levels of complex interactions and mechanisms. In **chapter 2** we use a mixture of FFA's mimicking the lipid-rich microenvironment of obesity *in vitro*, co-culturing these now insulin resistant (IR) adipocytes with iNKT cells demonstrated that the adipocyte IR phenotype causes a pro-inflammatory skew

in iNKT cell cytokine output, suggesting that cross-talk is an important target for further research [85]. Following this, in **chapter 3**, we hypothesized that AT-resident immune cells may also be detrimentally affected when exposed to a lipid-rich microenvironment. As LD's are found in many immune cells perhaps the elevated lipid environment would cause dysfunction in iNKT cells as well, creating a compound effect of dysfunctional communication between adipocytes and iNKT cells. We found iNKT cells are capable of taking up and storing lipids in droplets, even in "lean" conditions making them highly sensitive to their lipid environment as well as their lipid content. These findings highlight the importance of distinguishing between resident immune populations, as AT-resident iNKT cells respond differently to lipid environments compared to other iNKT cells in different locations, **Figure 1.**

In **chapter 4 and chapter 5** we show the nuanced nature of Tribbles 3 interactions with the available local partners to deliver site specific action. **Chapter 4** addresses the consequences of a global Tribbles 3 knock out, focusing on alterations –including the lipidome– in adipose tissue and adipocytes. We further investigated this in **chapter 5** focusing on the change in abundance of mitochondrial specific lipid species Cardiolipins. Here we find that changes in the ultrastructure of mitochondria results better stress tolerance under acute stress. Given its diverse interactions and implications in various diseases, understanding the interactions and functions in specific cellular contexts, such as Tribbles 3's role in adipose tissue lipidome modulation, offers potential avenues for targeted therapeutic interventions. The intricate interplay between local and global lipidomes and regulators or disruptors is an area of growing interest, as these conserved mechanisms appear to converge on crucial cellular processes. In **chapter 5** we present our data which suggests that pseudokinase Tribbles 3 may participate in location specific lipid metabolism and lipid signaling pathways, modulating the lipidome and thereby influencing responses to external cues.

In **chapter 6 part a** we reflect on the available adipose tissue model systems and the limitations they pose, showing their usefulness in specific contexts depending on the research question**,**. Finishing with **chapter 6 part b,** we review how lipidome species ratios may impact the local and global action of adipose resident iNKT cells.

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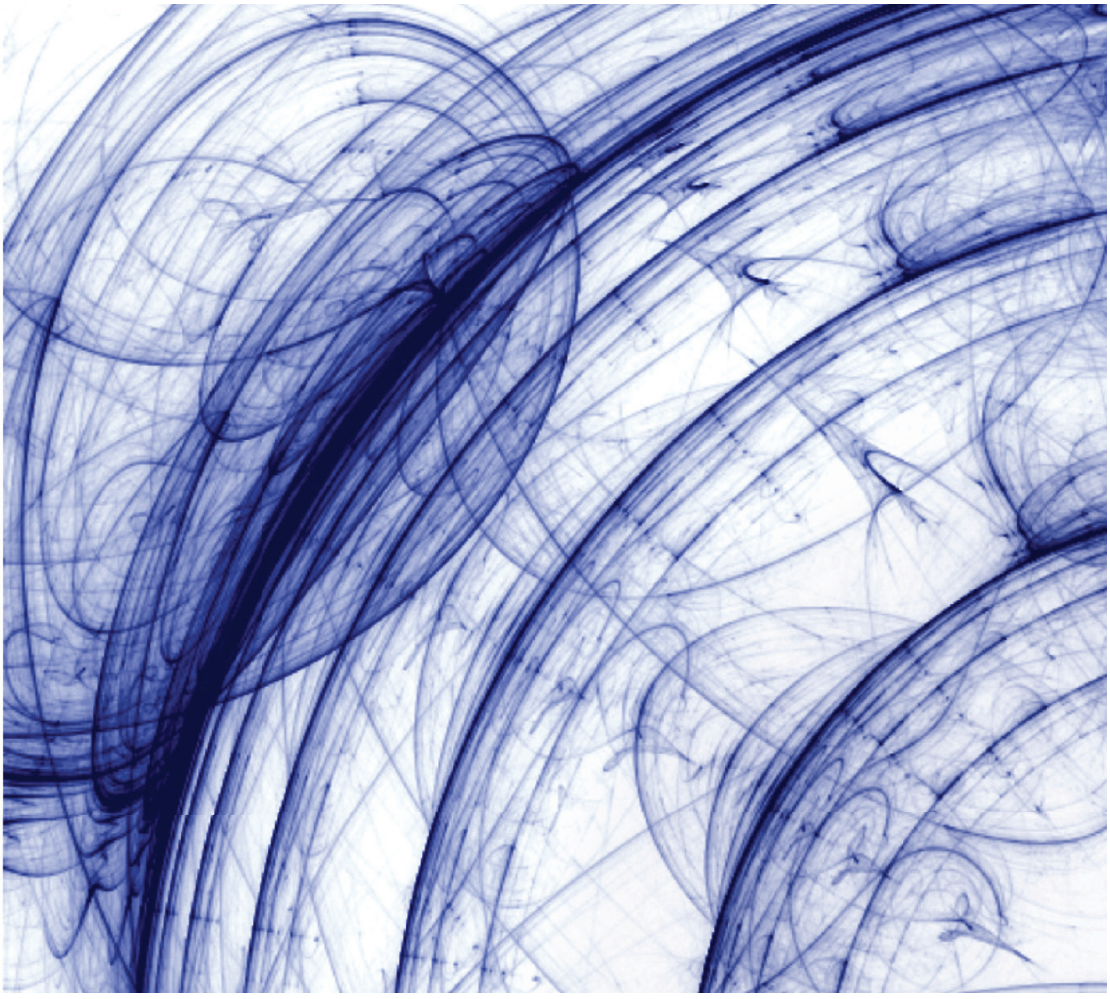
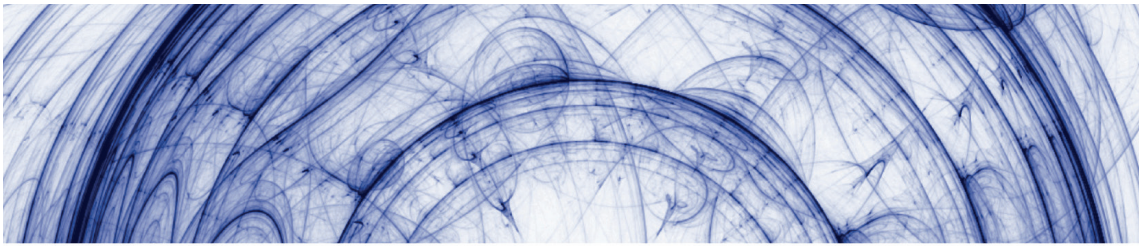
Chapter 2





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van Eijkeren, R.J*., **Morris, I*.,** Borgman, A., Markovska, A and Kalkhoven, E.

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Cytokine Output of Adipocyte-iNKT Cell Interplay Is Skewed by a Lipid-Rich Microenvironment

Robert J. van Eijkeren†, Imogen Morris†, Anouska Borgman, Angela Markovska and Eric Kalkhoven‡*

Molecular Cancer Research, Center for Molecular Medicine, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands

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****Correspondence:***
Eric Kalkhoven
e.kalkhoven@umcutrecht.nl

†These authors have contributed equally to this work

‡A list of members and affiliations appears in the Supplementary Note

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The complex direct and indirect interplay between adipocytes and various adipose tissue (AT)-resident immune cells plays an important role in maintaining local and whole-body insulin sensitivity. Adipocytes can directly interact with and activate AT-resident invariant natural killer T (iNKT) cells through CD1d-dependent presentation of lipid antigens, which is associated with anti-inflammatory cytokine production in lean AT (IL-4, IL-10). Whether alterations in the microenvironment, i.e., increased free fatty acids concentrations or altered cytokine/adipokine profiles as observed in obesity, directly affect adipocyte-iNKT cell communication and subsequent cytokine output is currently unknown. Here we show that the cytokine output of adipocyte-iNKT cell interplay is skewed by a lipid-rich microenvironment. Incubation of mature 3T3-L1 adipocytes with a mixture of saturated and unsaturated fatty acids specifically reduced insulin sensitivity and increased lipolysis. Reduced activation of the CD1d-invariant T-Cell Receptor (TCR) signaling axis was observed in Jurkat reporter cells expressing the invariant NKT TCR, while co-culture assays with a iNKT hybridoma cell line (DN32.D3) skewed the cytokine output toward reduced IL-4 secretion and increased IFNγ secretion. Importantly, co-culture assays of mature 3T3-L1 adipocytes with primary iNKT cells isolated from visceral AT showed a similar shift in cytokine output. Collectively, these data indicate that iNKT cells display considerable plasticity with respect to their cytokine output, which can be skewed toward a more pro-inflammatory profile *in vitro* by microenvironmental factors like fatty acids.

Keywords: adipocytes, iNKT cell, CD1d, lipolysis, insulin resistance

INTRODUCTION

Insulin resistance is one of the hallmarks of type II diabetes mellitus (T2DM) pathogenesis, with both overlapping and unique molecular mechanisms affecting different metabolic organs, including muscle, liver and adipose tissue (AT) (1, 2). AT has long been thought of as a simple storage organ, however it has more recently been shown to be an extremely complex tissue which plays a key role in global homeostasis (3, 4). Important and intertwined mechanisms through which AT can communicate with other metabolic organs and cells are facilitated by the production of adipokines (5), which can act locally but also enter into the circulation. From here the AT adipokine action, both locally and systemically, acts to regulate immune behavior in specific cytokine output

(3, 4, 6, 7). While the pathways leading from obesity to whole body insulin resistance and T2DM are complex and multifactorial (2), the increased uptake of nutrients leading to hyperplasia and hypertrophy of adipocytes is clearly an important early event, resulting in an altered adipokine secretion profile (5). In addition, hypertrophic AT displays a shift in AT-resident immune cells and cytokine output, where pro-inflammatory immune cells overwhelm the previously predominant anti-inflammatory immune cell populations (3, 6). The resulting chronic low-grade inflammation causes dysregulation of lipolysis, where adipocytes secrete higher levels of FFA, and glycerol (8, 9), which together with adipokines and cytokines can be viewed as an additional AT output. Combined these factors can have local or systemic effects and contribute to the development of whole-body insulin resistance and T2DM.

One of the AT-resident immune cell types that decrease dramatically with obesity both in mouse models and in humans are the invariant natural killer (iNKT) cells [reviewed in (10, 11)]. iNKT cells serve as a bridge between the innate and the adaptive immune system and are able to produce both pro-inflammatory cytokines, including IFNγ, and anti-inflammatory cytokines like IL-4 and IL-10 (10, 12, 13). IL-4 and IFNγ were initially thought to be on either end of the inflammatory spectrum, however recent research has shown that they also play a role in regulating other immune populations (14). Interestingly, the final steps of maturation of iNKT cells are thought to occur in the tissue where they reside, resulting in various tissue-defined subsets (10, 15). AT-resident iNKT cells for example have a Th2 cell phenotype and mainly produce anti-inflammatory cytokines IL-4 and IL-10 under lean conditions, which help to maintain AT homeostasis (16–20). iNKT cells are activated through a (semi)invariant TCR, that recognizes lipid antigens presented in the context of CD1d, a molecule that is expressed on the cell surface of various APC (13, 21, 22). In AT, iNKT cells can be directly activated by adipocytes, as they express not only CD1d itself but also possess a functional lipid antigen presentation pathway (16, 23–28), as well as a biosynthetic pathway for the production of lipid self-antigens (27, 28). Whilst in other biological settings the nature of lipid antigens, the type of APC and the microenvironment of the tissue have all been recognized as regulators of the secretion of Th1 and/or Th2 cytokines (10, 12, 29, 30), if and how the same parameters help to define AT-resident iNKT cell subsets and their cytokine output is largely unknown.

To study adipocyte-iNKT cell communication directly, i.e., without interference of other cell types, we and others have developed and characterized various co-culture assays, combining mouse or human (pre)adipocyte cell lines or primary adipocytes, with either reporter cells expressing the iNKT TCR (31), iNKT hybridoma cells (16, 24–28, 32) or primary iNKT cells isolated from AT or spleen (16, 24–28, 32). All these different assays strongly support direct adipocyte-iNKT cell communication, as they all show CD1d-dependent activation of the CD1d-iNKT TCR pathway upon co-culture with adipocytes, an activation boosted by the exogenous prototypical lipid antigen α-galactosylceramide (αGalCer), resulting in simultaneous production of both multiple pro- and anti-inflammatory cytokines (16, 24–28, 32).

Here we investigated the effects of FFA, as well as other obesity-associated components of the AT microenvironment, on the direct communication between adipocyte, and iNKT cells. First, we developed and characterized a cell culture model using mature murine 3T3-L1 adipocytes cell line treated with a commercially available and chemically-defined lipid mixture to mimic a high lipid microenvironment. Impaired insulin signaling and increased lipolysis was observed, without overall disruption of adipocyte-specific gene expression. Interestingly, while iNKT hybridoma cells, and primary iNKT cells produced both IL-4 and IFNγ under basal conditions, their cytokine output was skewed when adipocytes were pre-treated with the lipid mixture toward a low IL-4, high IFNγ profile. Taken together, these data indicate that iNKT cells display considerable plasticity with respect to their cytokine output, which can be skewed by microenvironmental factors like FFA.

MATERIALS AND METHODS

Materials

Dexamethasone (Sigma), 3-isobutyl-1-methylxanthine (IBMX)(Sigma), insulin (I9278, Sigma), Lipid mix 1 (sigma L0288), albumin conjugated linoleic (sigma L9530), and oleic acid (sigma O3008), sodium palmitate (sigma P9767), myristic acid (sigma M3128), stearic acid (sigma S4751), cholesterol (MP biochemicals, 219934230), αGalactosylceramide KRN7000 (Avanti, 867000), IFNγ (sigma SRP3058), TNFα (sigma H8916), Pam3Cys (Calbiochem), LPS (sigma L4516), rabbit-anti-AKT (also named PKB) (33), rabbit-anti-pAKT-ser473 (4060S Cell Signaling).

Cell Culture

The murine 3T3-L1 cell line (ZenBio) was cultured in Dulbecco's medium (DMEM) supplemented with 10% bovine serum (Invitrogen), penicillin and streptomycin (both 100 μg/ml; Invitrogen). For differentiation of 3T3-L1 cells to adipocytes, the cells were grown to confluence and after 2 days (day 0) stimulated with culture medium containing dexamethasone (250 nM), 3-isobutyl-1-methylxanthine (500 μM), and insulin (170 nM) for 2 days. On day 2, the medium was changed for culture medium containing insulin (170 nM) and maintained for 4–6 days. The murine iNKT cell hybridoma line DN32.D3 was cultured in RPMI-1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, penicillin and streptomycin (both 100 μg/ml), MEM Non-Essential Amino Acids Solution (100x; ThermoFisher Scientific), HEPES (100x; Sigma Aldrich), Glutamine (100x, Sigma Aldrich) and βMeOH (100 μM; Sigma Aldrich). The JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cell lines (31) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin (both 100 μg/ml).

Stimulation of 3T3-L1 adipocytes was done post differentiation for 3 days with lipid mix and individual fatty acids, and for 24 h with the residual obesogenic stimuli. One Millimolar of Palmitic, stearic and myristic acid was conjugated to fatty acid free BSA in a 6:1 ratio. Lipids were dissolved in 150 mM NaCl at 70°C while stirring. Before added to BSA

150 mM NaCl solution at 37°C while stirring. After adjusting pH to 7, 4 aliquots where stored at −20°C before further use (protocol adapted from Seahorse Bioscience).

Western Blot Analysis

3T3-L1 cells were grown in a six well format and differentiated accordingly. After stimulation cells were washed with ice cold PBS, scraped and lysed in 300 µl ice cold RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium DOC, 0.1 % SDS, 25 mM Tris pH 7.4, supplemented with protease inhibitor (Roche) and NaF acting as phosphorylase inhibitor) for 15′ at 4°C. After centrifugation on max speed at 4°C in table top centrifuge, supernatant was collected, supplemented with Laemmli Sample Buffer (LSB). All western blot samples were boiled for 5 min at 95°C before use. Samples were subjected to SDS-PAGE and transferred to PVDF membrane (Milipore). Blocking was done with 5% Skim Milk TBST. Primary antibody staining was performed overnight at 4°C (AKT & pAKT 1:2,000 in 5% BSA TBST). Secondary antibody staining was performed for 1 h at RT (1:1,000 in 5% milk TBST). After staining, membranes were washed for 1 h with TBST before being treated with ECL western blot substrate solution (Pierce). Protein expression was measured with LAS4000 ImageQuant.

Glycerol Measurements

Secreted Glycerol was measured using the Sigma-aldrich Glycerol Assay Kit (#MAK117-1KT) with 50 µl of media.

Triglyceride Measurements

Intracellular Triglycerides were measured using the kit Stanbio™ Triglycerides LiquiColor™ (#SB2200-225). Cells were washed with PBS and lysed in 50 µl cold PBS via syringe pull technique.

Co-culture Assays

3T3-L1 wild type (Zenbio) were plated in a 96-well format and differentiated according to the protocol mentioned above. Mature 3T3-L1 Cells were then treated with lipid mix or individual fatty acids for 4 days, or 24 h with residual obesogenic stimulants. After stimulation, either DN32.D3 iNKT hybridoma cells (50.000 cells/well) or JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells (50.000 cells/well) were added to the 3T3-L1 adipocytes and co-cultured for 24 h. Medium from DN32.D3 co-cultures (200 µl) was used to determine secreted IL-4 and IFNγ via the Invitrogen™ eBioscience™ Mouse IL-4 ELISA Ready-SET-Go!™ Kit (#501128931) and the IFNγ ELISA kit (BD-bioscience).

JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells (31) were collected after co-culture by resuspending and collection in round bottom 96-well plates. Cells were then centrifuged (1,600 RPM, 5′, RT) and resuspended in 200 µl PBS. Cells were analyzed for eGFP expression by flow cytometry with FACSDiva (BD) and FlowJo (Tree Star Inc.) software. Data is presented as geometric Mean Fluorescent Intensity.

RT-qPCR Analysis

RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized using the superscript first strand synthesis system (Invitrogen) according to manufacturer's protocol. Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to 36B4 expression. Primers for quantitative RT-PCR are described in **Table S1**.

Single Cell Suspension From Spleen and Adipose Tissue (eWAT)

Tissue collection was performed on animals sacrificed for other purposes and therefore exempted form ethical approval by the local Animal Welfare Body Utrecht, a body of Utrecht University and the University Medical Center Utrecht (https://www.ivd-utrecht.nl/en/). Spleens and epididymal white AT (eWAT) were dissected from male C57BL/6J mice aged between 11 and 14 weeks. Spleens were disintegrated through a 70 µM cell strainer into 50 ml cold PBS and centrifuged for 10 min at 400 g. After discarding the supernatant, the pellet was resuspended in 5 ml of 10x Red Blood Cell (RBC) Lysis Buffer (Abcam; ab204733) for 5 min at room temperature. Forty five milliliter of cold PBS was added and cells centrifuged for 10 min at 400 g. After discarding the supernatant, the pellet was resuspended in 10 ml of cold PBS and filtered through a 70 µM cell strainer. Cells were retained on ice.

Before processing blood vessels and lymph nodes were removed from the eWAT which was then minced in 10 ml cold digestion buffer (Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} supplemented with 0.5% bovine serum albumin). Per 1 g adipose, 1 ml of 10 mg/ml collagenase (Sigma-Aldrich; C6885) was added and incubated at 37°C for 15–20 min, with vigorous shaking every 5 min until AT was digested completely. Digested AT was then washed through a 100-µm cell filter with 20 ml of cold digestion buffer. Following centrifugation (500 g for 10 min) supernatant was removed and the cell pellet was resuspended in cold PBS. Cells were retained on ice.

iNKT Purification

Cells isolated from spleen and adipose mice were pooled, respectively, before purification in order to remove individual variation and to maintain consistent cell numbers during co-culture. iNKT cells were purified from the pooled populations using the NK1.1+ iNKT Cell Isolation Kit, mouse (Miltenyi Biotec; #130-0960513). Purified cells were then used in co-culture as described above.

RESULTS

Lipid Mixture Causes Insulin Insensitivity and Increased Lipolysis in Adipocytes

The increased concentration of circulating FFA observed in obesity contributes to the development of insulin resistance in adipose tissue (1, 2). To mimic this phenomenon in a cell culture model we used a chemically defined, commercially available lipid mixture containing cholesterol plus monounsaturated FFA (oleic acid), polyunsaturated FFA (arachidonic, linolenic, and linoleic acid) and saturated FFA (myristic, palmitic, and stearic acid), as reported previously (34). A significant increase in triglyceride (TG) storage was observed in mature 3T3-L1 adipocytes after

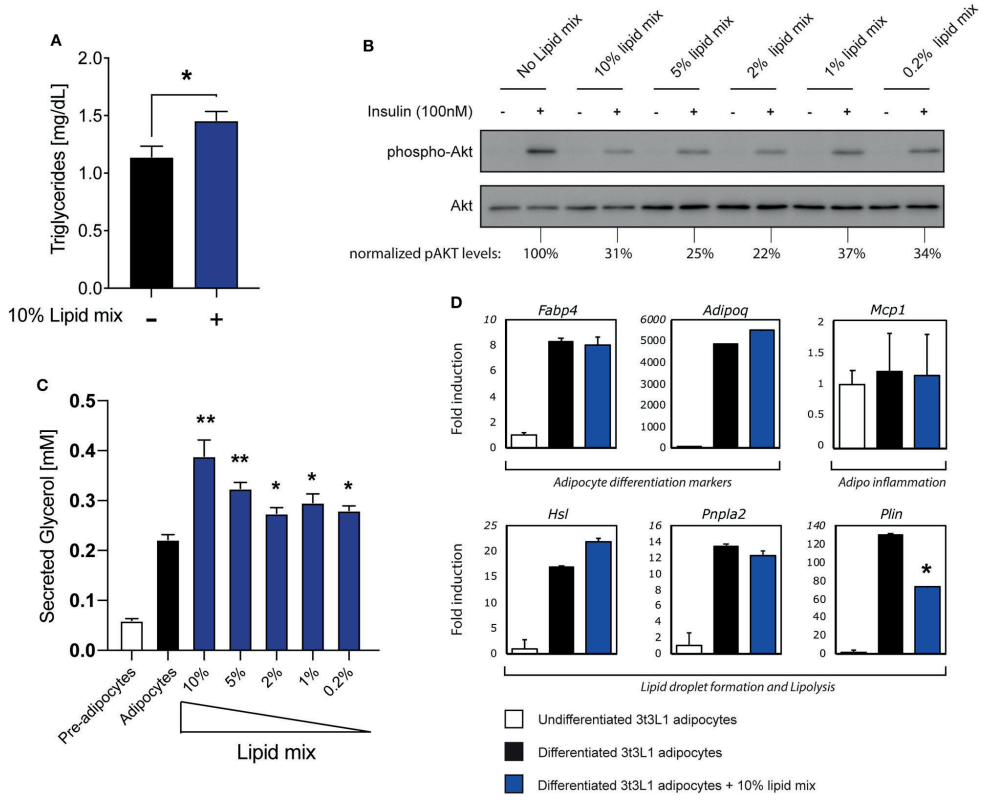

FIGURE 1 | Stimulation with a lipid mixture causes an insulin resistance phenotype in 3T3-L1 adipocytes. **(A)** Mouse 3T3-L1 preadipocytes were differentiated into mature adipocytes and cultured with 10% lipid mixture for 4 days and intracellular triglyceride levels were determined. Statistical analysis via Students *t*-test against adipocytes glycerol secretion (NS $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) ($n = 3$). **(B)** Mature 3T3-L1 adipocytes were treated with different concentrations (10–0.2%) Lipid Mixture for 4 days and deprived of insulin signaling before insulin stimulation (30 min). Lysates subjected to Western blot analysis. Western blot quantification of phospho-AKT (ser473) normalized to total AKT levels. **(C)** Quantification of glycerol secreted by 3T3-L1 adipocytes, and control pre-adipocytes, after 4 days in culture with titrated lipid mix dilutions. Statistical analysis via Students *t*-test against adipocyte glycerol secretion (NS $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$) ($n = 3$). **(D)** Mouse 3T3-L1 preadipocytes were differentiated into mature adipocytes and treated with 10% lipid mix for 4 days before being subjected to RNA isolation and quantitative RT PCR. Transcriptional activity of genes involved in adipogenesis, inflammation and lipolysis are depicted as fold induction relative to undifferentiated 3T3-L1 adipocytes. Data are normalized to housekeeping gene 36B4 and presented as mean +/– SD ($n = 6$).

culturing with 10% lipid mixture (**Figure 1A**), Next, the effects of different concentrations of lipid mixture on insulin sensitivity was assessed by re-stimulating insulin deprived mature 3T3-L1 adipocytes cultured in the absence or presence of various concentrations of lipid mixture. Insulin signaling decreased 3–4-fold compared to untreated adipocytes, with 10% lipid mixture displaying the maximal effect (**Figure 1B**). When tested individually, the inhibitory effect on insulin signaling by the different components of the lipid mixture was not observed when compared to the lipid mixture (**Figure S1A**). In addition, no inhibitory effect was observed after stimulation with cytokines TNFα and IFNγ (**Figure S1A**), which are both elevated in obesity (35, 36). To analyse functional effects of the reduced insulin signaling, we focused on lipolysis, a process that is highly regulated by insulin (2). As shown in **Figure 1C** a significant increase in lipolysis was observed when cells were subjected to the lipid mixture, as assessed by analyzing secreted glycerol concentrations. Again, stimulation with the individual components of the lipid mixture did not increase glycerol secretion significantly (**Figure S1B**). Additionally, stimulation with cytokines TNFα and IFNγ increases glycerol secretion (**Figure S1C**) but similar stimulation showed no effect on insulin

signaling (**Figure S1A**). Also, activation of TLR2 (Pam3Cys) or TLR4 (LPS), which has been shown to occur in obesity (37–39), has no outspoken effect on glycerol secretion (**Figure S1C**).

To characterize this cell model further, we analyzed the effects of the lipid mixture on mRNA expression of genes associated with adipocyte differentiation (adipogenesis), inflammation, and lipid storage by RT-qPCR (**Figure 1D**). Expression of the adipogenesis markers FABP4 and Adipoq (encoding adiponectin) were clearly upregulated during adipogenesis with expression remaining consistent irrespective of Lipid Mix (**Figure 1D**). The inflammation marker gene *Mcp1* was expressed in both undifferentiated and differentiated cells, and unaffected by the lipid mixture (**Figure 1D**). Furthermore, as we observed increased triglyceride storage and glycerol secretion in mature 3T3-L1 adipocytes subjected to the lipid mixture (**Figures 1A,C**), we analyzed the expression of genes involved in lipid storage and lipolysis. *HSL* and *PNPLA2* (encoding the ATGL protein) were more highly expressed in differentiated cells compared to undifferentiated 3T3-L1 cells, but no significant change in expression was observed upon lipid mixture treatment. The *Plin* gene, encoding Perilipin1, was the only gene tested here to show a significant decrease in expression. As also observed for insulin signaling (**Figure S1A**) and lipolysis (**Figure S1B**), none of the lipolysis related genes we tested increased upon stimulation with the main individual components that make up the lipid mix (**Figure S1D**). To verify that expression of the genes analyzed in general was not static but could be modified by other stimuli, we subjected the cells to other obesity-associated stimuli (TNFα, IFNγ) or inflammatory stimuli (Pam3Cys, LPS) and observed various changes in gene expression (**Figure S1E**).

Taken together, these data indicate that treatment of mature 3T3-L1 adipocytes with a chemically-defined lipid mixture results in a robust insulin resistance phenotype with increased lipolysis, without causing an overall disruption of cellular functionality, as the cells were clearly functional in terms of lipid metabolism and no dramatic changes in various key genes were observed. These observed characteristics support this cellular model as suitable for subsequent experimental approaches, including *in vitro* studies on adipocyte-iNKT cell communication.

Lipid Mixture Skews Cytokine Output in Adipocyte-iNKT Interplay

Having established a cellular adipocyte model with a high-lipid microenvironment (**Figure 1**), we next wished to investigate if and how adipocyte-iNKT cell communication is altered under these experimental conditions. For this we used different experimental co-culture approaches. First, we cultured mature 3T3-L1 adipocytes pre-treated with lipid mixture or left untreated together with the recently developed JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells (31). This reporter cell line is based on the Jurkat T cell line, stably transfected with an NFkB-eGFP reporter and the human Vα24-Jα18 TCRα chain and Vβ11 TCRβ chain (31). Co-culture of lipid antigen presenting cells followed by quantification by FACS analysis provides a sensitive fluorescence-based readout of iNKT TCR-lipid antigen interaction (31). In addition, the β2M gene was deleted from these cells using CRISPR/Cas9, to eliminate antigen-selfpresentation and self-activation (31). When JE6-1$^{REP-iNKT-\beta2M_KO}$ reporters were cultured for 24 h with lipid mixture- treated mature 3T3-L1 adipocytes, an increase in eGFP expression was observed compared to individually cultured reporter cells, which was boosted by the prototypical lipid antigen αGalCer (**Figures 2A,B**). These results expand the use of these reporter cells as a read-out for CD1d-iNKT TCR signaling to adipocytes (31). Furthermore, as similar observations were previously made using iNKT hybridoma cells (16, 24–28, 32), we conclude that these reporter cells provide a robust read-out system for 3T3-L1 adipocyte-iNKT cell interaction. When using this read-out system to investigate the effect of lipid mixture treatment, eGFP expression decreased when adipocytes were stimulated with αGalCer 24 h prior to co-culture (**Figure 2D**), a trend not observed in the absence of αGalCer (**Figure 2C**). Importantly, the lipid mixture or individual components did not alter eGFP expression when the reporter cells were tested in isolation, indicating that CD1d-iNKT TCR signaling is required (**Figure S2B**). Also, individual components of the lipid mix and the inflammatory stimuli IFNγ, Pam3Cys, and LPS did not alter eGFP expression after co-culture significantly (**Figures S2C–F**). It should be noted that TNFα did induce eGFP expression, as it can activate the NFkB reporter present in the JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells (**Figure S2A**).

To investigate potential mechanisms behind this change in adipocyte-iNKT reporter cell communication, we analyzed the expression of several genes that have previously been implicated in lipid antigen presentation in adipocytes (*Cd1d*, *MtpA,* and *MtpB*), or that could potentially play in role in this based on findings in other lipid APC (*Gla, Npc2, Psap*). In addition, we analyzed 3 genes implicated in the biosynthesis of potential endogenous lipid antigens in adipocytes (*Ugcg*) and other lipid APC's (*Glb1, Gba*). In agreement with previous reports (26, 27, 32), the *Cd1d* gene, encoding the actual lipid antigen presenting molecule, was upregulated during 3T3-L1 differentiation, here no effect of the lipid mixture was observed (**Figure 2E**). Also, expression of the 2 isoforms of Microsomal Triglyceride Transfer Protein [*MtpA* and *MtpB;* note that *MtpB* is predominantly expressed in 3T3-L1 adipocytes (27)], a factor we previously implicated in lipid antigen presentation in adipocytes (27), displayed < 2-fold change. The expression of other lipid antigen loading machinery genes which potentially play a role in lipid antigen presentation in adipocytes (27) like pro-saposin (*Psap*), Niemann Pick type C2 (*Npc2*), α-galactosidase (*Gla*), were also not altered dramatically (**Figure 2E**). The *Ugcg* gene, encoding ceramide glucosyltransferase and implicated in the biosynthesis of endogenous lipid antigens in adipocytes and other APC (28, 40), also displayed a limited change upon lipid mixture treatment (**Figure 2E**), as did two other genes potentially involved in the biosynthesis of endogenous lipid antigens, *Glb1*, encoding β-galactosidase, and *Gba*, encoding beta-glucocerebrosidase (**Figure 2E**). Stimulation with individual lipid mix components

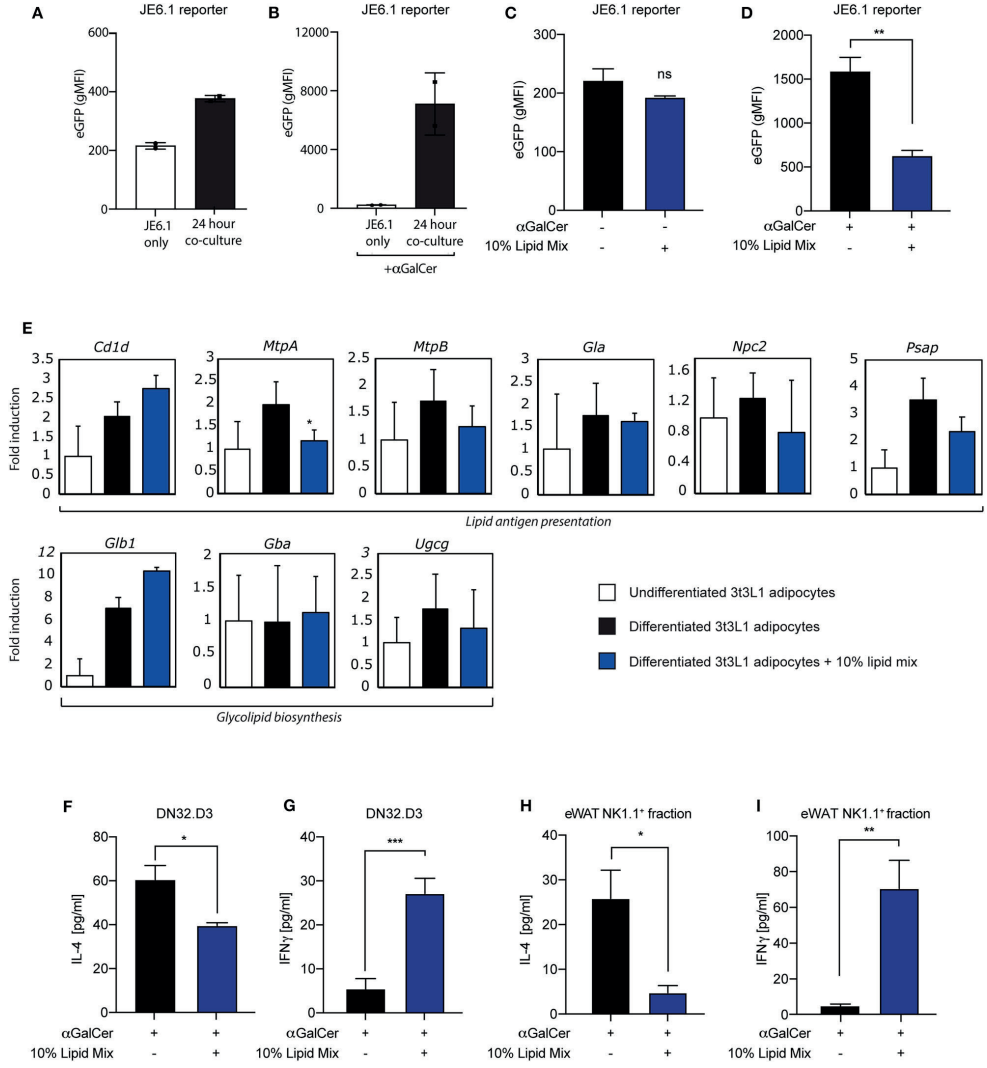

FIGURE 2 | Lipid mixture treatment skews iNKT cell cytokine secretion toward a pro-inflammatory profile after co-culture. **(A,B)** Co-culture between JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells and mature 3T3-L1 adipocytes stimulated with **(B)** or without **(A)** αGalCer (0.5 μg/ml) 24 h prior to co-culture ($n = 2$) Data is presented as geometric Mean Fluorescent Intensity (gMFI). **(C,D)** Co-culture between JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cell (5×10^4 cells per well in a 96 well-format) and mature 3T3-L1 adipocytes treated with or without 10% lipid mix and stimulated with **(D)** or without **(C)** αGalCer (0.5 μg/ml). JE6-1$^{REP-iNKT-\beta2M_KO}$ cells were collected after 24 h co-culture and analyzed for GFP expression by FACS. Data is presented as geometric Mean Fluorescent Intensity (gMFI) +/− SD ($n = 3$, $P = 0.0044$). **(E)** Mouse 3T3-L1 preadipocytes were differentiated into mature adipocytes and treated with 10% lipid mix for 4 days before being subjected to RNA isolations and Quantitative RT PCR. Transcriptional activity of genes involved in adipogenesis, glycolipid biosynthesis, lipid antigen presentation, and lipolysis are depicted as fold induction relative to undifferentiated 3T3-L1 adipocytes. Data is normalized to housekeeping gene 36B4 and presented as mean +/− SD ($n = 6$). **(F,G)** ELISA analysis of IL-4 and IFNγ secreted by DN32.D3 cells after 24 h co-culture with mature 3T3-L1 adipocytes treated with or without 10%

(Continued)

FIGURE 2 | lipid mix ($n = 3$, $p = 0.0319$ and 0.0004 for IL-4 & IFNγ, respectively) Each data point represents 5×10^4 cells. **(H)** IL-4 secretion following 24 h co-culture of NK1.1 positive *ex-vivo* fraction extracted from visceral eWAT, cultured with αGalCer stimulated (0.5 μg/ml) mature 3T3-L1 adipocytes treated with and without 10% lipid mix. Each data point represents 5×10^4 cells taken from a pooled population, statistical analysis via Students *t*-test against (NS $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) ($n = 3$) **(I)** IFNγ secretion following 24 h co-culture of NK1.1 positive *ex-vivo* fraction extracted from eWAT, cultured with αGalCer stimulated (0.5 μg/ml) mature 3T3-L1 adipocytes treated with and without 10% lipid mixture. Each data point represents 5×10^4 cells taken from a pooled population. Statistical analysis via Students *t*-test against (NS $P > 0.05$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) ($n = 3$–6).

also shows no dramatic differences in gene expression of lipid antigen presentation and glycolipid biosynthesis genes (**Figure S1D**). Taken together, these data indicate that a lipid-rich microenvironment can alter communication through the CD1d-iNKT TCR axis, but that changes in genes implicated in lipid antigen presentation are not likely to present the underlying mechanism.

The second read-out system used was co-culture of mature 3T3-L1 adipocytes with mouse iNKT hybridoma cells followed by cytokine analysis, a robust system previously used by us and others (26–28, 32). In line with the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter-based system (**Figure 2D**), we saw a significant decrease in secretion of the anti-inflammatory cytokine IL-4 by the iNKT cell hybridoma DN32.D3 when adipocytes were subjected to the lipid mixture (**Figure 2F**). In contrast, a significant increase in secretion of the pro-inflammatory cytokine IFNγ was observed upon lipid mixture treatment (**Figure 2G**), indicating plasticity of the iNKT cell in terms of cytokine output depending on the microenvironment. Again, in line with the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter-based system, individual components of the lipid mix and inflammatory stimuli did not alter cytokine secretion by DN32.D3 iNKT cells in a co-culture setting (**Figures S2G,H**). Also, 3T3-L1 adipocytes treated with 10% lipid mixture did not produce high levels of IFNγ that may conflict with the outcome of 3T3-L1-DN32.D3 co-cultures (**Figure S2I**).

Whilst the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter cells and DN32.D3 iNKT cell hybridoma represent useful proxies for iNKT cells they do not fully reflect all iNKT cell characteristics, which are in part tissue-specific (10). Therefore, as a third read-out system we replaced the DN32.D3 hybridoma with primary iNKT cells isolated from either spleen or eWAT, based on the NK1.1 marker (NK1.1.+ iNKT cells) as reported previously (16). Similar to the DN32.D3 based co-culture system (**Figures 2F,G**), NK1.1$^+$ eWAT iNKT cells co-cultured with mature 3T3-L1 adipocytes treated with lipid mixture show a significant reduction ($p = 0.0233$) in IL-4 (**Figure 2H**) and an significant increase ($p = 0.0086$) in IFNγ secretion (**Figure 2I**). On the other hand, the response of NK1.1$^+$ iNKT cells isolated from mouse spleens was far less pronounced for both IL-4 and IFNγ secretion (**Figures S3A,B**). Furthermore, the NK1.1$^-$ fraction from both eWAT and spleen followed the same trend as their positive selection counterparts, but to a lower extent (**Figures S3C,D**). These data indicate that the eWAT iNKT cytokine secretion profile has a specific plasticity and is highly responsive to the adipose environment, with a key role for free fatty acids in this process.

DISCUSSION

Aside from their CD1d restricted TCR, iNKT cells can have divergent functions, surface markers and cytokine secretion preferences, all of which seem to be determined by the nature of lipid antigens, the type of APC and the microenvironment of the tissue (21, 41, 42). Despite their environmental priming, they also appear to retain plasticity, enabling them to respond swiftly and dynamically to changes in their surroundings (10, 12, 29, 43). Given the complex nature of AT and the various interactions between the different cell types present in this tissue, we decided to develop assay systems where it would be possible to study adipose—iNKT crosstalk in isolation. To facilitate this, we optimized a lipid enriched adipose cell line 3T3-L1 which exhibits several obesity-associated characteristics without disturbing functionality (**Figure 1E**). Using two iNKT model cell lines, DN32.D3, and JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$ reporter cells, we show that cross-talk with the lipid enriched 3T3-L1 adipocytes influences output following TCR stimulation (**Figure 2**). Interestingly, iNKT cells extracted from eWAT, like DN32.D3 hybridoma cells, display an IFNγ secretion skew over IL-4 in lipid rich environments. On the other hand, splenic iNKT cells do not show the same capacity in this context, highlighting the divergence between the two populations. Based on this rapid response to a lipid rich environment, we conclude that eWAT iNKT cells harbor significant plasticity in terms of their cytokine output, which is pre-primed by their tissue micro-environment. Interestingly, a short term HFD diet intervention in which skewing of cytokine output by AT-resident iNKT cells was reported by Li et al. (20). It should be noted however that while we observed a skew toward IFNγ production upon treatment with a defined lipid mixture, Li et al. (20) reported skewing toward higher IL-4 production in AT upon HFD feeding. Although further research is needed to establish how the *in vivo* and *in vitro* interventions can be translated into each other, both studies support the view that the regulatory function of iNKT cells is dynamic and complex, exemplified by their ability to rapidly switch their cytokine preference based on their micro-environment.

In the present study we compared several co-culture systems to address the delicate cross-talk between adipose and iNKT cells in a lipid-rich environment. Under our experimental conditions, we obtained similar results from both DN32.D3 hybridoma cells and primary iNKT cells form eWAT, suggesting that DN32.D3 hybridoma cells represent an appropriate model for iNKT cells in the context of eWAT (**Figure 2**). In our co-culture assays, the response of the JE6-1$^{\text{REP}-\text{iNKT}-\beta2\text{M_KO}}$

reporter cells (31) reflected the effects observed in the other systems on IL-4 secretion, i.e., reduced output when adipocytes were pre-treated with lipid mixture, but not the increased IFNγ secretion. It should be noted that the NFkB-eGFP reporter in the JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells provides a single read-out (31); our data suggest that the intracellular pathways ultimately leading to IFNγ and IL-4 secretion are wired differently at some level, but the underlying molecular mechanisms remain to be established. The JE6-1$^{REP-iNKT-\beta2M_KO}$ reporter cells nonetheless clearly present a very valuable tool for identification and verification of (endogenous) lipid antigens (31).

In contrast to iNKT cells from eWAT, we have also shown in the present study that splenic iNKT cells do not exhibit an IL-4/IFNγ preference in co-culture assays upon lipid mixture treatment. Splenic iNKT cells have been previously reported to have an IL-4 and IFNγ cytokine secretion capacity (10, 43), we show that this capacity is not significantly altered by lipid enrichment. As previously stated, we focused on IL-4 and IFNγ because of their duel nature, as both are linked to inflammatory balance as well as their regulatory implications for the surrounding immune populations (44–49). Therefore, the contrast between AT and splenic iNKT populations emphasizes that isolating immune cells from the tissue that is being studied for subsequent analyses is recommendable. This is particularly true for iNKT cells, as iNKT populations for the most remain in one tissue environment with only few cells in circulation, in contrast to for example the much more mobile macrophages (17, 41, 43, 46).

Immune cell plasticity refers to immune cells which have a flexible, context-dependent inflammatory phenotype. This flexibility allows for rapid response to stimulus without being permanently defined by it (41, 43, 48, 50). We can draw parallels with other more defined immune populations. A good example of this is M1/M2 macrophages, where the macrophage population is capable of being on either end of the inflammatory spectrum and anywhere in between (19, 48, 49, 51). Similarly, iNKT cells are able to secrete a multitude of cytokines but in a context dependent pre-primed manner (6, 10, 14, 41, 52–54). Therefore, we propose that iNKT cells have a plasticity in response to their surroundings, and that their response is potentially tailored to the specific requirements of their micro-environment. How the plasticity observed in our proof of principle studies translates into the complex interplay between adipocytes and various AT-resident immune cell types *in vivo*, and ultimately into whole body energy homeostasis will be the topic of future studies. Indeed, very recent *in vivo* studies by LaMarche et al. already underscored the microenvironmental impact on iNKT cell output (55).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because it was exempted by the local animal ethics committee.

AUTHOR CONTRIBUTIONS

RE, IM, AB, AM, and EK designed the experiments. RE, IM, AB, and AM performed experiments and analyzed the data. RE and IM drafted the manuscript. RE, IM, and EK edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00479/full#supplementary-material

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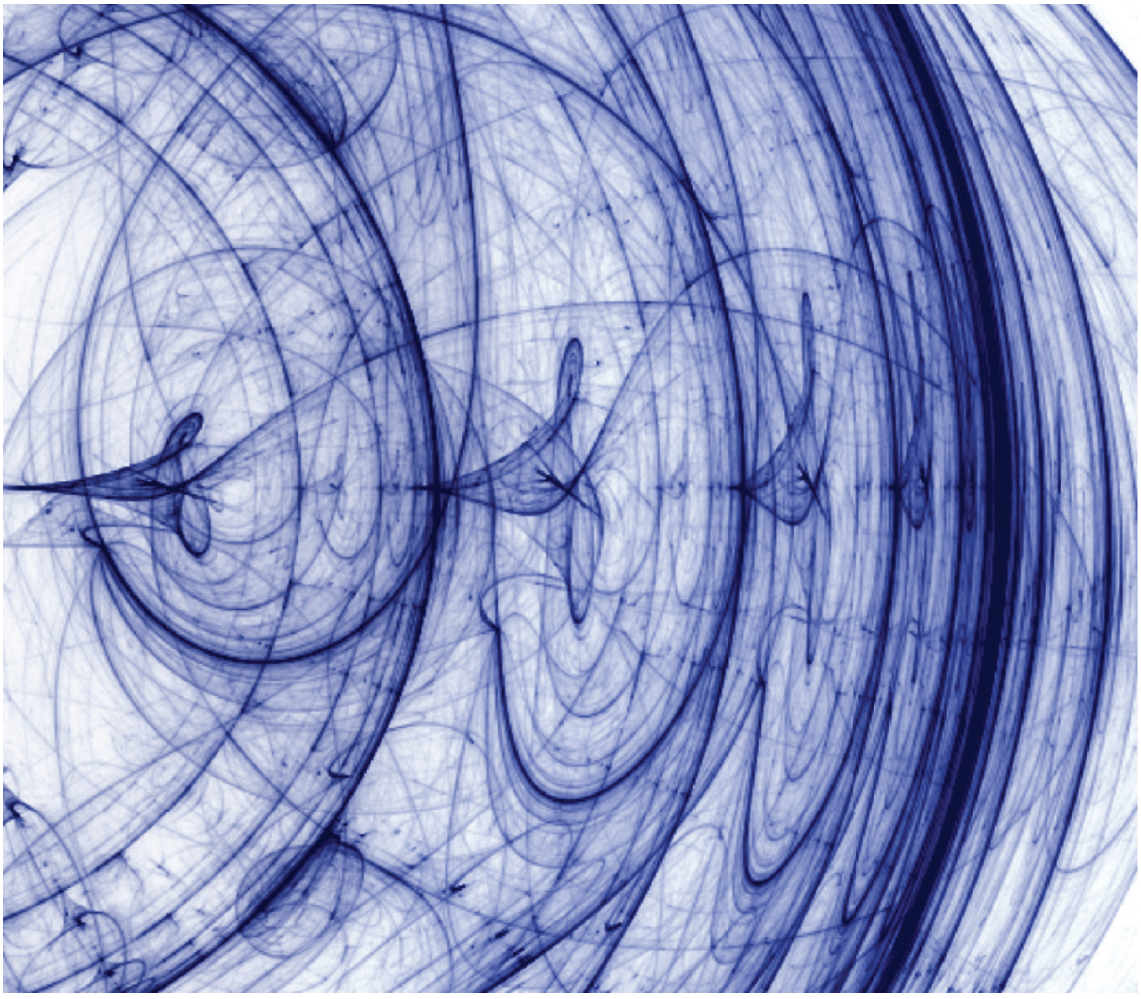
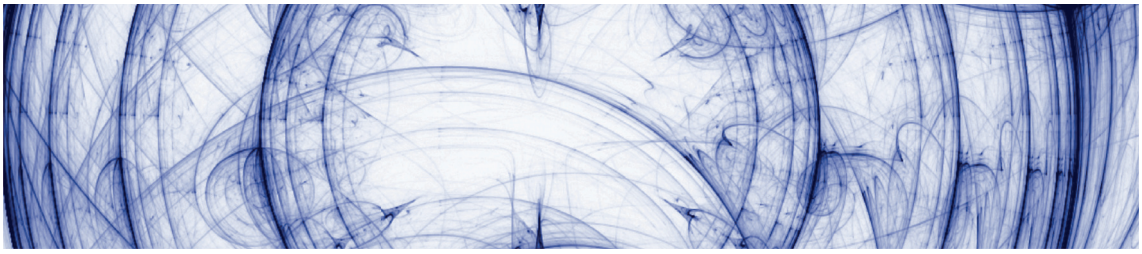
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 3





Lipid accumulation in Adipose Tissue-resident iNKT Cells Contributes to Inflammatory phenotype

Imogen Morris[1], Frank Vrieling[2], Annemieke Bouwman[1], Rinke Steinstra[2,3] and Eric Kalkhoven[1]

Abstract

Reciprocal communication between adipocytes and immune cells is essential to maintain optimal adipose (AT) tissue functionality. Amongst others, adipocytes directly interact with invariant NKT cells (iNKT), which in turn secrete various cytokines. A lipid-rich microenvironment, as observed in obesity, skews this adipocyte-driven cytokine output towards a more inflammatory output. Whether a lipid- rich microenvironment also affects iNKT cells directly, however, is unknown. Here, we show that primary mouse iNKT cells isolated from AT can accumulate lipids in lipid droplets (LDs), more so than liver- and spleen-resident iNKT cells. Furthermore, a lipid-rich microenvironment increased production of the proinflammatory cytokine IFNγ. Next to an indirect, adipocyte-mediated cue, iNKT cells can directly respond to environmental lipid changes, supporting a potential role as nutrient sensors.

Keywords: iNKT cell; adipose tissue; lipids; lipid droplet; inflammation; IFNγ.

Introduction

Metabolism and immunology interact at numerous levels, leading to the emergence of the novel field of immunometabolism [1]. An important immunometabolic interface is formed by cell-cell communication between adipocytes and immune cells. One side of the immunometabolic interface is formed by adipocytes, a highly specialized cell type characterized by the ability to store lipids as triglycerides in so-called lipid droplets (LDs). During times of increased energy demand, lipolysis hydrolyzes the TG stored in the LDs back into FFAs. LDs have a highly conserved structure and proteome and are present in many organisms, from bacteria to algae and plants, insects to humans [2]. It has even been suggested that LDs are so highly conserved and ubiquitous that they could be one of the most ancient organelles to have formed [3], [4]. LDs are very common in all eukaryotes and can be found in many organelles, including the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, secretory vesicles, and others [1]. The ER and Golgi have been long-recognized as mammalian cells' main LDs processing organelles [2]. Lipid droplets are comprised of a core of neutral lipids surrounded by a phospholipid monolayer and associated proteins (class 1 and 2). These small organelles have been shown to have a highly dynamic life cycle in adipocytes, providing protection from lipotoxicity and enabling the cell to function, grow, and divide (reviewed [5], [6]).

Interestingly, adipocytes share or can adopt some phenotypic aspects of immune cells: adipocytes express the machinery for both peptide antigen and lipid antigen presentation, they have secretory capacities (cytokines and adipokines), and the adipocyte gene expression profile is regulated by transcription factors like PPARs and LXRs, which also play an essential role in various immune cells. On the other hand, immune cells, which form the other side of the immunometabolic interface, share features with adipocytes. One example of this is the storage of triglycerides in LDs, first reported in macrophages/monocytes, dendritic cells, and lymphocytes [7]. Catabolism and storage of lipids in these immune cells have been shown to impact their functional phenotype [8], [9]. For instance, excessive storage of lipids in macrophages can lead to a pro-inflammatory phenotype and promote pro-inflammatory cytokine secretion, which can further recruit more immune cells to the inflammatory sites [10], [11].

One particular type of immune cells, invariant Natural Killer T-cells (iNKT cells), has attracted attention as (i) in healthy adipose tissue they can make up to 20% of the immune cell population [53]–[55], (ii) AT-resident iNKT cell numbers decline in obesity and various mouse models indicate that they support optimal AT function, (iii) iNKT cells can be activated by lipid antigens presented in the context of CD1d by adipocytes [12]–[14]. While iNKT cells can secrete both anti- and pro-inflammatory cytokines and

thereby regulate other AT-resident immune cell types, they predominantly secrete anti-inflammatory cytokines in healthy AT [15] and can be skewed towards a more pro-inflammatory phenotype under obese conditions, both in vitro and in vivo [refs]. Furthermore, the overall phenotype of iNKT cells can vary substantially by the tissue they reside in (e.g. AT vs liver), but if and how the lipid rich environment of AT directly influences iNKT cells is largely unexplored. Here we examined the effects of a lipid rich environment on iNKT cells i from different tissues (AT, liver, spleen) in vitro and report their ability to store lipids in LDs. Furthermore, lipid storage resulted in the secretion of higher levels of the pro- inflammatory cytokine IFNγ, most pronounced in AT-resident iNKT cells. We conclude, therefore, that next to an indirect, adipocyte-mediated cue, iNKT cells can directly respond to environmental lipid changes, supporting a potential role as nutrient sensors.

Results

The iNKT hybridoma cell line DN32.D3 can store environmental lipids as lipid droplets

To investigate the potential lipid loading capacity of iNKT cells and subsequent effects on their phenotype, we first developed a protocol based on our previous work [16], where we exposed mature 3T3-L1 adipocytes to a mix of exogenous lipids, including different FFAs and cholesterol (Supplemental Fig. 1). To allow extensive optimization in these initial stages, we used the murine iNKT cell hybridoma line DN32.D3 [17], which has served as an iNKT cell model in various studies [14], [18], [19]. To visualize lipid accumulation, cells were stained for neutral lipids with BODIPY and analyzed with confocal microscopy, and F-actin and DNA were stained with Phalloidin and DAPI, respectively, to identify individual cells. In parallel, lipid accumulation was quantified by colorimetry, and cell viability was assessed. Using standard growth media as control condition, we consistently observed a few lipid droplets in a subset of DN32.D3 cells, suggesting that these cells harbor the intracellular pathways required for proper LD formation (Fig. 1a and b). Incubation with 0.5%, 2%, and 10% lipid mixture for 12h showed a dose-dependent increase in the amount of lipid droplets (Fig. 1a-l and Fig. S1). To assess how quickly lipids accumulate in this cell system, we incubated with lipid mix for different time periods (0.5h-24h), and elevated TG content was already observed after 0.5h-2h with all concentrations of lipid mix (Fig. 1b, e, h, k). In the control setting, TG uptake remained stable until 24h, where we observed a significant decrease in TG content (Fig. 1b) accompanied by increased viability (Fig. 1c). Upon incubation with 0.5% and 2% lipid mix, the initial TG accumulation was accompanied by a modest increase in viability, especially at 24h (Fig1. f and i). Incubation with 10% lipid mix

gave the highest level of TG accumulation (Fig. 1k) but at the same time reduced cell viability (Fig. 1l). Taken together, these results show that exposure to exogenous lipids rapidly results in TG accumulation in DN32.D3 over a range of concentrations, with prolonged incubations at high concentrations resulting in reduced cell viability. Based on these findings, subsequent experiments were performed with 1-2% lipid mix for 1 hour. Finally, we tested flow cytometry as a method to assess immune cell populations for the presence of lipid droplets using BODIPY or LipidTox (see supplemental). Flow cytometry data (Fig. 1m and n) concurred with our imaging and TG quantification (Fig. 1b and h), showing this to be a reliable additional readout method.

AT-resident iNKT cells store lipids and display lipid-induced IFNγ production

Having observed that the iNKT hybridoma cell line DN32.D3 can store exogenous lipids in LDs (Fig. 1), we next wished to investigate this in primary mouse iNKT cells. As the phenotype of iNKT cells depends on the tissue they reside in [14], [20], [21], we set out to compare iNKT cells from spleen, liver, and AT. As iNKT cells are part of the lymphocyte population, which includes many immune cell types whose LD content has been shown to modulate their IFNγ response [22]–[24], we first examined the lymphocyte population as a whole and subsequently CD3+ lymphocytes before focusing on tissue-resident iNKT cells. We used a stepwise strategy, first passing the tissue single cell suspensions through a MACS iNKT enrichment. We employed a gating strategy based on known iNKT and other immune population markers (see Fig. 2a strategy overview).

After isolation of lymphocyte populations from the tissues and subjecting them to the same lipid staining protocol as for DN32.D3 cells (Fig. 2a), we found that lymphocytes from spleen, liver and AT contain lipid droplets (Fig. 2a). Lymphocytes extracted from AT contained significantly more lipids than either spleen or liver lymphocytes (Fig. 2b). These findings support the view that lipid droplets are a generic organelle present in many immune cell populations and that immune cells residing in higher lipid environments are adapted to import and store higher concentrations of environmental lipids.

CD3+ populations don't exhibit lipid loading tissue specific differences

Before assessing the specifically marked iNKT population, we took advantage of the general immune cell population markers, such as CD3, to gauge the effects of various lipid environments on comparable populations. We examined CD3+ cells, a diverse branch of lymphocytes with distinct functions, including T cells. Previously, the composition of their lipid environment has been reported to heavily influence the

metabolism of extracellular lipids and the IFNγ response of CD3+ cells [24]. In contrast to the general lymphocyte populations (Fig. 2), we observed no significant difference in stored lipid content between CD3+ cells from different tissues (Fig. 3a and b). However, CD3+ cells from liver and AT contained a significantly higher proportion of IFNγ producing cells (Fig. 3c). Furthermore, when exposing these cells to exogenous lipids, no clear differences in lipid accumulation were observed between the tissues (Fig. 3e), but CD3+ cells from AT responded significantly more pronounced with respect to IFNγ production (Fig. 3f).

Adipose resident iNKTs contain elevated lipid levels and respond rapidly to high lipid environments

Having observed phenotypic differences between immune cell populations from different tissues (Fig. 2 and 3), we finally examined the role of tissue environment in lipid storage and IFNγ production of iNKT cells. We therefore gated the immune cells further using the iNKT cell specific glycolipid loaded CD1d tetramer [25], [26]. Lipid staining revealed that AT-resident (CD3+CD1d+) iNKT cells contain significantly higher lipid levels when compared to cells isolated from spleen and liver (Fig. 4a and b) but share a similar percentage of IFNγ+ cells when compared to liver-resident iNKT cells (Fig. 4c). After 1 hour of 1% lipid mix conditioning, both liver-resident and AT-resident iNKT cells contained significantly more lipids than spleen-resident iNKT cells, with nearly all gated INKT cells now producing IFNγ (Fig. 4f-i). We assessed CD44 levels, as this is a maturation marker in iNKT cells with higher levels linked to higher IFNγ production [20]. We found no significant changes in CD44+ low or high subpopulations upon 1% lipid mix exposure (sup Fig.4 a and b), indicating that the lipid mix-induced increase in IFNγ production is not an indirect effect of iNKT cell maturation.

Taken together, these data show that AT-resident iNKT cells display a tissue-specific phenotype, including high lipid storage under basal conditions and increased IFNγ production upon exposure to exogenous lipids.

Discussion

Adipose-resident iNKT cells, likely pre-adapted to a high lipid environment, show heightened responsiveness to changes in the environmental lipidome. LaMarche et al,. previously showed splenic iNKT uptake of palmitic acid, elevated LipidTox signal, resulting in upregulation of E4BP4 and downregulation of PLZF [21]. Our study demonstrates that various iNKT cell populations and others can uptake environmental

lipids, storing them in a tissue-specific manner (Fig. 4). iNKT cells immunometabolism regulation relies heavily on their lipid environment (reviewed [27], [28]). We propose that iNKT cells, particularly adipose resident, are highly sensitive to lipid environments and can more rapidly uptake and store environmental lipids when compared to other immune populations (Fig. 2 and 3). Furthermore, Adipose-resident iNKT cells, likely pre-adapted to a high lipid environment, show heightened responsiveness to changes in the environmental lipidome setting them apart from other iNKT populations in an adipose tissue specific manner.

Adipocytes can tolerate high levels of environmental FFAs as well as sustained uptake and storage during obesity. Our understanding of adipose tissues has drastically changed in the last decade, transforming from an inert storage place to a highly dynamic vascularized, innervated hub where metabolism and immunity intersect [29]–[33]. Like adipocytes, many other cell types can take up environmental lipids and store them in droplets, enabling rapid nutrient supply and a buffer for Lipotoxisity (reviewed [5]). Co-stimulation through CD40/CD40L interaction or cytokines can contribute to iNKT cell activation [34], CD1d-mediated lipid antigen presentation remains the primary driving force. Genetic loss of CD1d in hepatocytes, increased iNKT abundance [35] adipocytes, reduced iNKT abundance [36], [37], and the intestine, no change in iNKT abundance [38], illustrates the crucial but varied role of CD1d in shaping iNKT cell populations. However, even in the absence of CD1d- mediated lipid antigen presentation, external stimuli can directly influence iNKT cell phenotypes [34]. AT-resident iNKT cells exhibit a specific requirement for AMPK and fatty acid metabolism, linking nutrient sensing to immune function. AMPK, a nutrient sensor, plays a pivotal role in regulating glucose and fatty acid uptake, with consequences for lipogenesis, lipolysis, and fatty acid oxidation [39].

Adipose iNKT cells, being more sensitive to lipid uptake, particularly free fatty acids (FFAs), exhibit a distinct response compared to other resident iNKT populations. This sensitivity is further underscored by the observed alterations in iNKT cell phenotype during the weight gain/iNKT decline phase of obesity, transitioning from anti-inflammatory to pro-inflammatory states [14], [21], [40], [41]. iNKT cells regulation of local immune cells and their sensitivity to environmental changes have put them as the fore font of research from cancer to diabetes (reviewed but not limited to: [27], [42]–[45]). While much progress has been made in understanding the evolution and conservation of lipid droplets (LDs) in various species and cell types, the role of LDs in immune cells and inflammation remains an area of active investigation. Here, we highlight the importance of LDs in modulating immune responses and inflammation, suggesting that LDs may be a potential target for therapeutic intervention.

Materials and methods

Cell culture and lipid loading assays

The murine iNKT hybridoma cell line DN32.D3, kindly donated by the Brennan lab, was cultured in Roswell Park Memorial Institute (RPMI) medium (Sigma) supplemented with 10% fetal bovine serum (Bodinco BV), 1% Penicillin-Streptomycin (Sigma), 1% L-Glutamine (Sigma), 2% HEPES (Gibco) and beta- mercapto-ethanol (100 µM). Media was filtered before use. DN32.D3 were grown in suspension flasks up to a confluence of $1x10^6$ cells/mL, and the live/dead ratio was maintained at 95-99% live as evaluated using the Countess II Automated Cell Counter (Invitrogen). Lipid loading was performed by adding Lipid Mixture 1, Chemically Defined (Sigma, L0288) to a regular medium. Before experiments, DN32.D3 was counted, centrifuged (300G for 5 minutes), and resuspended in media containing lipid mix at a confluence of 2-3x10^5 cells/mL. After indicated time points, cells were washed in PBS, fixed in 4% PFA for 30 minutes at room temperature, washed twice in PBS, and stored at 4°C until further analysis. The HeLa: CD1d cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) - high glucose (Sigma) supplemented with 10% FBS (Bodinco BV) and 1% Penicillin-Streptomycin (Sigma).

Lipid loading DN32.D3

Fixed DN32.D3 were stained for neutral lipid using HCS LipidTox Green Neutral Lipid Stain (1:1000, Invitrogen, H34475) or BODPIY 493/503 dye (1:1500, Invitrogen, D3922) for 30 minutes at room temperature. Acquisition was done within 2 hours using a FACSCelesta (BD) 488 laser with a BB515 filter using FACSDiva Software (BD Life Sciences). Flow cytometry results were analyzed using FlowJo v10.8 Software (BD Life Sciences).

Triglyceride Measurements

Intracellular Triglycerides were measured using the kit Stanbio™ Triglycerides LiquiColor™ (#SB2200- 225). Cells were washed with PBS and lysed in 50µl cold PBS via syringe pull technique, then analyzed as per kit instructions.

Confocal microscopy

Samples were stained with Hoechst 3342 (1:1000), BODPIY 493/503 dye (1:500, Invitrogen, D3922), and Phalloidin 647 (1:1000). Within 2 hours, samples were imaged on a Zeiss laser scanning microscope (LSM)880, using a 40x water immersion objective. Brightness and contrast were adjusted using Fiji version 1.52g. (National Institutes of Health, USA).

Flow cytometry and analysis

Spleen, liver, visceral, and subcutaneous adipose tissue were collected and pooled from four B6 mice of 9- 11 weeks old. Spleens and livers were degenerated through a 70 µm cell strainer into 50 mL ice- cold PBS and centrifuged at 400G for 10 minutes. Red blood cells were lysed in 5 mL Red Blood Cell Lysis Buffer for 5 minutes at room temperature, washed in PBS, and filtered through a 70 µm filter. Visceral adipose tissue was minced after removal of blood and lymph nodes and kept in ice-cold digestion buffer (Hanks' balanced salt solution (HBSS) with Ca^{2+} and Mg^{2+} supplemented with 0.5% bovine serum albumin) Per 1 g adipose, 1 ml of 10 mg/ml collagenase (Sigma-Aldrich; C6885) was added and incubated at 37°C for 15–20 minutes with vortexing every 10 minutes until a single cell suspension was achieved. Adipose tissue single cells were then passed through a 100 µm filter. iNKT cells were isolated from the single-cell suspensions using the $NK1.1^+$ iNKT Cell Isolation Kit (Miltenyi Biotec, 130-0960513) in which the Anti-NK1.1-APC antibody was replaced for mCD1d tetramer-APC (NIH Tetramer Core Facility). NK1.1 kit enrichment cocktail, CD115, CD8a, CD45R, Nkp46 (CD33) and TCR (gamma lambda yd). The enriched single cell suspensions were then resuspended in 100µl PBS, following FIX & PERM™ Cell Permeabilization Kit (GAS003), with the antibodies, CD3 (BioLegend 100203), CD44 (BioLegend 103031), IFNγ (BioLegend 502527), OHC loaded tetramer CD1d (NIH Tetramer Core Facility) and LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen™ L34957) on ice. Cells were washed twice with staining buffer, resuspended in 200µl staining buffer, and analyzed within 1 hour using a CytoFLEX S cytometer (Beckman Coulter).

Data representation and statistics

Flow cytometry data were analyzed using FlowJo software version 10.8.1 (Becton Dickinson). All graphs and statistical analyses were generated using GraphPad Prism 9.1.0.

Data availability statement

All datasets generated for this study are included in the article/Supplementary material.

Declarations and ethics statements

All mouse study protocols were approved by the Utrecht University Ethical Committee for Animal Experimentation (protocol 2010. III.07.083 and 2011.III.06.061) and were in accordance with Dutch laws on animal experimentation.

Competing interests

The authors have no competing interests to declare.

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Author contributions
IM, FV, RS, and EK designed the experiments. IM, FV, and AB performed experiments and analyzed the data. IM drafted the manuscript. IM, FV, RS, and EK edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Figure

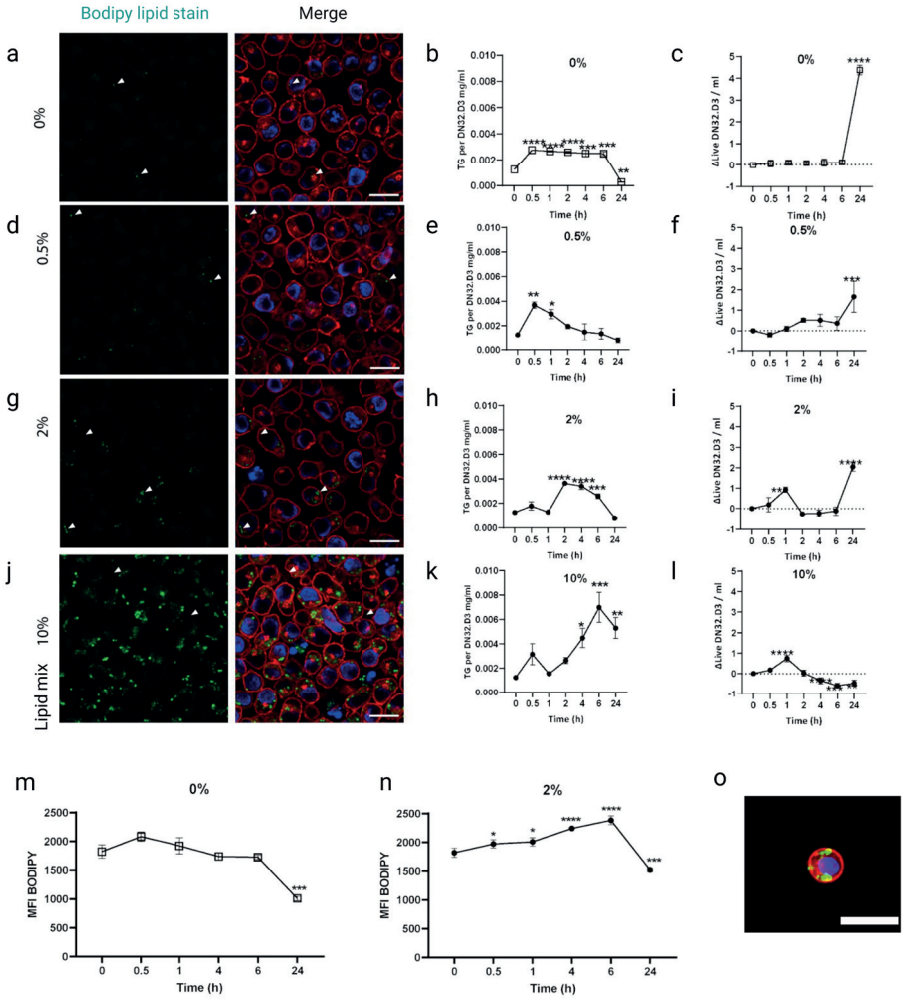

Figure 1: DN32.D3 iNKT cell line loads and stores environmental lipids as lipid droplets in a dose dependent manner

(a)DN32.D3 cells cultured in growth medium for 12h stained for lipids (Bodipy in green) DNA (DAPI in blue) and membrane (Phalloidin in red). (b) intracellular triglyceride content of DN32.D3 (mg/ml). (c) delta change of live dead ratio of DN32.D3 over a 24h period of culture. (d-f) 0.5% Lipid mix. (g-i) 2% Lipid mix. (j-l) 10% Lipid mix. (m) 24h culture of DN32.D3 were cultured in growth media (n) or 2% lipid mix over a 24h period, stained for lipid content via Bodipy then analyzed for mean fluorescent intensity (MFI)by flow cytometry (10.000 events/run). (o) Lean visceral adipose iNKT stained as DN32.D3 above. (ns p >0.05, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001), white scale bar is 20µm, n=3.

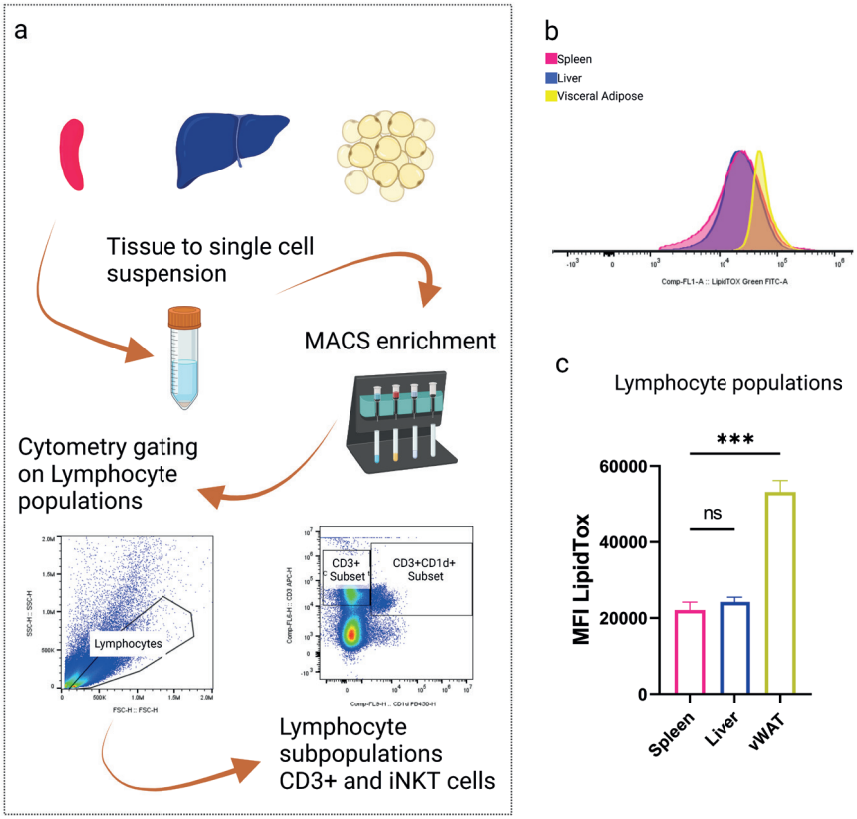

Figure 2: Ex-vivo gated lymphocytes show a lipid loaded adipose phenotype
Representative histogram of lipid stained lymphocyte populations from spleen, liver and vWAT. (b) MFI of lipid stained lymphocyte populations (ns p >0.05, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001), n=3.

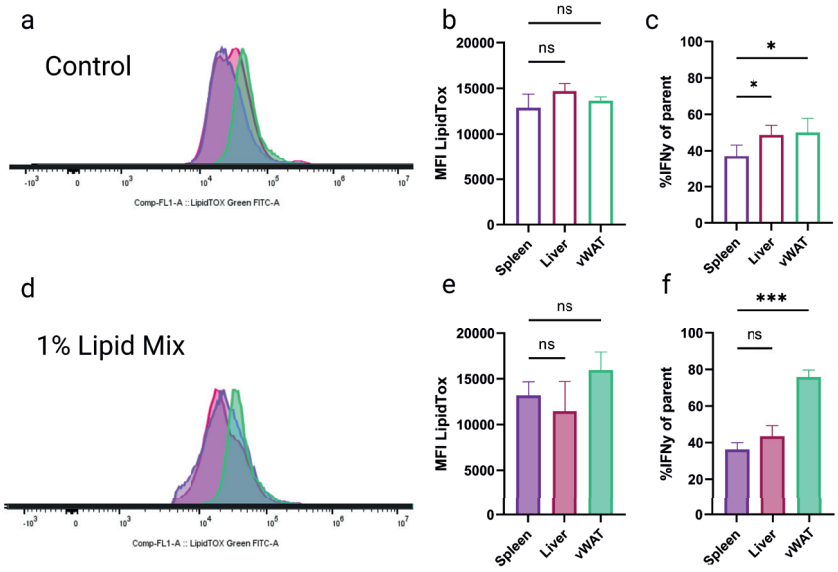

Figure 3: Ex-vivo gated CD3+ enriched cells do not load lipids in an organ dependent phenotype
Representative histogram of lipid stained ex-vivo CD3+ populations from spleen, liver, and vWAT. MFI of lipid stained CD3+ populations. (c) CD3+ cells %IFNγ of parent populations. (d) Representative histogram of ex-vivo CD3+ populations conditioned for one h in 1% lipid mix spiked growth media. (e) MFI of lipid stained CD3+ populations. (c) CD3+ cells %IFNγ of parent populations. (ns p >0.05, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001), n=3.

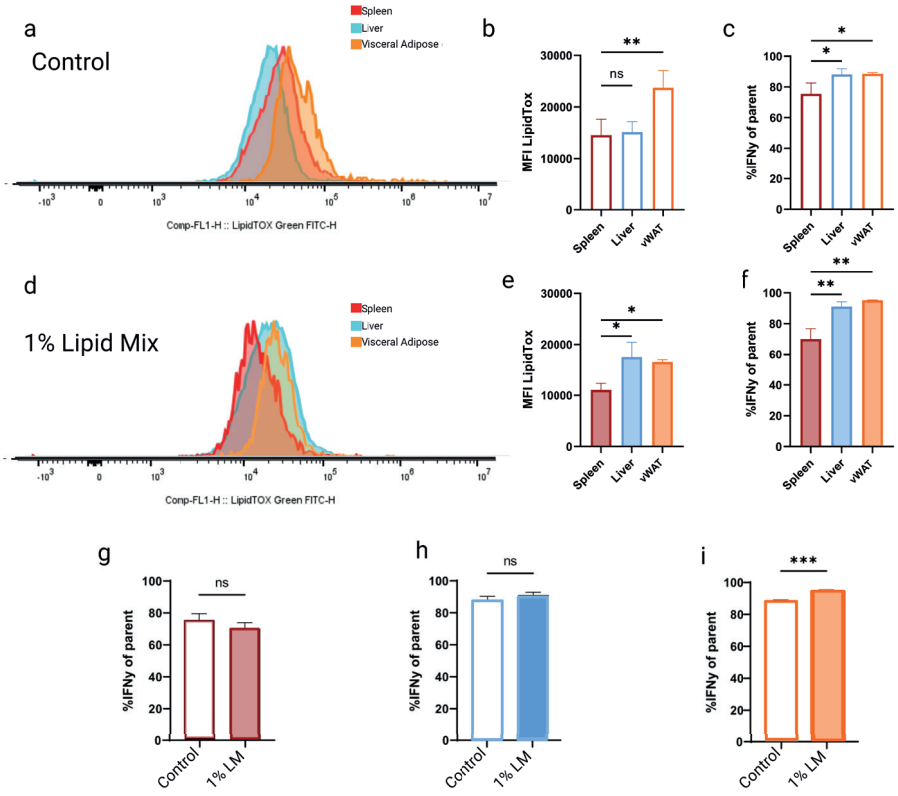

Figure 4: iNKT populations conditioned in 1% lipid mix significantly elevate their IFNγ in an adipose specific manner

(a)Representative histogram of lipid stained ex-vivo CD3+CD1d+ (iNKT) populations from spleen, liver, and vWAT. (b) MFI of lipid stained CD3+CD1d+ (iNKT) populations. (c) CD3+CD1d+ (iNKT) cells %IFNγ of parent populations producing the cytokine IFNγ. (d) Representative histogram of ex-vivo CD3+CD1d+ (iNKT) populations conditioned for 1h in 1% lipid mix spiked growth media. (e) MFI of lipid stained CD3+ populations. (c) CD3+ cells %IFNγ of parent populations. (ns p >0.05, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001), n=3.

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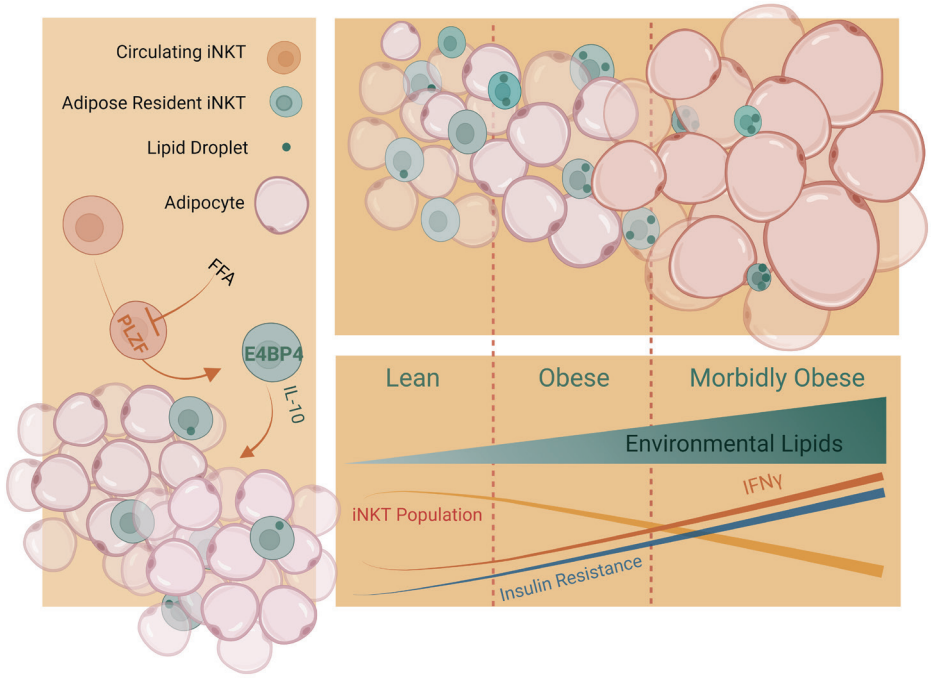
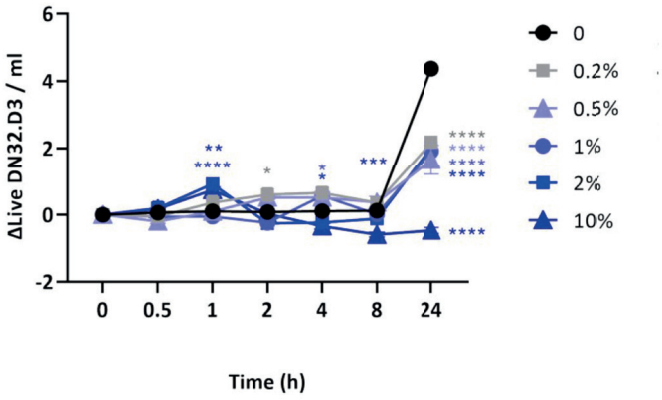
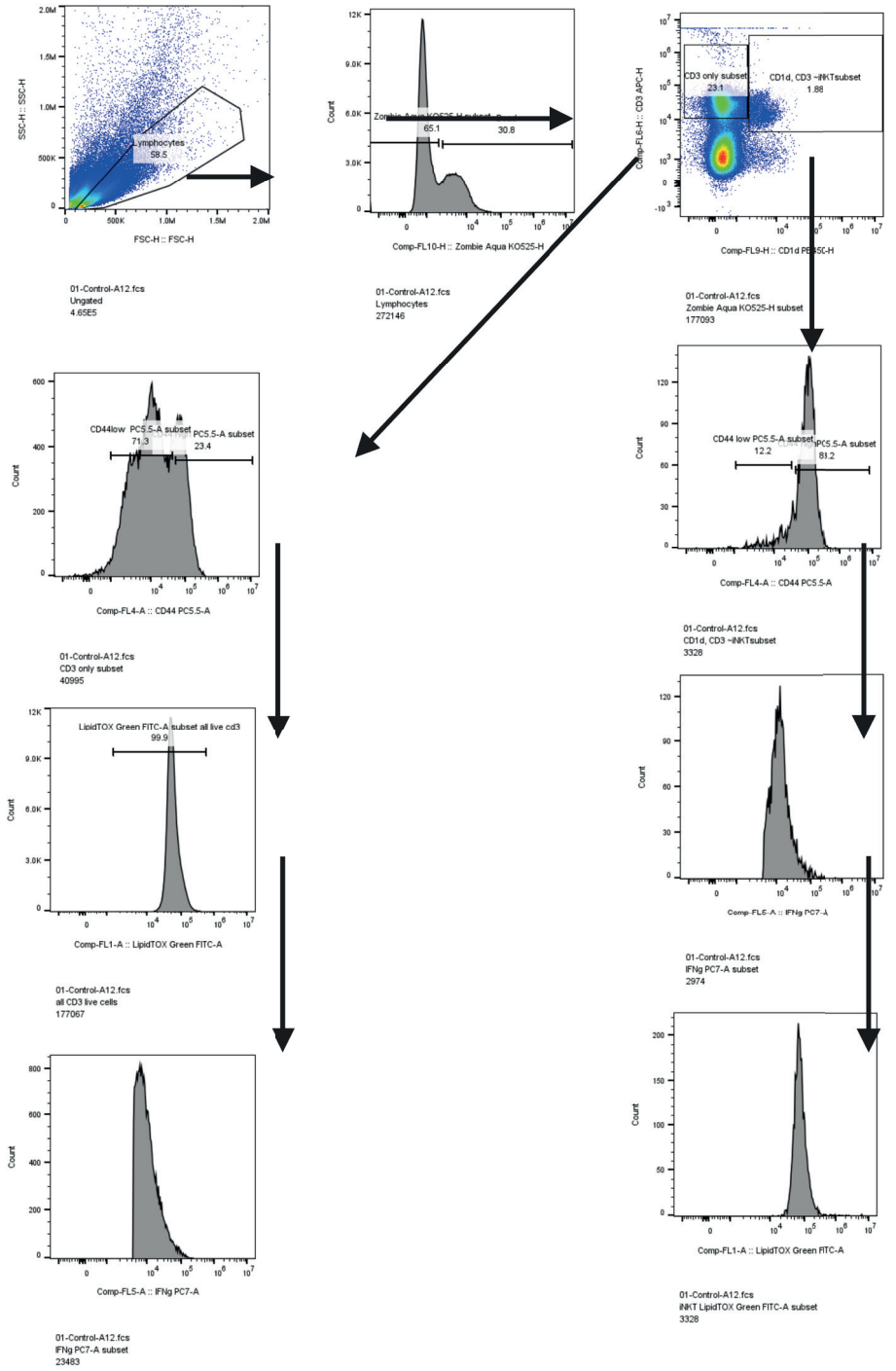
Graphical Abstract



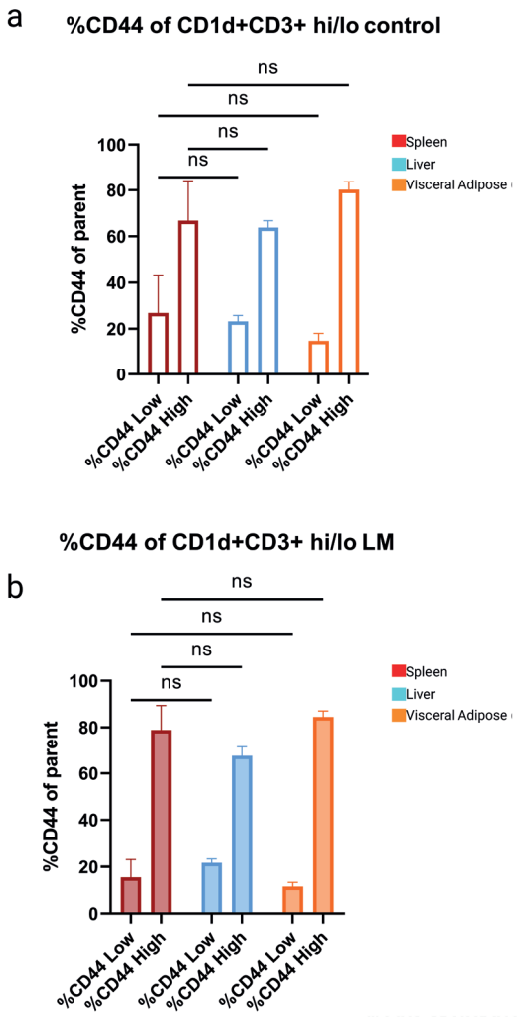
Increased Lipid Droplet Accumulation in Adipose-Localized Invariant Natural Killer T Cells Contributes to Inflammatory Imbalance and Impaired Proliferation in Obesity



Supplemental Figure 1:
Delta change live dead ratio of DN32.D3 cells cultured in various doses of lipid mix spiked growth media over a 24-hour period. (ns p >0.05, * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001), n=3.



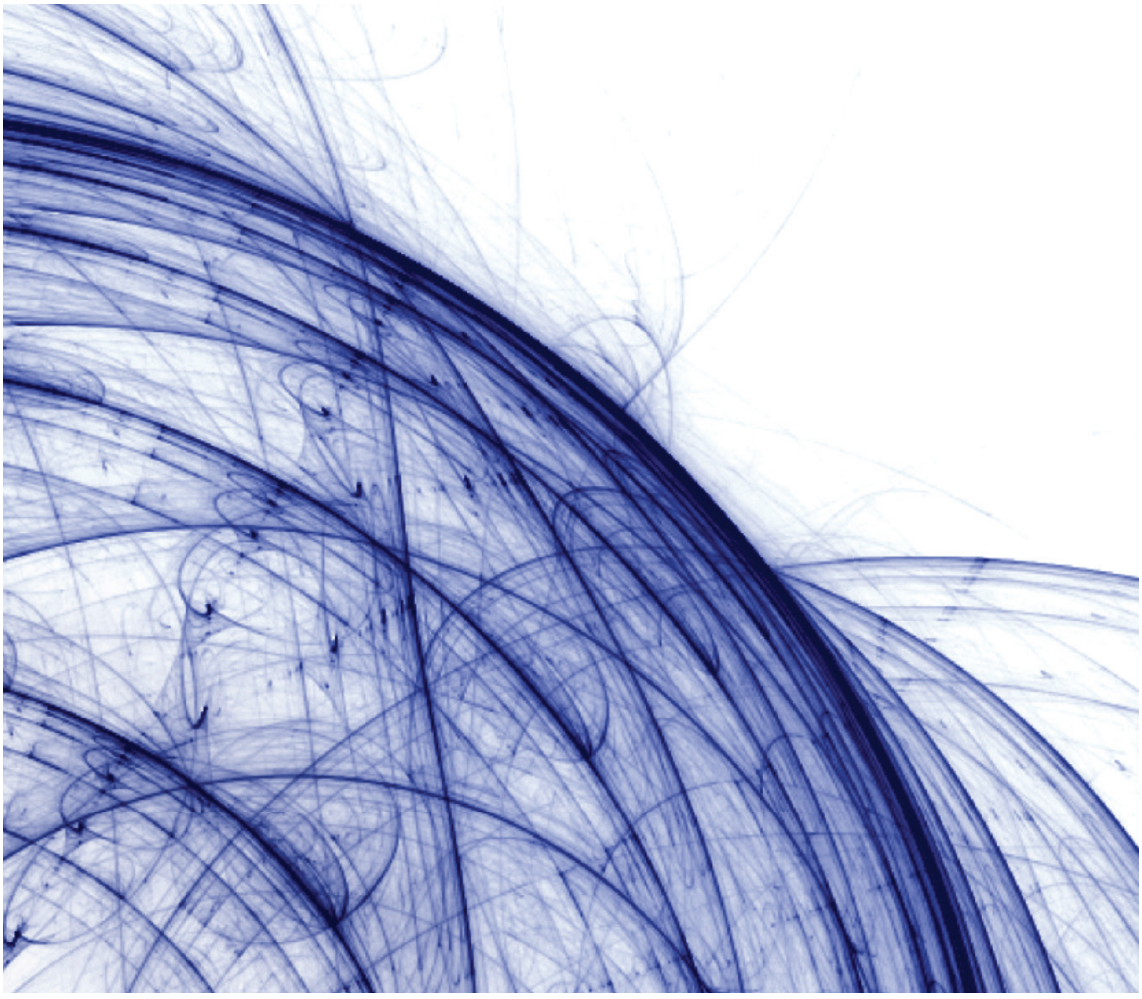
Supplemental Figure 2: Gating strategy

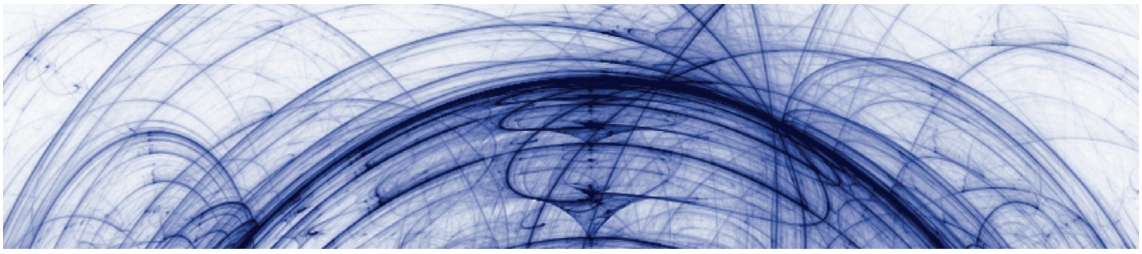


Supplemental Figure 3: CD44, a maturation and activation marker of iNKT cells is not affected by lipid loading ex-vivo iNKT with -/+ 1% lipid mix

ex-vivo CD3+CD1d+ (iNKT) from spleen, liver and vWAT cultured in control growth media and (b) ex- vivo CD3+CD1d+ (iNKT) cultured in 1% LM stained for CD44. (ns p >0.05, * p <0.05, ** p <0.01, *** p<0.001, **** p <0.0001), n=3.

Chapter 4





The pseudokinase TRIB3 controls adipocyte lipid homeostasis and proliferation *in vitro* and *in vivo*

Hernández-Quiles, M*., Martinez Campesino, L*., **Morris, I.,** Ilyas, Z., Sobrevals Alcaraz, P., van Es, R., Vos, H., Wilson, H., Kiss-Toth, E. and Kalkhoven, E.

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ABSTRACT

Objective: *In vivo* studies in humans and mice have implicated the pseudokinase Tribbles 3 (TRIB3) in various aspects of energy metabolism. Whilst cell-based studies indicate a role for TRIB3 in adipocyte differentiation and function, it is unclear if and how these cellular functions may contribute to overall metabolic health.

Methods: We investigated the metabolic phenotype of whole-body *Trib3* knockout (*Trib3*^{KO}) mice, focusing on adipocyte and adipose tissue functions. In addition, we combined lipidomics, transcriptomics, interactomics and phosphoproteomics analyses to elucidate cell-intrinsic functions of TRIB3 in pre- and mature adipocytes.

Results: *Trib3*^{KO} mice display increased adiposity, but their insulin sensitivity remains unaltered. *Trib3*^{KO} adipocytes are smaller and display higher Proliferating Cell Nuclear Antigen (PCNA) levels, indicating potential alterations in either i) proliferation-differentiation balance, ii) impaired expansion after cell division, or iii) an altered balance between lipid storage and release, or a combination thereof. Lipidome analyses suggest TRIB3 involvement in the latter two processes, as triglyceride storage is reduced and membrane composition, which can restrain cellular expansion, is altered. Integrated interactome, phosphoproteome and transcriptome analyses support a role for TRIB3 in all three cellular processes through multiple cellular pathways, including Mitogen Activated Protein Kinase- (MAPK/ERK), Protein Kinase A (PKA)-mediated signaling and Transcription Factor 7 like 2 (TCF7L2) and Beta Catenin-mediated gene expression.

Conclusions: Our findings support TRIB3 playing multiple distinct regulatory roles in the cytoplasm, nucleus and mitochondria, ultimately controlling adipose tissue homeostasis, rather than affecting a single cellular pathway.

Keywords Tribbles; Adipocyte; Omics analyses; Metabolism

1. INTRODUCTION

The prevalence of obesity and its related comorbidities have tripled since 1990 and the global incidence of type II diabetes is projected to reach 350 million cases by 2030 [1]. Obesity is a chronic, multifactorial disease, developed through the interaction between genetics and environmental factors, such as nutrition, physical activity and cultural influences [2—4]. The World Health Organization (WHO) defines obesity as an unhealthy state characterized by excessive and abnormal adiposity. This adipose alteration represents the first step into the development of chronic inflammation and insulin resistance, resulting in metabolic dysfunction [5]. Adipose tissue (AT) is responsible for the storage and release of free fatty acids in response to different metabolic needs as well as the regulation of whole-body metabolism through the production and secretion of adipose-specific chemokines [6,7]. Adipocyte differentiation and function are tightly controlled by a set of pro- and anti-adipogenic factors, such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBP) as pro-adipogenic factors [8] and Wnt signaling as an anti-adipogenic pathway [9,10]. Prolonged and excessive exposure to a high-caloric diet together with a sedentary lifestyle result in an increase in adipocyte number and size. These hypertrophic adipocytes become dysfunctional, leading to a reduction in insulin sensitivity and overall metabolic health [11,12]. In this context, understanding the mechanisms that govern adipocyte function and expandability is crucial for the development of new targeted therapies to improve insulin resistance and adipose metabolic health.

TRIB3 is a member of the Tribbles family of serine/threonine pseudokinases that functions as regulatory/scaffold proteins, controlling a plethora of metabolic and cellular functions (reviewed in [13—15]).

[1]Center for Molecular Medicine, University Medical Center Utrecht, Utrecht University, 3C584 CG Utrecht, The Netherlands [2]Division of Clinical Medicine, School of Medicine and Population Health, University of Sheffield, Sheffield S10 2TN, UK [3]Oncode Institute and Molecular Cancer Research, Center for Molecular Medicine, University Medical Center Utrecht, Utrecht University, 3C584 CG Utrecht, The Netherlands [4]Department of Dermatology and Allergology, University of Szeged, H-6720 Szeged, Hungary [5]Lee Kong Chian School of Medicine, Nanyang Technological University Singapore, Clinical Sciences Building, 11 Mandalay Road, 308232 Singapore, Singapore [6]School of Biological Sciences, Nanyang Technological University Singapore, 60 Nanyang Drive, 637551 Singapore, Singapore

[7] These authors contributed equally to this work.

*Corresponding author. E-mail: e.kalkhoven@umcutrecht.nl (E. Kalkhoven).

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Tribbles act as interaction platforms promoting and inhibiting post-translational modification, such as ubiquitination and phosphorylation, and affecting protein-protein interactions [16] by preventing or enhancing specific interactions. Thus, Tribbles have been shown to play a critical role in pathways that control cellular differentiation, lipid metabolism and immune cell activation among others [17–19]. Yet, the role of TRIB3 in adipocytes is incompletely understood. Previous *in vivo* studies have pointed to TRIB3 as a critical regulator of glucose tolerance and insulin sensitivity [20–23], with inhibition of insulin-mediated AKT activation as the prime underlying mechanism in hepatocytes and adipocytes [23–26], and in vitro studies have suggested a role for TRIB3 in adipocyte differentiation and function by regulating C/EBPβ [27] and PPARγ transcriptional activity [28]. Also of interest is the ability of TRIB3 to promote Wnt signalling by stabilizing the interaction between β-Catenin and TCF4 observed in colorectal cancer stem cells [29], as the Wnt pathway inhibits adipogenesis [10,30]. To determine the functional importance of TRIB3 in adipose tissue we therefore characterized the AT phenotype in a full-body TRIB3 knockout mouse model and combined lipidomics, transcriptomics, interactomics and phosphoproteomics analyses to elucidate cell-intrinsic functions of TRIB3 in pre- and mature adipocytes. We found that TRIB3 ablation impairs adipocyte expandability and their lipid profile, resulting in an increased adipose tissue mass, composed of smaller adipocytes. In addition, we found that TRIB3 functions as an intermediate between molecules that regulate a number of critical signalling cascades in response to external stimuli. Our study presents TRIB3 as a critical signalling mediator that regulates AT expansion and homeostasis.

2. MATERIALS AND METHODS

2.1. Mouse experiments, licensing, husbandry and care

All experiments were performed in accordance with UK legislation under the Animals (Scientific Procedures) Act 1986. The University of Sheffield Project Review Committee approved all animal experiments which were carried out under the UK Home Office Project License 70/7992 held by Professor S.E. Francis, and Personal License to L. Martinez Campesino ID645D5F9. Mice were kept in an optimal and controlled environment to reduce stress. Mice were subjected to 12 h light/12 h dark cycle, at 22 °C with 40-60% of humidity. Their diet consisted of a standard chow (Harlan, 18% protein rodent diet) and it was unrestricted.

2.2. Mouse strains and genotyping

Development of full body *Trib3* knock out mice ($Trib3^{KO}$) strain was previously described [31]. Genomic DNA isolated from mouse ear clips was amplified by PCR (Supplementary Table 1), using specific primers for the *Trib3* WT and KO alleles (Supplementary Table 2). Samples were qualitatively analyzed using a trans-illuminator with EtBr/UV filter (Supplementary Figure 1). All the genotyping process was performed by the Genomics Core facility. Mice were weighed and culled via pharmacological overdose of 0.2 ml sodium pentobarbital (200 mg/ml) applied into the peritoneal cavity and cervical dislocation. Body as well as tissue weights were recorded.

2.3. Magnetic resonance imaging

Fifteen-week-old chow-fed male $Trib3^{KO}$ ($n = 4$) and $Trib3^{WT}$ mice ($n = 3$) were subjected to magnetic resonance imaging (MRI). Images were obtained using a 9.4 T, Bruker Avance III MRI scanner (Bruker Biospin MRI GmbH, Ettlingen, Germany) with a 25 mm 1H volume coil. Each sacrificed mouse was placed in the center of the coil aligned with the abdomen and with the hips oriented at the top of the image. Structural MRI scans were performed using an MSME spin echo sequence (FOV 3.0 × 3.0 cm, 512x512 matrix, TE/TR 16 ms/1000 ms, Number of averages 16). A stack of contiguous axial slices of 1 mm thick were acquired for each mouse (35 ± 1 slices per mouse in total) and processed using Bruker Paravision 5.1 software. The slice package of MRI images was segmented and analyzed in FIJI/ImageJ. To align the fat measurements in the histograms, the location of the hips was used as standardized reference point. Intensity and threshold adjustment were performed for fat identification. Total adipose tissue, as well as subcutaneous (inguinal and dorsolumbar) and visceral (epididymal, mesenteric and perirenal) depots were distinguished based on their location.

2.4. Lipid profiling

Plasma was separated from isolated blood by centrifugation (1500×*g* for 5 min at room temperature) and immediately stored at -80 °C. For analysis, 150 μl of plasma was sent to the Department of Clinical Chemistry at the Royal Hallamshire Hospital (Sheffield Teaching Hospitals) to assess a full lipid profile measuring: total cholesterol, low (LDL) and high (HDL) density lipoproteins, triglycerides and glucose, using a Roche Cobas 8000 modular analyser series. For definitions of lipoprotein profiling see Supplementary table 3.

2.5. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

Mice were fed on chow diet for at least 8 weeks and then fasted overnight. Fasting mice were weighted and blood was collected from the tail. A 20% glucose solution was prepared and filtered through 0.2 μm filter. For GTT, 2 mg of glucose were administrated per g of body weight and blood was collected at 0, 30, 60, 90 and 120 min after glucose challenge by tail sampling method. For ITT, mice were faster for at least 2 h and then weighted. Then 0.75 mU insulin per gram of body weight was injected intraperitoneally. Using an insulin syringe. Blood glucose was measured at 0, 20, 40, and 60, 90 and 120 min after injection.

2.6. Semi targeted lipidomics

3T3-L1 cells stably transfected with shScramble or shTRIB3 plasmids were differentiated in 6-well plates and once differentiated, cells were washed with 300 μL of ice-cold PBS solution three times. 500 μL of dry-ice cold methanol/water mix (80%/20%,v/v) was added and cells and scraped from the well. Cells were then collected and stored on dry ice or at -80 °C for lipidomic analysis.

2.7. Lipidomics

A volume of 50 μL homogenized cells was subjected to Liquid-Liquid extraction (LLE). The sample was vortex-mixed with methanol-methyl-tert-butylether (containing one internal standard per lipid class and an amount of antioxidant — BHT - to prevent lipid oxidation) after which an amount of water was added to induce phase separation. After incubation, the sample was centrifuged and the organic top layer containing all lipids was transferred to a clean sample vial. This lipid fraction was dried in a vacuum concentrator. Prior to analysis the lipid residue was dissolved in acetonitrile, thoroughly vortex mixed and transferred to an injection vial. LC-MS/MS sample analysis was conducted on an Ultimate 3000 UHPLC with LTQ-Orbitrap XL high resolution mass spectrometry detection. For chromatographic separation an Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm) positioned in a 60 °C column oven was used. Upon injection of 5 μL sample a 10 min gradient was started (total runtime 20 min per sample). Sample analysis was conducted in both positive mode and negative mode.

Generated data were submitted to MZMine for alignment and data analysis. Raw data was uploaded and normalized via Metaboanalist 5.0. Principal component analysis (PCA) analysis and heat map generation was also performed via Metaboanalist 5.0 software [32]. Enrichment analysis of normalized lipidomic data, using 'ranking mode', was performed via LIONweb [33]. Data has been deposited using Metabolights (www.ebi.ac.uk/metabolights/mtbls8891).

2.8. Isolation and culturing of primary mouse and human (pre) adipocytes

Adipose tissue from *Trib3*^{KO} and *Trib3*^{WT} was dissected, minced and digested using a collagenase buffer (HBBS medium (Gibco), 2% (v/v) BSA (Sigma) and 1.4 mg/ml collagenase type II (Sigma)) to facilitate the dissociation between adipocytes and stromal vascular cells (SVC). To separate and discard adipocytes from SVCs, the digested tissue was filtered and centrifuged, and the supernatant containing adipocytes was removed. The cell pellet corresponding to the SVC fraction was then incubated with red blood cell (RBC) lysis buffer, neutralized and centrifuged again. The resulting cell pellet containing SVCs was used for preadipocyte culture. The isolated SVC fraction from the AT digestion was cultured in complete DMEM media, changing media every 2 days. At 90% cell confluency, cells were treated with differentiation media, consisting of complete DMEM media supplemented with 1 μg/ml of Insulin (Sigma), 2.5 μM Dexamethasone (Sigma) and 0.5 μM 3-Isobutyl-1-methylxanthin (IBMX) (Sigma). After 4 days of differentiation, cells were maintained with complete media supplemented with 1 μg/ml of Insulin (Sigma) for 7 days, until complete differentiation.

Human liposuctions were isolated from subcutaneous adipose tissue of consented unrelated healthy volunteers aged between 20 and 50 years under ethical approval from the national Ministry for Human Resources in Hungary (Ref: FAT-H2020-001). Approximately 25 ml of fat was taken from liposuction material, centrifuged twice (430×*g*, 10min) in sterile PBS with the infranatant discarded. Enzymatic digestion was performed using collagenase (Sigma, 0.5 mg/ml in PBS) at 37 °C for 30-40 min with gentle rotation. The resultant homogeneous emulsion was filtered with a 100 μm strainer. To isolate adipocytes, warm culture media (DMEM with 10% FBS) was added to the filtered mixture for 5min to inhibit the enzymatic activity, then centrifuged (800×*g*), where the upper fat layer containing mature adipocytes was collected for further RNA isolation and gene expression analysis (RNeasy UCP kit, Qiagen). The remaining supernatant was discarded, and the cell pellet was collected for stromal vascular cell fraction (SVF) isolation. Red blood cell lysis was performed by resuspending the pellet with 10 ml of RBC lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 M EDTA in H2O) for 10min. The cell pellet following centrifugation (800×*g*) containing the SVF was resuspended in sterile PBS + 2%(w/v) BSA. SVF was cultured to obtain a purified population of pre-adipocytes in DMEM with 10% FBS for 3-4 days at 37 °C and 5% CO2. Media was removed after 1 day of culture to discard non-adherent cells, then replaced twice weekly, until 80-90% confluent where cells were detached using trypsin/EDTA. Cell pellets were collected for further RNA isolation and gene expression analysis (RNeasy UCP kit, Qiagen).

2.9. RNA extraction and reverse transcription (RT)-qPCR

Total RNA was isolated from pre-adipocytes and mature adipocytes was performed by using the RNeasy lipid tissue mini kit (Qiagen) following manufacturer's protocol. cDNA was synthesized using the Precision nanoScriptTM 2 RT kit (Primer design) according to manufacturer's instructions. Quantitative PCR was carried out using Precision PLUS SYBR-Green master mix (Primer design) in a Bio-Rad i-Cycler machine. Specific primers were designed with NCBI BLAST and all assays were performed in triplicate and normalized to the expression levels of *cyclophilin A* as a suitable housekeeping gene. Fold changes compared to the house-keeping genes were calculated using ΔΔCt method. Amplification and melting curves were checked for each reaction to ensure specific single products were amplified with >90% efficiency.

2.10. RNA sequencing and bioinformatic analyses

RNA was isolated from differentiated adipocytes from *Trib3*^{WT} and *Trib3*^{KO} mice (N = 5, per group) using the RNeasy lipid tissue mini kit (Qiagen) according to manufacturer's instructions. Samples were then sent to Novogene Co. Ltd (https://en.novogene.com) and used for messenger RNA sequencing. For cDNA library construction, mRNA was enriched using oligo(dT) beads, randomly fragmented following cDNA synthesis. Then cDNA libraries were assessed for quality control and qualified libraries were used for sequencing using Illumina sequencers (NovaSeq platform). The obtained raw data files were analyzed and quality controls on the fastq files, principal component analysis and differentially expressed gene analysis was performed. Data was analyzed using Ingenuity Pathway Analysis software (IPA, Qiagen). Data will be deposited in the NCBI GEO database and accession number will be provided upon submission of the manuscript.

2.11. Tissue sections and staining

Tissue sections were dewaxed in xylene, rehydrated in graded alcohols (100%—75% v/v) and rinsed in water following incubation in Gills hematoxylin solution for 5 min. The slides were then rinsed in running water, submerged in Scott's Tap-Water for 30 s and rinsed in water again. Eosin-phloxine was used for counter-staining following water rinse and dehydration in graded alcohols and xylene. Slides were then mounted with coverslips using DPX mounting solution (Sigma-Aldrich, UK). Images to assess tissue morphology were taken using a brightfield microscope (Nikon Eclipse E6000) at 10x and 20x magnification. Three fields of view per tissue per mouse were captured and analyzed using Image J software. Adiposoft software [34] was used to asses adipocyte area.

2.12. Cell culture

The immortalized, murine-derived brown pre-adipocyte cell line (IBA) [35,36] was cultured in high-glucose (4.5 g/L D-glucose) DMEM medium (Life technologies, Carlsbad, CA) supplemented with 10% bovine serum and 1% penicillin and streptomycin. Cells were incubated in 5% CO_2 incubator at 37 °C and 95% humidity. Generation of inducible TRIB3-tGFP IBA cells was done using third-generation lentiviral constructs using supernatants form HEK293T (ATCC CRL-3216, Manassas, VA, USA) cells transfected with lentiviral packaging plasmids. HEK293T cells were transfected using X-treme gene 9 DNA transfection reagent (Roche) following manufacturer's instructions.

2.13. Western blot analysis

Western blotting was performed as described before [37]. In short, after induction with doxycycline, cells were lysed in iced-cold lysis buffer (150 mM NaCl, 1% NP40, 0.5% sodium DOC, 0.1% SDS, 25 mM Tris pH7.4 and supplemented with protease inhibitors). Protein concentrations were measured and samples were supplemented with Laemmli sample Buffer (LSB). Samples were run in SDS-PAGE gels and transfer to PVDF membranes. Blocking was performed in 5% milk in TBS-T for 45 min at room temperature. ECL western blot solution was used to detect protein expression using a LAS4000 Image Quant.

2.14. Immunoprecipitation for mass spectrometry

Immunoprecipitation was performed as described previously [38] using turboGFP-Trap beads (Chromotek) after 24/48 h induction with doxycycline. Samples were prepared in triplicates and Label Free Quantification (LFQ) was used to determine the interactors of TRIB3 and day 0 and day 6. Turbo-GFP only was used as control in both cases to filter out possible contaminants or proteins sticking to the beads or the tGFP tag.

2.15. Mass spectrometry

Mass spectrometry methodology was extensively described previously [38]. In short, precipitated proteins were digested with trypsin (250 ng/μL) and peptides were separated from beads using a C-18 stage tip (3 M, St Paul, MN, USA). After separation peptides were electrosprayed directly into an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Scientific). The MS was run in DDA mode with one cycle per second. Full scan (400−1500 mass range) at a resolution of 240,000 Ions was performed reaching an intensity threshold of 10,000. Ions were isolated by the quadrupole and fragmented with an HCD collision energy of 30%. The obtained data was analyzed with MaxQuant [Version 1.6.3.4] using the Uniprot fasta file (UP000000589) of *Mus musculus* (Taxonomy ID: 10090).

2.16. Phosphoproteomics

Enrichment of phospho-peptides for SILAC labeling, IBA cells with inducible tFGP-TRIB3 overexpression were cultured in high-glucose (10% dialyzed FBS (BioWest)) DMEM (Thermo) lacking lysine and arginine supplemented with Lys-0/Arg-0 or Lys-8/Arg-10 (Silantes). Cells were lysed in 8 M Urea, 1 M Ammonium-BiCarbonate (ABC) containing 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 40 mM 2-chloro-acetamide supplemented with protease inhibitors (Roche, complete EDTA-free) and 1% (v/v) phosphatase inhibitor cocktails 2 and 3 (Sigma, Cat. No. P5726 and Cat. No. P0044). After ultra-sonication, Heavy and light cell lysates were mixed 1:1 and proteins (20 mg total) were over-night in solution digested with trypsin (1:50) (Worthington). Peptides were desalted using SepPack columns (Waters) and eluted in 80% acetonitrile (ACN). To enrich for phospho-peptides, 200 mg Calcium Titanium Oxide (CaTiO3) powder (Alfa Aesar, 325 mesh) was equilibrated 3 times with binding solution (6% Acetic acid in 50% ACN pH = 1 with HCl) after which the phospho-peptides were allowed to bind at 40 °C for 10 min on a shaker. After 6 times centrifugation and washing, phospho-peptides were eluted twice with 200 μl 5% NH3. The peptides were dried using a SpeedVac and the dissolved in buffer A (0.1% FA) before loading on in-house made C18 stage-tips and divided with high PH elution into three fractions (100 mM NH3/FA PH = 10 in 5%, 10% or 50% ACN).

2.17. Data analysis

Raw files were analyzed with the Maxquant software version 1.6.3.4 (Cox and Mann, 2008) with phosphorylation of serine, threonine and tyrosine as well as oxidation of methionine set as variable modifications, and carbamidomethylation of cysteine set as fixed modification. The Mus Musculus protein database of Uniprot (January 2019) was searched with both the peptide as well as the protein false discovery rate set to 1%. The SILAC quantification algorithm was used in combination with the 'match between runs' tool (option set at 2 min). Peptides were filtered for reverse hits and standard contaminants. Forward and Reverse ratios were plotted in R (www.r-project.org). The mass spectrometry proteomics data has been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (http://www.ebi.ac.uk/pride, identifier: PXD046703).

2.18. F2 intercross mice study (BHF2) dataset analysis

Dataset generated at The university of California Los Angeles (UCLA) from the F2 intercross between C57BL/J6 and C3H/Hej mice (BHF2 population) on a ApoE insufficient background ($ApoE^{-/-}$) is publicly available at http://www.genenetwork.org. Data was retrieved and Pearson correlations between male mice adipose tissue mRNA expression and the described phenotypes were obtained.

3. RESULTS

3.1. *TRIB3* expression is increased in hypertrophic adipocytes and its expression correlates with different metabolic traits

First, we used publicly available human datasets from the Gene Expression Omnibus (GEO) repository to assess the expression of *TRIB3* in adipose tissue (AT). The expression of TRIB3 was evaluated as normalized signal intensity in three different datasets: GSE23699, GSE12050 and GSE61302. In GSE23699, the expression of genes in human subcutaneous adipose tissue was compared to the expression of human subcutaneous adipocytes. The study from GSE12050 evaluates the differential gene expression of tissue samples from lean and obese subjects. And GSE61302 contains microarray data from the analysis performed for the comparison of stromal stem cells before and after 7 days and 21 days of adipocyte differentiation in vitro.

We found that TRIB3 is highly expressed in human AT, and in particular in the adipocyte fraction (Figure 1A). In addition, TRIB3 expression was elevated in AT of individuals with obesity compared to lean individuals (Figure 1B). Furthermore, TRIB3 expression was elevated in fully mature adipocytes (day 21) compared to undifferentiated adipose tissue-derived stromal stem cells (ASCs) and early differentiation state (day 7) (Figure 1C). In addition, we used a well-characterized mouse hybrid panel [39] to assess whether adipose tissue-specific TRIB3 expression correlates with any phenotypic trait [40]. We found that TRIB3 expression in AT positively correlates with leptin levels ($r = 0.174$; $p = 4.1*10^{-2}$) and negatively with weight ($r = -0.170$; $p = 3.9*10^{-2}$) and non-abdominal fat ($r = -0.190$; $p = 2.1*10^{-2}$) (Supplementary Figure 2); it should be noted that these traits are not independent (e.g. body weight positively correlates with leptin levels [41]). Altogether, these data point to TRIB3 as a potential regulator of AT function(s) and metabolic health in humans.

3.2. *Trib3* deficiency leads to an increased body weight, altered cholesterol and glucose homeostasis

While publicly available data show correlations between TRIB3 expression and adipocyte and AT function (Figure 1 and Supplementary Figure 2), such correlations may be indirect and either causal or consequential. Therefore, we investigated the role of TRIB3 in the AT more directly using *Trib3* full body knockout mice (*$Trib3^{KO}$*). Fifteen-week-old chow-fed *$Trib3^{KO}$* mice had an increased body weight (26.16 ± 0.71 g), compared to wild-type littermates (*$Trib3^{WT}$*) (23.92 ± 0.74 g) (Figure 2A), in line with the negative correlation with body weight and the positive correlation with leptin levels observed in the mouse hybrid panel [39] (Supplementary Figure 2). However, when stratified by sex, only males had an increased body weight compared to wild-type littermates (Figure 2B), hence the focus on male mice for the rest of this study. *$Trib3^{K0}$* mice showed a significant increase in plasma total cholesterol (Figure 2C) but not in plasma triglycerides (Figure 2D) or fasting glucose levels (Figure 2E). When analyzing plasma lipoproteins, HDL levels were similar and LDL profiles showed some subtle yet significant differences, including LDL IVa, IIa and I (Supplementary Figure 3). In addition, glucose tolerance test (GTT) indicated no difference in glucose clearance in *$Trib3^{K0}$* mice compared

to wild type animals (Figure 2F). Next, we performed an insulin tolerance test to assess insulin sensitivity in these animals and observed that *Trib3*^{KO} mice recover circulating glucose levels similar to the wild-type littermates after the insulin bolus (Figure 2G). Taken together, *Trib3*^{KO} mice display increased body weight and cholesterol levels that were not accompanied by altered plasma triglyceride levels or overt insulin resistance.

3.3. Adipose tissue mass and adipocyte size is affected in the *Trib3*^{KO} mice

To gain an insight into the potential anatomical reasons for the observed difference in body weights of male *Trib3*^{KO} *vs.* *Trib3*^{WT} mice, we performed magnetic resonance imaging (MRI) to assess body fat composition. Image slices were aligned at the hip level and the total adipose area per section was quantified, shown as white area within the image (Figure 3A and Supplementary Figure 4). The total fat volume per mouse showed a trend towards increased adiposity (Figure 3A), which was significant when fat depots were analyzed separately. In fact, we found that the difference in body weight is mainly due to an increase in the inguinal fat depot (subcutaneous white adipose tissue, subWAT; Figure 3B) and a moderate increase in epididymal WAT (visceral white adipose tissue, visWAT; Figure 3C). This difference in adiposity was also accompanied by a change in adipocyte size. Quantifications from H&E staining of sections from inguinal and epididymal fat depots (Figure 3D) confirmed that *Trib3* deficiency results in significantly smaller adipocytes in the subWAT with a shifted frequency distribution towards smaller cell size (Figure 3E). Similar differences, albeit less pronounced were found in visWAT (Figure 3F). Furthermore, the expression of Proliferating Cell Nuclear antigen (PCNA), a well-known marker of proliferating cells [42], was increased in the subWAT of *Trib3*^{KO} mice when compared to wild type littermates, both at the mRNA and protein levels (Figure 3G). Together these data suggest that TRIB3 plays a role in adipose tissue expansion and remodeling, with the strongest effects in subWAT where the absence of TRIB3 increases adipocyte proliferation and/or constrains cell size.

3.4. TRIB3 regulates the overall lipid profile of adipocytes

The reduced adipocyte size observed upon *in vivo* *Trib3* ablation (Figure 3) could have different causes, including impaired expansion after cell division, an altered balance between lipid storage and release, or a combination thereof. To investigate these possibilities and exclude the influence of other cell types and/or organs we generated mouse 3T3-L1 (pre)adipocyte cell lines with stable shRNA-mediated knockdown (KD) of *Trib3* (Supplementary Figure 5). First, we measured the consequences of *Trib3* silencing on intracellular TG levels. Mature *Trib3* KD adipocytes displayed lower intracellular TG levels (Figure 4A) and higher levels of FFA in the media (Figure 4B), suggesting that *Trib3* is required to maintain a proper balance between lipid storage and release. To obtain a deeper understanding of the role of *Trib3* in lipid handling, we performed semi-targeted lipidomics, which allows in-depth total lipidome profiling by using lipid class standards to define the individual species within that class. *Trib3* KD in mature 3T3-L1 adipocytes resulted in changes in the lipid profile of these cells, as shown by initial principal component analysis (PCA), where the lipidome in KD cells clusters separately from control counterparts (Figure 4C). While intrinsic variability was observed for some lipid classes, the reduction of *Trib3* levels had consistent and significant effects on the levels of ceramides (Cer), phosphatidylethanolamines (PE), Glycosyldiradylglycerols (DG) and Glycerophosphates (PA) (Figure 4D and Supplementary Table 4). Both Cer and PE present membrane components, potentially pointing to altered membrane composition and thereby altered expandability. We further analyzed these data using LIONweb, an online ontology enrichment tool specifically designed to associate lipid species to biological features and functions [33]. Several processes and lipid classes linked to membrane functions were enriched (e.g. 'Glycerophosphoethanolamines' and 'Very high lateral diffusion' respectively); Figure 4E). Interestingly, the LIONweb category 'Mitochondrion' was most significantly enriched, suggesting a yet unidentified role of TRIB3 in cell biology (see Discussion). In summary, the reduction of *Trib3* levels alters the balance between lipid storage and release, and potentially also their expandability through altered lipid composition of membrane components,

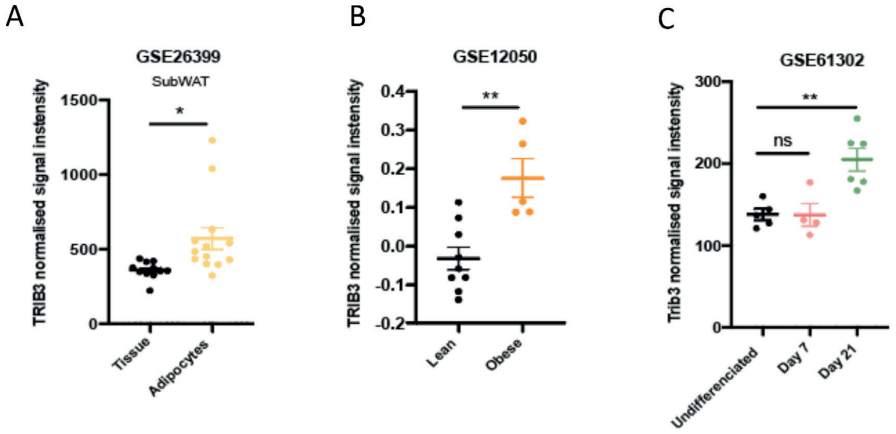

Figure 1: Expression of TRIB3 in human adipose tissue and adipocytes. (A) TRIB3 expression in human subcutaneous adipose tissue (GSE26399). (B) TRIB3 expression is higher in obese individuals compare to lean (GSE12050). (C) TRIB3 expression in ASCs (GSE61302). Graphs are presented as mean ± SEM, unpaired T-test (*$p \leq 0.05$. **$p \leq 0.01$, N.S = not significant).

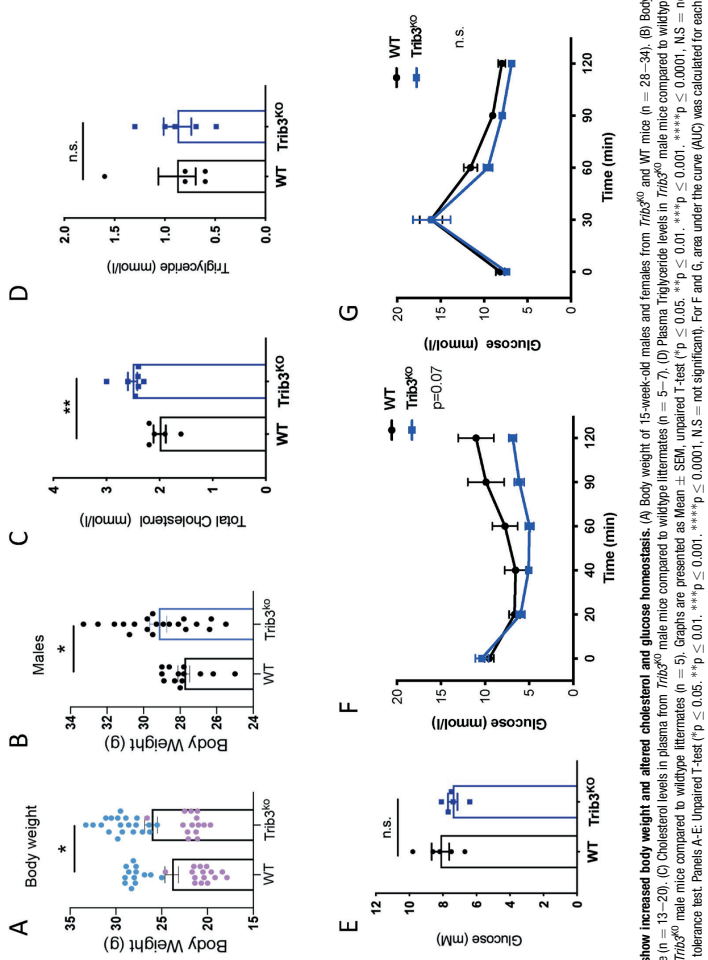

Figure 2: *Trib3*^{KO} mice show increased body weight and altered cholesterol and glucose homeostasis. (A) Body weight of 15-week-old males and females from *Trib3*^{KO} and WT mice (n = 28–34). (B) Body weight of 15-week-old *Trib3*^{KO} and WT male mice (n = 13–20). (C) Cholesterol levels in plasma from *Trib3*^{KO} male mice compared to wildtype littermates (n = 5–7). (D) Plasma Triglyceride levels in *Trib3*^{KO} male mice compared to wildtype littermates (n = 5). (E) Fasting glucose levels in *Trib3*^{KO} male mice compared to wildtype littermates (n = 5). Graphs are presented as Mean ± SEM, unpaired T-test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, N.S = not significant). (F) Insulin tolerance test. (G) Glucose tolerance test. Panels A-E: Unpaired T-test (*p ≤ 0.05. **p ≤ 0.01. ***p ≤ 0.001. ****p ≤ 0.0001, N.S = not significant). For F and G, area under the curve (AUC) was calculated for each sample and an unpaired T-test was carried out to compare the impact of Trib3 deficiency on glucose handling.

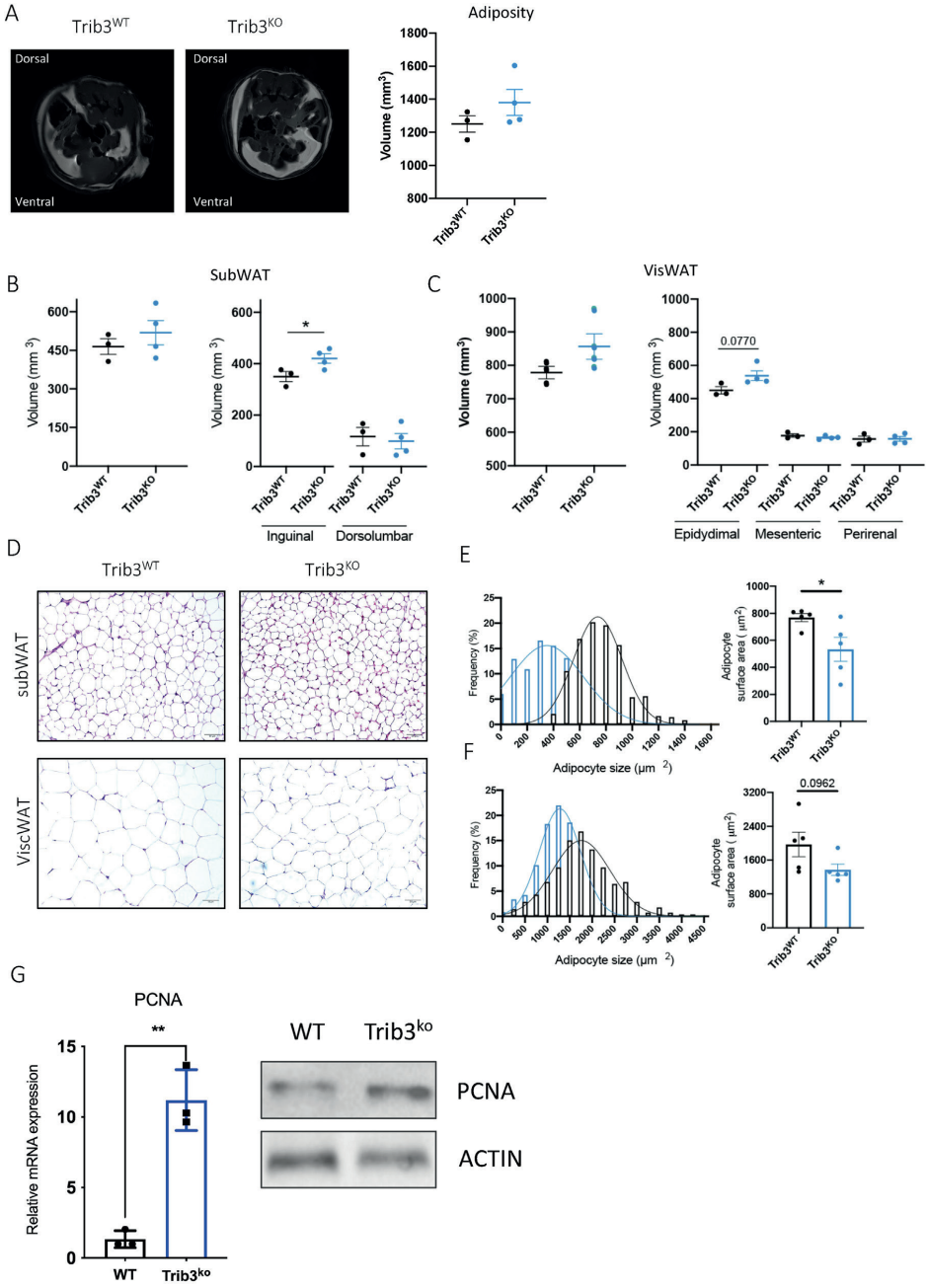

Figure 3: *Trib3*^{KO} mice display increased body weight and adiposity with smaller adipocytes. (A) Representative MRI cross-sectional images showing the fat distribution at the abdominal region and total adipose quantification. (B) Quantitative analysis of the total volume and the individual depot volumes of the subcutaneous (B) and visceral (C) adipose tissue by MRI (n = 3–4). (D) Representative hematoxylin & eosin (H&E) stained subcutaneous (inguinal) and visceral (epididymal) adipose tissue sections. Scale bar, 50 µm. Quantification of the mean adipocyte surface area and frequency distribution of adipocyte size of the H&E sections from the subcutaneous (E) and visceral (F) described depots (n = 5). Graphs are presented as Mean ± SEM, unpaired student's T test, *p < 0.05. (G) PCNA expression and protein levels in subWAT.

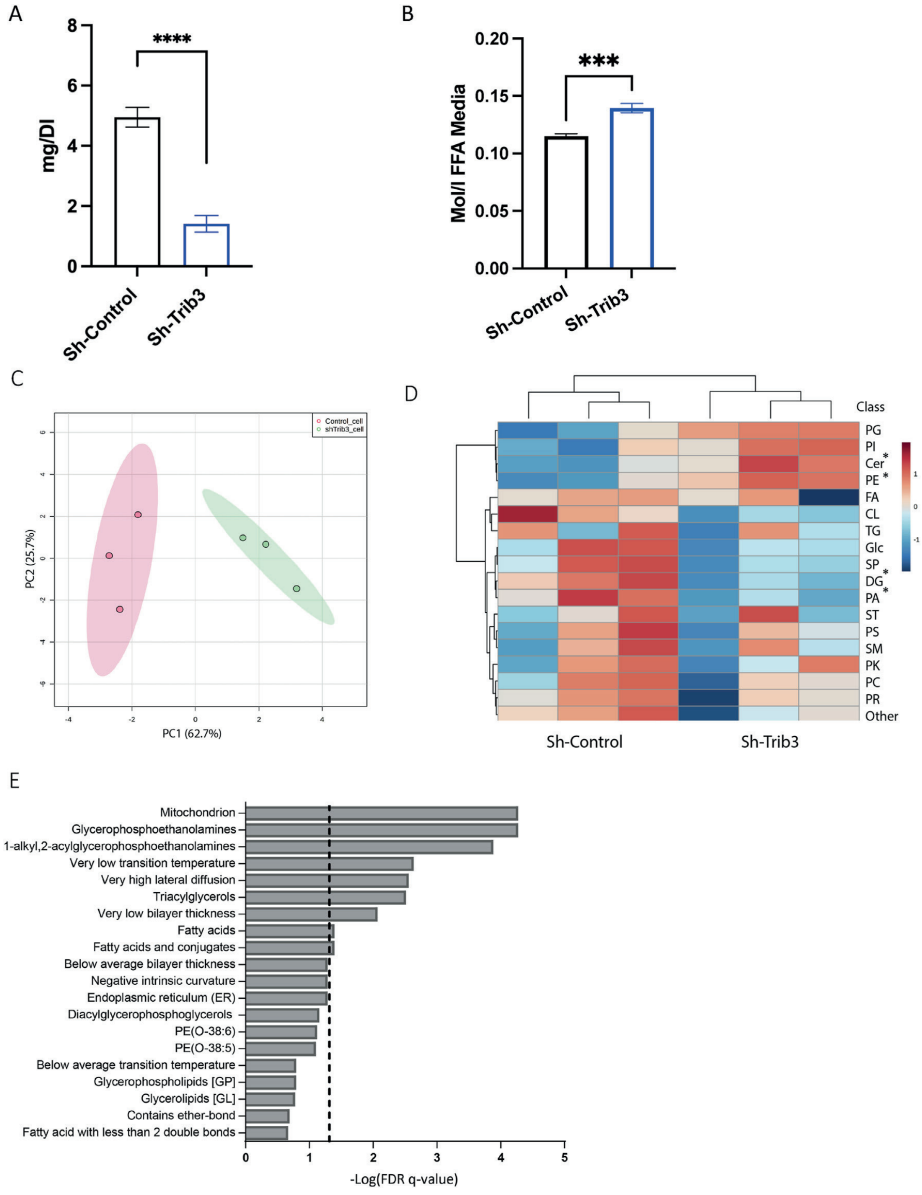

Figure 4: Knockdown of *Trib3* in 3T3-L1 adipocytes alters lipid profiles. (A). TG content of scrambled and *Trib3* shRNA KD 3T3-L1 adipocytes. (B) FFA levels in media of scrambled and *Trib3* shRNA KD 3T3-L1 adipocytes (C) Principal component analysis of Sh-*Trib3* and Sh-Control lipidome. (D) Semitargeted lipidomics analysis, identifying 577 lipid species total. Heat map of average lipid class abundance highlight *Trib3*'s total adipose lipid profile alteration. Statistical significance was calculated using unpaired T-test with Welch's correction. (E) LIONweb enrichment analysis of semi-targeted lipidomic data ranking mode showing the top 20 enrichment hits, black line designating threshold for significance.

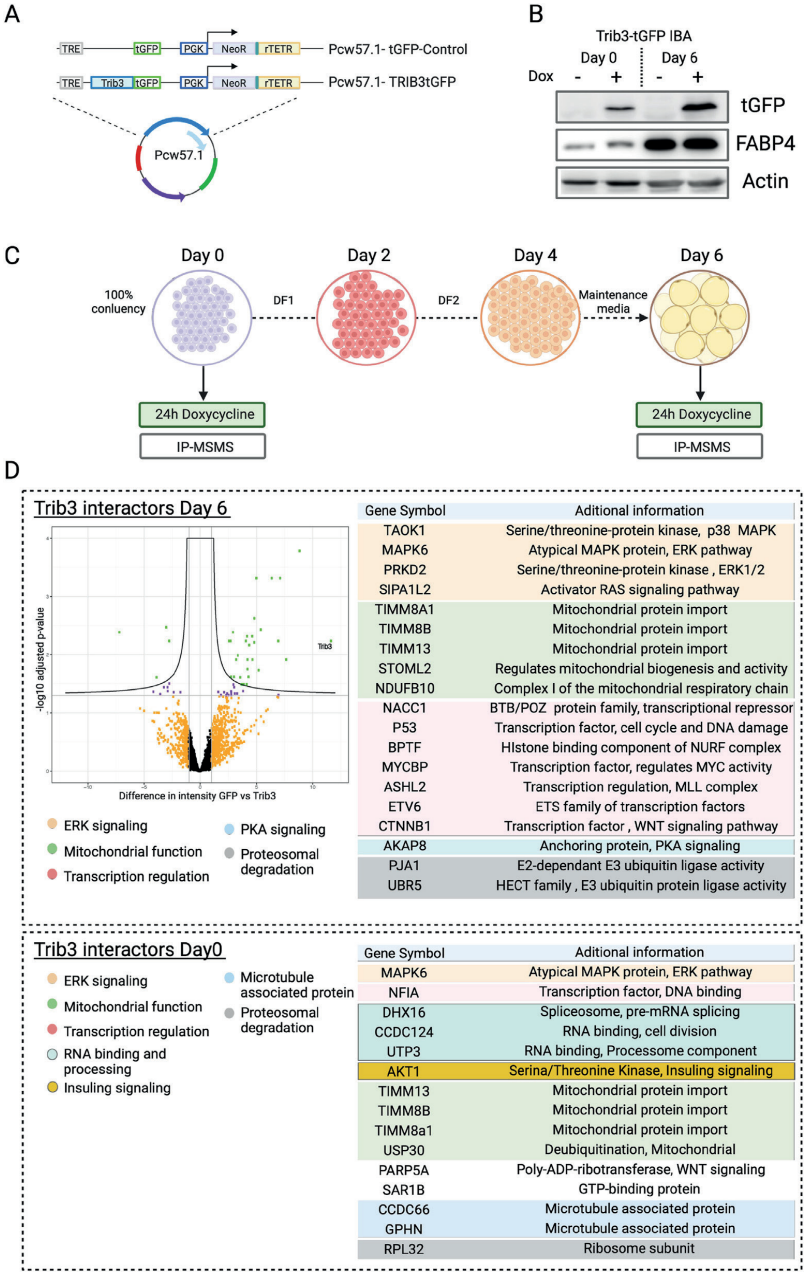

Figure 5: Trib3 interacting proteins in (pre)adipocytes. (A) Doxycycline inducible constructs used to generate TRIB3-tGFP and tGFP control cell lines in IBA cells. (B) Representative Western blots showing induction of TRIB3-tGFP upon doxycycline, together with FABP4 as an adipocyte differentiation marker and actin as loading control, at day 0 and day 6 of adipocyte differentiation. (C) Schematic representation of adipocyte differentiation. In short, cells were cultured until 100% confluency was reached then cells were treated with differentiation media DF1 (1 µg/ml of Insulin, 2.5 µM Dexamethasone and 0.5 µM IBMX). After 2 days, cells were treated with DF2 (complete media supplemented with 1 µg/ml of Insulin) for 2 days and finally at day 4 cells were kept in maintenance media (DMEM high glucose, 10% FBS). (D) Table of TRIB3 interacting proteins in un-differentiated (Day 0) and differentiated (Day 6) IBA (pre)adipocytes. Additional interactome data are provided as Supplementary file 1.

which together may present cell-autonomous mechanisms contributing to the smaller adipocyte size, observed in *Trib3*^{KO} mice (Figure 3).

3.5. Integrated omics identifies multiple TRIB3-dependent pathways regulating lipid handling and proliferation-differentiation in (pre)adipocytes

To identify cellular pathways that may mechanistically underpin the effects of *Trib3* silencing on the lipidome (Figure 4), ultimately resulting in altered adiposity and reduced adipocyte size *in vivo* (Figure 3), we employed a combination of omics approaches, including protein-protein interactome, phosphoproteome and transcriptome analyses.

We first analyzed the TRIB3 interactome, employing our previously described mass spectrometry-based approach [38]. We generated inducible TRIB3-tGFP cell lines in mouse IBA (pre)adipocytes, an accessible and versatile model to study adipocyte biology [36], and induced TRIB3-tGFP expression with doxycycline for 24 h at day 0 and day 6 of adipocyte differentiation (Figure 5A); differentiation was monitored by expression of Fabp4 (Figure 5B). Subsequently, we identified TRIB3 interacting proteins by immunoprecipitating TRIB3-tGFP with a nanobody against tGFP, coupled to agarose beads, followed by mass spectrometry analysis of the immunoprecipitated proteins (Figure 5D and Supplementary file 1). Among the interactors found at day 0, two serine/threonine protein kinases are of particular interest: the Serine/Threonine kinase 1 (AKT1), a previously reported TRIB3 interacting protein that regulates —amongst others— insulin signalling [26], and MAPK6/ERK3, an atypical MAP kinase that is member of the extracellular-regulated kinases. Other interacting proteins included a number of proteins involved in RNA binding and processing (DHX16, CCDC124 and UTP3) and microtubule associated proteins such as CCDC66 and GPHN. Interestingly, we also detected mitochondrial importer proteins of the TIMM/TOMM complex that we have previously identified as TRIB3 interacting proteins in cancer cells [38] and USP30, a deubiquitylation enzyme involved in mitochondrial fusion [43]. Similar to the interacting proteins identified prior to differentiation, we found that TRIB3 interacts with ERK/MAPK pathway proteins in mature adipocytes, such as TAOK1, MAPK6, PRKD2 and SIPA1L2, confirming the central regulatory role of TRIB3 on ERK/MAPK signalling described by us and others in multiple cellular contexts [44–47]. In addition, we found the same mitochondrial transporters (TIMM13, TIMM8A1 and TIMM8B), together with two other mitochondrial proteins (STOML2 and NDUFB10) interacting with TRIB3 in mature adipocytes. In contrast with the interacting proteins detected at day 0, at day 6 we found a high number of proteins that have been linked to transcriptional regulation such as NACC1, ASHL2, ETV6 and CTNNB1/β-catenin. NACC1 and ETV6 are associated with transcriptional repression and have been described in ovarian cancer progression [48,49] and leukemia [50] respectively, neither of these proteins have been reported as an interactor of TRIB3 before. In contrast, ASHL2 and CTNNB1/β-catenin have been previously described as TRIB3 interacting partners. ASHL2 is a member of the WRAD complex, responsible for histone-3 lysine-4 methylation in mammalian cells, and we and others have previously reported the interaction between TRIB3 and subunits of the MLL-WRAD complex [38,51,52]. Previous studies have also reported the interaction between TRIB3 and β-catenin, showing that TRIB3 promotes Wnt signalling by interacting with CTNNB1/β-catenin and TCF4 and enhances their transcriptional activity [29,53,54]. In adipocytes, Wnt signalling regulates the balance between proliferation and differentiation, promoting pre-adipocyte proliferation whilst inhibiting terminal differentiation into mature adipocytes [10,30]. Taken together, the (pre) adipocyte TRIB3 interactome spans many different protein classes (e.g. kinases and transcriptional regulators) as well as cellular localizations (e.g. cytoplasm, nucleus and mitochondria), which may directly or indirectly link to proliferation, cell size and lipid storage and release.

Since interactome analyses may be limited by the strength and stability of protein-protein interactions, we complemented the TRIB3 interactome studies with TRIB3-dependent phosphoproteome analyses. While TRIB3 is not able to phosphorylate target proteins due to the lack of the metal binding motif in the kinase domain, it can interact with canonical kinases and regulate their function [47,55], as also observed here (Figure 5). We again used the inducible TRIB3-tGFP IBA cells described above and subjected these to SILAC-based quantitative proteomics. Cells were maintained in media containing heavy or light amino acids (lysine and arginine) for 5 passages and incorporation of labelled amino acids was assessed prior to the beginning of the experiment (data not shown). TRIB3-tGFP lines were induced with doxycycline for 24 h in differentiated IBA cells, and then the phosphoproteome of induced vs uninduced differentiated TRIB3-tGFP cells was compared, together with the reverse experiment (Figure 6A). Among the top 15 canonical pathways that were identified, dysregulated mTOR and insulin receptor signaling were the most significant (Figure 6B and Supplementary file 2), in line with the interactome data presented above (Figure 5) and previous studies [26,56]. In addition, cellular pathways that control whole-body energy balance were found to be dysregulated, including the ERK/MAPK, AMPK and PKA pathways. AMPK (AMP-activated protein kinase) inhibits fatty acid and cholesterol synthesis in adipocytes upon low levels of nutrients [57]. On the other hand, PKA is a major regulator of mitochondrial biogenesis and lipolysis, enhancing browning of WAT and the release of fatty acids from the lipid droplets by phosphorylation of lipases and perilipin [58,59]. Moreover, the induction of TRIB3 affects the G2/M DNA damage checkpoint and ATM signaling, which, together with the result from the previous section where p53 was found as an interacting partner of TRIB3, situates the pseudokinase as a pivotal regulator of the cell cycle in adipocytes. Analysis of the upstream regulators reveals kinases and other protein complexes that are altered by TRIB3 in adipocytes (Figure 6C). Among the most significant upregulated are EGF, AKT1, GH1 and MAPK1, it is also worth mentioning the downregulation of p53, PTEN and PTPN11 according to the Z-score generated. We also found several transcription factors that have changed their phosphorylation status upon TRIB3 induction, including ATF4, ATF7 and STAT3. These results indicate that TRIB3 is able to regulate adipocyte function, modulating the activation of mTOR, insulin signalling and ERK/MAPK pathways, resulting in changes in transcription factor activation that drive adipocyte function.

Finally, we analyzed the effect of *Trib3* depletion on adipocyte gene expression. To focus on cell-intrinsic effects rather than changes caused by interplay of adipocytes with other AT-resident cells, the stromal vascular fraction (SVF) from subcutaneous and visceral WAT depots from *Trib3*^{KO} and *Trib3*^{WT} male mice was isolated, expanded and differentiated into mature adipocytes as shown before [60](Figure 7A). Adipocyte differentiation was evaluated by mRNA expression of markers such as *Pparγ, Lpl*, *Fabp4 and Adipoq* (encoding adiponectin). We found that *Trib3* deficient cells displayed significantly higher levels of *Pparγ* and *Fabp*4 when compared to WT cells, while *Adipoq* and *Lpl* showed a strong but not significant trend of upregulation in the *Trib*3 deficient cells (Figure 7B). When the same markers were assessed in *ex-vivo* differentiated SVF from visceral WAT similar trends were observed albeit to a lesser extent (Supplementary Figure 6), in line with the stronger effect of *Trib3* ablation on subcutaneous AT volume and adipocyte size compared to vWAT (Figure 3B—F). We also analyzed correlations between TRIB3

mRNA expression and various markers in *ex vivo* differentiated human adipocytes, starting from the SVF of subcutaneous AT. Similar trends were observed to mouse AT, which did not reach statistical significance due to the low sample size or larger individual variation in humans compared to mice (Supplementary Figure 7).

To gain a more comprehensive insight into *Trib3*-dependent changes in adipocyte transcriptional programmes, transcriptome analyses of *ex vivo* differentiated adipocytes from subcutaneous WAT of $Trib3^{KO}$ and $Trib3^{WT}$ mice were performed. Pathway analysis of differentially expressed genes (Ingenuity Pathway Analysis (Qiagen)) was performed and revealed 41 altered canonical pathways and 64 diseases and functions significantly affected by *Trib3* deficiency (Supplementary Table 5). The top altered canonical pathways in $Trib3^{KO}$ adipocytes include downregulated pathways involved in lipid homeostasis (TG degradation and PKA signaling) in line with our lipidome, interactome and phosphoproteome data (Figures 4-6). Among all the molecules that were found significantly dysregulated (p-value <0.05) 'transcription regulator' was the most frequent molecule type identified (Figure 7D). Within this category, the top activated molecule was TCF7L2 while HOXA10 was the most inhibited (Figure 7E). TCF7L2 (also known as TFC4) is the endpoint of the Wnt/β-Catenin signalling cascade which has been implicated in adipocyte differentiation and function [61,62] as well as the Wnt/β-Catenin pathway being associated with TRIB3 in multiple other cell types [29,53,54]. The homeobox transcription factor HOXA10 has also been implicated in adipocyte differentiation and function [63].

Taken together, our integrated omics approaches indicate that i) TRIB3 can interact with a large set of proteins in adipocytes, including various kinases, such as AKT and MAPK6, and transcriptional regulators, such as CTNNB1//β-catenin, ii) TRIB3 can directly or indirectly alter signaling pathways in adipocytes, such as mTOR, MAPK and PKA signaling, iii) depletion of *Trib3* affects the adipocyte transcriptome, with dominant effects on the transcription factors TCF7L2 and HOXA10. Rather than pointing to a single cellular pathway being affected, these findings support TRIB3 playing multiple roles, both in the cytoplasm and the nucleus and potentially also in mitochondria, in

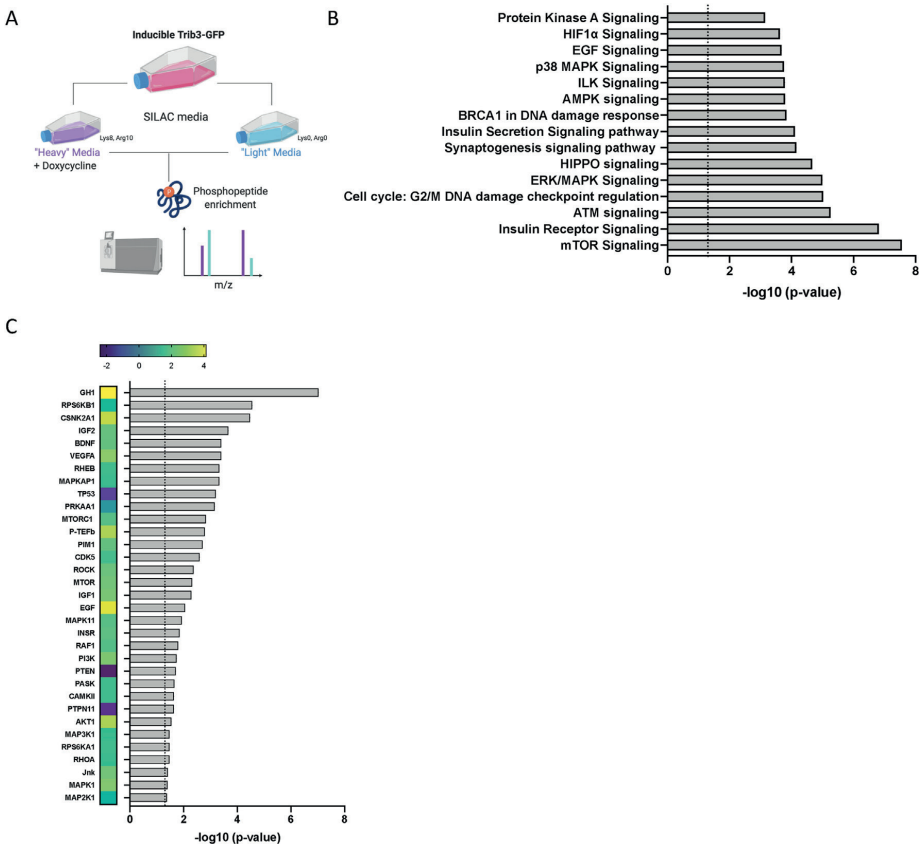

Figure 6: TRIB3 induction alters mTOR and MAPK signalling and affects adipocyte cell cycle progression. (A) Schematic representation of phospho-proteomics experiments using TRIB3-tGFP IBA cells in heavy and light SILAC media. (B) Top 15 altered canonical pathways found dysregulated upon TRIB3 induction in phospho-proteomics experiment in IBA cells. The dotted line indicates a p-value of 0.05. (C) Altered upstream kinases, protein complexes and enzymes according to Ingenuity pathway analysis (Quiagen) indicating p-value (dotted line indicates p-value of 0.05) and activation Z-score (colour coded). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

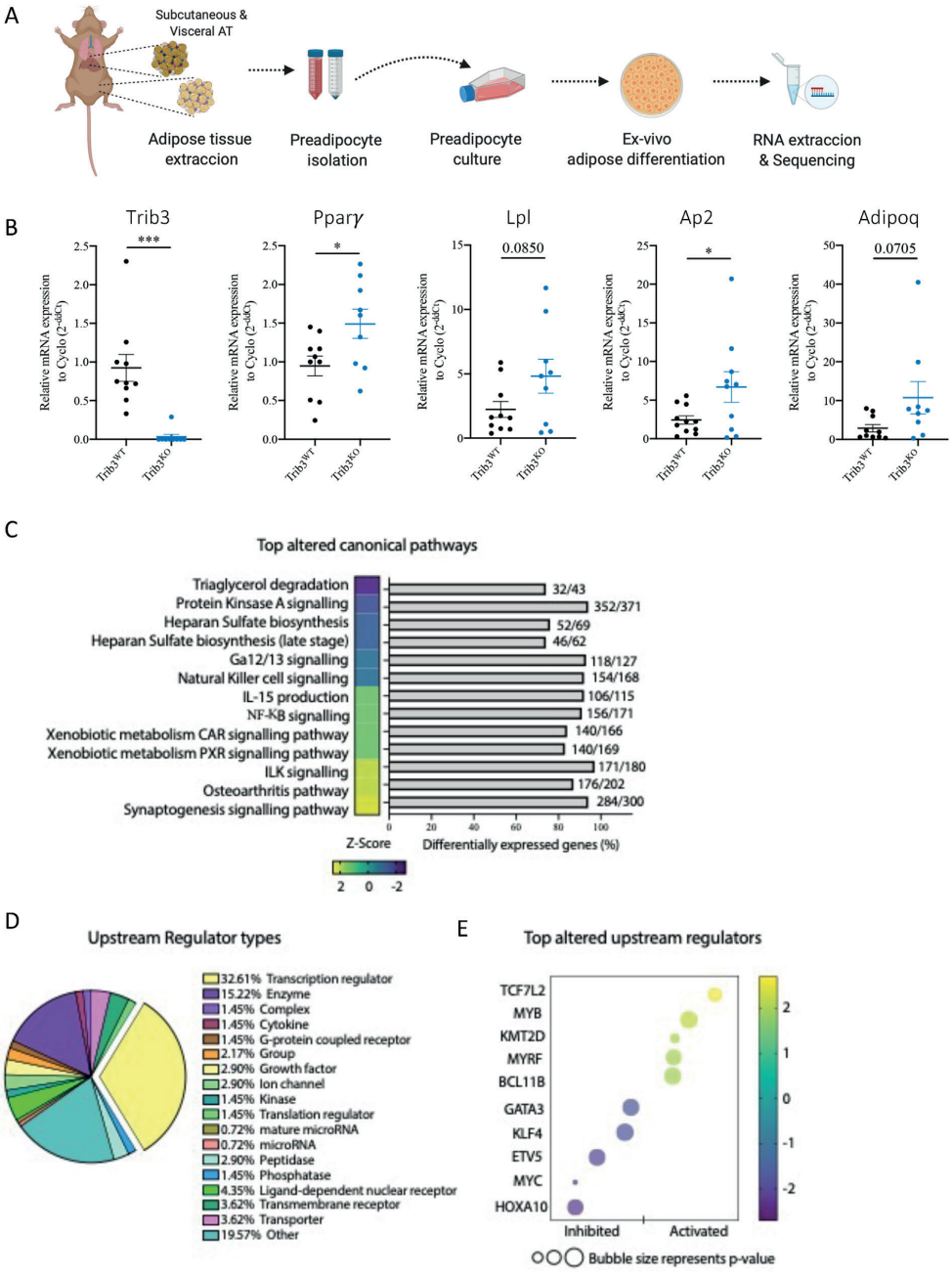

Figure 7: RNA sequencing of *ex-vivo* differentiated adipocytes reveals differences in gene expression profiles between *Trib3*^{KO} adipocytes and wild type. (A) Schematic representation of preadipocyte isolation and ex-vivo differentiation. (B) Expression of *Trib3*, *Pparγ*, *Lpl*, *Fabp4* and *Adipoq* in ex-vivo differentiated adipocytes. Cyclophilin A expression was used as an internal control. Graphs are presented as mean ± SEM, unpaired student's test (*$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$. ****$p \leq 0.0001$, N.S = not significant) (C) Top altered canonical pathways found dysregulated in the RNAseq of the ex-vivo differentiated subWAT of the Trib3^{ko} mice compared to WT littermates. (D) Upstream regulators analysis of RNA-seq dataset using Ingenuity pathway analysis (Qiagen). (E) Top altered upstream regulators indicating p-value and activation Z-score.

intertwined pathways, ultimately contributing to an optimal balance in proliferation vs. differentiation capacity, and proper lipid storage. As a consequence, ablation of *Trib3* affects the proliferation-differentiation balance and net lipid storage in the AT of *Trib3* full body knockout mice.

4. DISCUSSION

In vivo studies in humans and mice have indicated that the pseudokinase TRIB3 regulates energy metabolism [64,65], and cell-based studies suggest a role in adipocytes [28]. However, it was unknown if these observations are causally linked. In our study, we investigated the impact of whole-body ablation of Trib3 in mice and found that it leads to adipose tissue expansion while maintaining insulin sensitivity. The KO animals exhibited an increase in the number of smaller adipocytes, a phenotype often associated with insulin sensitivity [66–68]. Through a combination of lipidome, interactome, phosphoproteome and transcriptome analyses, we uncovered a multifaceted role for TRIB3 in proliferation, TG storage and cellular expansion. These functions are potentially regulated through multiple cellular pathways, including MAPK/ERK and PKA signalling and TCF7L2/beta catenin-mediated gene expression. In support of these various molecular roles, TRIB3 has been reported to be localized in the cytoplasm and the nucleus [44], and to interact with a large set of cellular proteins in various biological settings [29,69]. Interestingly, the TRIB3 interactome in adipocytes reported here as well as the interactomes reported earlier in MCF7 breast cancer cells and HEK297T cells [38], all suggest a potential role for TRIB3 in mitochondria, which will be the focus of future studies.

Our combined interactome and phosphoproteome analyses indicate that TRIB3 functions as an integrator of signalling pathways in adipocytes, capable of regulating kinases downstream of membrane receptors, such as AKT1, mTOR or ERK3, as well as transcription factors such as CTNNB1 or ASH2L that drive transcription of key factors of adipose biology. Regarding the interactions with kinases and proteins that regulate kinases, TRIB3 appears to influence their substrate specificity, redirecting these kinases in certain directions [70,71]. The effect of TRIB3 on transcription factor activity is also complex. TRIB3 has been shown to interact and modulate the activity of a number of transcription factors [29,72], and can repress transcription through recruitment of repressor proteins like ZBTB1 [38] or interference with recruitment of transcriptional activators like the MLL complex [73]. In agreement with other studies [29] we describe an interaction between TRIB3 and CTNNB1/β-catenin (Figure 5) and also report upregulation of TCF7L2-mediated transcription upon ablation of *Trib3* (Figure 7E). Together, these findings suggest that TRIB3 may negatively regulate Wnt signalling in adipocytes, potentially recruiting aforementioned repressor proteins or preventing CTNNB1//β-catenin from entering the nucleus. However, further experimental studies are necessary to fully understand this model, particularly since Wnt activity impairs adipogenesis [10,30] and TRIB3 has been described as a positive regulator of Wnt signalling in various cancer types [29,53,54]. Smaller adipocytes, as observed here in *Trib3*^{KO} mice, have been associated with insulin sensitivity [66–68]. In fact, various therapeutic approaches have aimed to increase adipocyte proliferation to generate more small adipocytes [74]. While this suggests that targeting TRIB3 in adipocytes for degradation may represent a rational therapeutic approach; it should be noted that drug targeting of Tribbles and specifically TRIB3 presents both an exciting area of development and a significant challenge, as discussed elsewhere [15,75–77]. Furthermore, it is important to consider that adipocyte-specific targeting of TRIB3 may be required and the role of TRIB3 in AT resident immune cells, which can also affect adipocyte size and functionality [78], has not been addressed directly yet. While the current study supports the view that TRIB3 is a critical regulator of adipocyte proliferation, homeostasis and function, future studies are clearly needed to elucidate its therapeutic potential.

AUTHOR CONTRIBUTION STATEMENT

MHQ, LMP, IM, ZI, SR, PSA, ECAS, RE and HV performed the experiments, data-analysis, and wrote the manuscript; AV and JV performed patient inclusion and detailed clinical assessment. NST, HLW, EKT and EK designed and supervised the study. All authors reviewed the manuscript.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY

Data will be publicly available upoin acceptance of manuscript.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101829.

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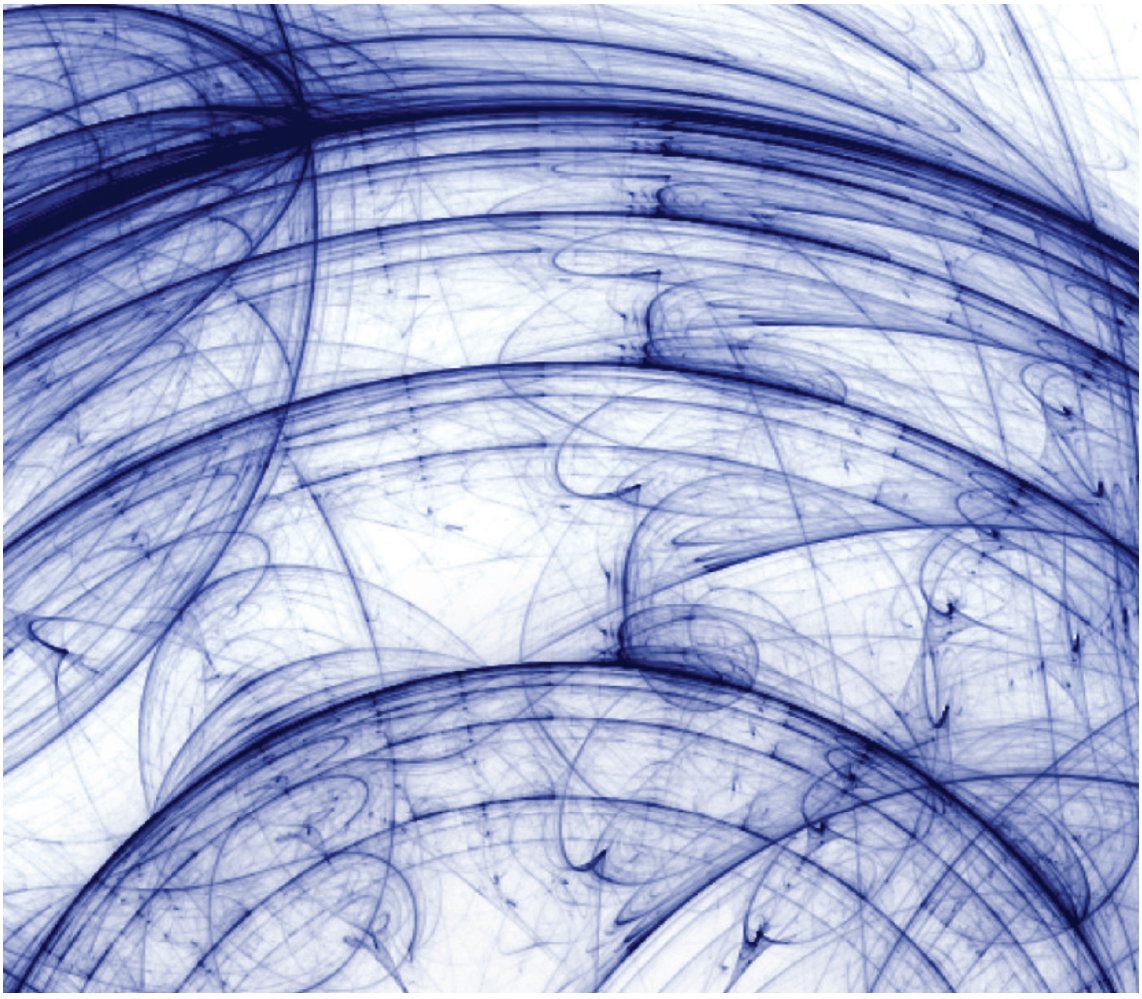
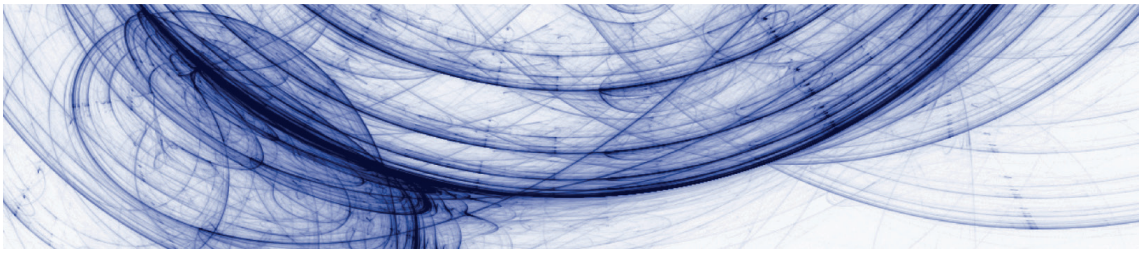
Chapter 5





The pseudokinase Tribbles 3 regulates mitochondrial lipid species and function in white adipose tissue

Morris, I*., Stigter, E., Hernández-Quiles, M., Bhutia, K., Westland, S., de Heus, C., Velasco, G., Liv, N., and Kalkhoven, E.

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Abstract

Recent advancements in lipidomic, metabolomic, and proteomic techniques have provided insights into biomarkers and disease phenotypes, allowing for more effective treatment options. The lipidome is a dynamic environment of biosynthesis and remodeling, with organ- and tissue- and even organelle- specific lipid species essential for correct function. Lipid species ratio alterations have been associated with or used as biomarkers for diseases, with cardiolipin abundance linked to NASH, diabetes, and heart failure. Modern machine learning techniques are now being deployed to identify easily druggable targets, with pseudokinases, such as the TRIBBLES family, being an attractive target for drug development. Tribbles 3 (Trib3), a member of the TRIBBLES family, has been associated with metabolic diseases and aggressive cancers, suggesting a role in metabolic flexibility. Trib3 has further been linked to lipid metabolism, with elevated expression during cellular stress found in tumors. In this study, we investigate the impact of TRIB3$^{-/-}$ on the WAT lipidome and report its significant impact on lipid species abundance and ratios associated with mitochondria structure and function, resulting in enhanced acute mitochondria response stress. Our findings suggest a potential role for Trib3 in lipidome homeostasis and metabolic flexibility.

Introduction

Recent advances in lipidomic, metabolomic and proteomic techniques allow probing for biomarkers and disease phenotypes, aiding in more effective treatment options (reviewed [1–4]). Lipidomes present a complex mixture of different lipid classes, which differ between organs and tissues, cell types and even cell organelles, and are the dynamic net product of biosynthesis, modification, degradation and remodeling [5]. In addition, many organ's lipidomes are highly plastic and impacted by environment (e.g. liver and blood), whereas organs like the brain have a much more rigid lipidome, and are predominantly dictated by genetics or sex [5]. Even on the level of basic cellular stuctures, such a cell mebranes and cell organelles, differences in lipid composition occur. For example, eukaryotic cell membranes are composed of many different kinds of glycerophospholipid species with >1000 different species per cell [6], most of which are synthesized in the ER [7], but the species composition would be different in muscle cells compared to spleen cells [5] [8]. Moreover, the organelles that make up the cells of each organ or tissue also have specific lipid species and class ratios associated with them, thus specifying functionality. For example, high concentrations of sphingolipids are detected in the renal cortex while prenol lipids are most prevalent in the heart (reviewed in [1]). Alterations in lipidomes are increasingly being associated with or used as biomarkers for diseased states, For example, NASH, diabetes and heart failure are associated with altered abundance of cardiolipins [10,11], a unique class of lipids primarily found in the mitochondria, with their four-legged arrangement defining the shape and abundance of mitochondrial cristae, an essential structure which determines the efficiency of the respiratory chain [12].The sheer diversity and number of lipid species identified is expanding rapidly [6], and whilst the functions and interactions of many species and classes are well defined, many new functions remain to be discovered [13,14].

Adipose tissue (AT) is dedicated to handling of lipids and represents one of the largest organs of the human body [15]. Adipose tissue is composed of a variety of cells that includes mature adipocytes, fibroblastic pre-adipocytic cells, endothelial cells, various types of immune cells and neurons. There are two major types of adipose tissue: white and brown adipose tissue (WAT and BAT, respectively). White adipocytes are specialized cells that import and store lipids to create a readily accessible reserve of energy in a single large lipid droplet; the release of lipids as an energy source for other tissues in times of need is reffered to as lipolysis. In addition, white adipocytes can synthesize and present lipid antigens, to modulate the activity of immune cells present in AT [16]. In contrast, brown adipocytes burn lipids, stored in multiple smaller lipid droplets, in uncoupled mitochondria to produce heat during non-shivering thermogenesis in response to cold [17]. In addition, both WAT and BAT are active

endocrine organs, whereby they are able to communicate with other metabolic organs like muscle and liver to regulate whole body energy metabolism [18,19]. Given the central role of AT in metabolic health [20], it is no surprise that all aspects of lipid handling in adipocytes are highly regulated, with insulin playing a key role [21]. In obesity, where adipocytes both increase in number (hyperplasia) and lipid content per cell (hypertrophy), adipocytes become insulin resistant, resulting amongst others in deregulated lipolysis [21]. One of the cellular proteins implicated in insulin resistance is the pseudokinase Tribbles 3 (Trib3) [22]. Elevated *TRIB3* expression in AT is associated with obesity and insulin resistance in humans [23–25], while the Q84R polymorphism of *TRIB3* in humans is associated with dyslipidemia, insulin resistance and cardiovascular risk [53] (reviewed [54]). On a molecular level, Trib3's effect on insulin sensitivity has been largely attributed to its direct interaction with and inhibitory effect on AKT, an essential kinase in insulin signalling [26]. However, TRIB3 can also directly or indirectly regulate insulin signalling through other mechanisms. For example, in hepatocytes Trib3 interacts with the ubiquitin E3 ligase COP1, leading to increased degradation of the deactylase SIRT1 which results in increased acetylation and reduced activity of AKT [27,28]. When looking at the boader picture of lipid metabolism, Trib3 can act independently of AKT: the aforementioned Trib3-COP1 interaction increases degradation of acetyl–coenzyme A carboxylase (ACC) in adipocytes, and thereby impairs fatty acid synthesis during fasting [29]. Given that Trib3 interacts with a broad range of cytoplasmic and nuclear proteins [30–32], this protein may also affect lipid metabolism directly or indirectly through yet undiscovered mechanisms.

Here, we examined the metabolic role of Trib3 in visceral AT, by combining lipidome and metabolome analyses with proteome analyses and high-resolution electron microscopy. We found that loss of Trib3 has a significant impact on the visceral AT lipidome, particularly on lipid species associated with mitochondrial structure and function. Indeed, our data suggest that loss of Trib3 increases the respiratory capacity of mitochondria, especially when cells are challenged with excess exogenous lipids, implying a yet undiscovered role for this pseudokinase in lipid metabolism in adipocytes.

Results

Loss of Trib3 reduces lipid abundance in visceral adipose tissue

Previously we reported that shRNA-mediated knockdown of Trib3 in mature 3T3-L1 adipocytes results in a significant change in the cellular lipidome [32]. Enrichment analysis significantly linked these changes to membrane components, mitochondria and ER function [32]. These features are also apparent in *Trib3*$^{-/-}$ mice, specifically ER stress

and reduced adipocyte size [32]. To further probe this lipidome-associated phenotype, we preformed untargeted lipidomic analyses on visceral adipose tissue (vWAT) of WT and *Trib3*$^{-/-}$ mice. We identified 504 individual lipid species in 18 lipid classes (Sup Fig. 1). The relative abundance of all lipid species combined was significantly reduced in vWAT from *Trib3*$^{-/-}$ mice when compared to control littermates (Fig. 1a) (p=<0.0001). Also when the lipid species identified were split into lipid classes, the majority of lipid classes displayed significantly reduced abundance in vWAT from *Trib3*$^{-/-}$ mice when compared to control animals (Fig. 1b). Most significantly reduced were lipid classes associated with membrane or organelle structure and functionality, including ceramides (CER; p=<0.0001), cardiolipins (CL; p=<0.0001), fatty acyls (FA; p=<0.0001), glycerophosphoethanolamines (PE; p=<0.0001) glycerophosphoinositols (PI; p=<0.0001) and glycerophosphoserines (PS p=<0.0001). In particular, the reduced cardiolipin abundance is of interest (Fig. 1b), as these phospholipids are primarily found in the inner mitochondrial membrane (IMM) making up to 20% of the phospholipid content [12]. Their chemical nature dimeric structure with two negative charges (from two phosphatidic acid groups) is important for mitochondrial structure and function, particularly for the curvature of the crista essential for cellular respiration [33], as well as the dynamic nature of their structure [34]. Glycerophosphates (PA) and glycerophosphoglycerols (PG) [6] serve as precursors for CL's (reviewed [35,36]), and also displayed reduced levels in *Trib3*$^{-/-}$ vWAT. In addition, other glycerophospholipid species that contribute to mitochondrial functions [37–39], including glycerophosphocholines (PC), glycerophosphoethanolamines (PE), glycerophosphoinositols (PI), were also reduced in our analyses (Fig. 1b). Other lipid classes that were reduced but to a lesser extent included DG (p=0.0001), PC (p=<0.0002) and TG (p=<0.002). To compare the differences between WT and *Trib3*$^{-/-}$ vWAT on the level of individual lipid species, we utilized LIONweb, an online tool that enables Lipid ontology (LION)-terms to be associated with lipidomic subsets based on single lipid species interactions within lipidomes [40]. Fig. 1c shows the top-10 LION enriched terms ranking mode. The most enriched LION term was "Glycerophosphoethanolamines" (-logFDR= 0.00015), a group primaily composed of PE lipids, which are essential membrane components that play a role in its fluidity and structure through their "positively charged head group", which was the second highest enrichment term (-logFDR=0.00029). Following this are LION terms that also link Trib3 to impaired membrane integrity and function (e.g. "negative intrinsic curvature" (-logFDR=0.0003), "mitochondrion" (-logFDR=0.0003), "membrane component" (-logFDR=0.0003), "diacylglycerophosphoethanolamines" (-logFDR=0.00035), "endoplasmic reticulum" (- logFDR=0.00073), "glycerophospholipids" (-logFDR=0.01898), "high lateral diffusion" (- logFDR=0.03726), and "low bilayer thickness" (-logFDR=0.03726)). Interestingly, also the categories "mitochondrion" (-logFDR=0.0003) and "endoplasmic reticulum" (-logFDR=0.00073) showed up.

In summary, vWAT of Trib3$^{-/-}$ mice displays an overall reduction in lipid abundance. The most affected lipid classes (e.g cardiolipins) and individual lipid species suggest that Trib3 plays a specific role, directly or indirectly, in cellular membrane integrity and function, and also point to a role in mitochondria and ER.

Trib3 ablation alters the metabolite profile of visceral adipose tissue

To extend our Trib3-dependent lipidome analyses (Fig. 1) to other metabolites, we performed semi- targeted metabolomics analyses and detected a total of 177 individual metabolites (Sup. 2). Comparative analysis showed specific metabolites to be enriched or reduced in Trib3$^{-/-}$ vWAT (Fig. 2a- b). We found a highly significant reduction in L-Arginine, an amino acid whose catabolism is linked to dysfunctional mitochondria [41] (reviewed [42]) and mitochondrial biogenesis [43]. We ran pathway analyses (Metaboanalyist), to identify Trib3's impact on adipocyte metabolism. Enrichment analysis showed "steroid hormone biosynthesis" (p= 0.015) to be the most significant pathways enriched in the data set. Closer inspection of the data indicates that this is due to the high levels of cortisone present in the TRIB3$^{-/-}$ mice (Fig. 2a). While the underlying mechanism in the context of Trib3 ablation remains to elucidated, cortisone has been linked to adipose tissue expansion, lipolysis and responsiveness to stress [44–46]. We also found "galactose metabolism" (p= 0.042), "Neomycin, kanamycin and gentamicin biosynthesis" (p=0.042), "One carbon pool folate" (p= 0.160) which regulates stability of Complex I of the respiration chain [47]. "Taurine and hypotaurine metabolism" (p= 0.175) and "Glutamine and glutamate" (p= 0.200), all of which have been linked to mitochondrial (dys)function [48,49].

Trib3 interacts with Mitochondrial proteins

Our lipidomic and metabolomic data (Fig. 1 and 2) suggest that one of the ways in which TRIB3 affects cellular metabolism is through mitochondrial function. While Trib3 has been implicated in the regulation of signal transduction and transcription through direct interactions with various cellular proteins [50], functional interactions that may explain a role in mitochondria in adipocytes suggested by the lipidomic and metabolomic analyses here have not been reported extensively so far. Furthermore, most studies so far have indicated that Trib3 resides primarily in the nucleus and the cytoplasm [50]. Therefore we first performed cell fractionation studies in IBA adipocytes with doxycycline-inducible GFP-tagged Trib3 overexpression [32]. With this approach we detected Trib3 in the mitochondrial fraction (Fig. 3a). Next we analyzed the Trib3 interactomes that we previously identified in HEK293T embryonic kidney cells and MCF7 breast cancer cells [31] as well as in adipocytes [32] for mitochondrial

proteins. Within the large sets of Trib3 interacting proteins identified in each cell type, mitochondrial proteins were identified in each case (Fig. 3b-c and Sup. 3). These interactions implicate Trib3 in mitochondrial biogenesis and activity regulation (interaction Stoml2), mitochondrial import (interactions Timm8a1, Timm8b, Timm13) and mitochondrial respiratory chain (interaction comlex 1 protein Ndufb10) (Fig. 3c). In conclusion, together with the alterations in lipid classes associated specifically with mitochondria (Fig. 1), these data support a potential role for Trib3 in mitochondrial structure and function.

Trib3 ablation results in smaller mitochondria in adipose tissue

Having established that *Trib3* ablation is associated with lower lipid abundance (Fig. 1a), including significantly lower cardiolipins (Fig. 1b), and having observed an association of the Trib3 protein with several mitochondrial proteins (Fig. 3), we examined the role of Trib3 on the abundance, shape and size of mitochondria, as mitochondrial structure has been linked to optimal function [12]. We used high-resolution electron microscopy to analyze ultrastructural and size alterations in the adipose tissue of WT and KO mice. The ultrastructure of mitochondria was comparable to WT with no directly visible defects in both white and brown adipose tissue of the KO mice. We did not observe any clear ultrastructural alterations in the outer and inner membranes, inter-membrane space, or in the cristae. We then used the same high-resolution images to quantify the size of mitochondria in WT and KO samples. Considering the small number of mitochondria present in WAT cells to reach a solid statistical measure for mitochondria size (Fig. 4a and b), we decided to perform size quantification of mitochondria on the BAT tissue from the same animals, which contain higher numbers of mitochondria per cell. Representative images of mitochondria of the wild type vWAT and BAT are shown in Fig. 4a and c, respectively. The analyses of Trib3$^{-/-}$ BAT clearly showed that this tissue contains mitochondria that have a significantly smaller area (Fig. 4c-d). We also observed a skewing in the size distribution compared to control (Fig. 4c-d), with smaller mitochondria being more abundant (Fig. 4g-h). Apart from this size difference, the number of cristae per area of the mitochondria was comparable between wt and Trib3 KO adipocytes (Fig. 4e) as was the roundness of their structure (Fig. 4f).

Reduced levels of Trib3 enhance mitochondrial respiratory capacity in adipocytes

Finally, we assessed the role of Trib3 on mitochondrial function, focusing on the role of mitochondrial respiration as Trib3 ablation impacts cardiolipin levels (Fig. 1) and cardiolipins have been implicated in respiratory chain function [12]. We subjected

previously developed 3T3-L1 mature adipocyte shTrib3 lines to the Seahorse XF Mito Stress test, which measures changes in mitochondrial function via the oxygen consumption rate (OCR) [51]. Reduced Trib3 levels resulted in a slight increase in basal respiration, maximal respiration and spare respiration compared to the scramble control cells, which failed to reach statistical significance (Fig. 5a-d). Next, we challenged the cells with an excess of exogenous lipids (lipid mix; LM) [52], to assess the impact of reduced TRIB3 on mitochondrial respiration under stressed conditions. While LM treatment significantly reduced maximal respitration and spare respiration in both control cells and KD cells (Fig. 5a), maximal respitration and spare respiration was significantly higher in knock down cells compared to control cells (Fig. 5f-g). In fact, knock down cells subjected to LM treatment could not be distinguished from unchallenged control cells (Fig. 5a, h-j; light blue vs dark red). Taken together, these data suggests that Trib3 ablation may partially protect mature adipocytes from the deleterious effects of excess lipids on mitochondrial respiration.

Discussion

Adipocyte function, rather than simply adipocyte numbers or adipocyte size, may be the dominant factor in adipocyte- and adipose tissue-mediated effects on metabolic health and is therefore coming to the fore of scientific research [53]. We and others have previously implicated the pseudokinase Trib3 in adipocyte function [23,29,54] and in the current study we analyzed its role in defining the adipocyte lipidome, as lipidomes can provide valuable information on cellular structure and function [9], energy metabolism and storage, signal transduction and health status from cell to organism [1]. We found a significant reduction in lipid class and species abundance in vWAT from *Trib3*$^{-/-}$ mice, particularly those associated with mitochondrial membranes. Furthermore, our analyses of the Trib3 interactome in adipocytes (and other cell types) revealed multiple mitochondrial proteins, and loss of Trib3 resulted in smaller mitochondrial size. When examining mitochondrial respiration, adipocytes with reduced Trib3 levels responded to stress more favorably when exposed to exogenous lipids. Taken together, these findings reveal a novel role for the pseudokinase in mediating mitochondrial function.

Proteomics shows TRIB3 interacts with SLP-2 (Fig. 3), known to regulate biogenesis of mitochondria and responds to stress by forming and interacting with various complexes and proteins within the mitochondria IMM [55–57]. SLP-2 is required to stabilize complex PHB1 and PHB2, enabling them bind to, mature and stabilize Cardiolipin rich membrane domains [58] as well as complex I and IV of the electron transport chain [58]. Upregulation

of SLP-2 increases mitochondrial synthesis of CL as well as mitochondria biogenesis [55,59]. This increased membrane stability in turn stabilizes OPA1, a GTPase involved in sequestering cytochrome C and respiratory chain super-complex formation. SLP-2 also interacts with the i-AAA protease YME1L and PARL to for the SPY complex, this complex activates both OPA1 and Cardiolipin synthase[60–62]. These interactions within the IMM facilitate respiratory super- complex formation within the CL rich membrane domain bringing the complex subunits closer together [63], essentially becoming a proton trap and maintaining the pH gradient of the IMM essential for oxidative phosphorylation [64]. We also show evidence of TRIB3 interacting with Ndufb10 of Complex I in the electron transport chain, this chain is comprised of several complexes with optimal function relying on close proximal interactions ultimately facilitated by Cardiolipin rich membrane domains of the IMM [65]. Specifically, cardiolipin interacts with Complex I inducing conformational changes, modulating the accessibility of quinone and the coupling mechanism of complex I [66] (Fig. 3c). Tribbles 3 adipose ineractome reveals that it interacts with several key mitochondrial protiens and complexs, many of which rely on cardiolipin disribuion throughout the mitochondrial membranes to function optimaly [67]. Strikingly, TRIB3 interacts spesificaly with mitochondrial proteins which either modulate cardiolipin content or rely on optimal cardiolipin distribution for correct function, reflected in our lipidomic data and enrichment analysis (Fig. 1). Moreover, these dynamically interacting lipid classes provide information on the nutrient and metabolic status of the cell (reviewed [68]). With more refined lipidomic techniques the many roles of cardiolipins are now being explored including, but not limited too; once oxidized CL acts as apoptotic trigger via cytochrome C release, substrate carrier, mitochondrial import, oxidized CL can even stimulate TLR4 and activate pro-inflammatory signaling [69,70]. Cardiolipin abundance in humans, animal and cell models of obesity is consistently reduced, resulting in a decreased overall mitochondrial mass (reviewed [71]) whereas, weight loss and exercise have been shown to restore CL abundance along with glucose control [72]. We can see that in lean mice the cardiolipin content is significantly altered (Fig. 1b), however it is only during stress we observe the full implications of its action (Fig. 5). Showing us that there is an inherent level of flexibility to cardiolipin (and other membrane lipids) content that is tolerated, unless the mitochondria are put under stress.

Trib3 has already been associated with ER stress, alterations in cell membrane structure and CHOP-10, adaption to mitochondrial stress [73], in brown adipose, we postulated a metabolic flexibility phenotype due to mitochondrial membrane changes. Sustarisc et al. have shown that depletion of CL in adipose promoted whole body metabolic inflexibility and therefore insulin resistance via ER stress response factors CHOP-10's repression of UCP1 [74]. Confirming this mechanism reduced CL mouse model (AdCKO) found that reduced CL caused ER stress and elevated Ero1l activated by

CHOP-10, which in turn significantly upregulates *Trib3*. A phenotype only exacerbated when exposed to a 60% HFD leading the researchers to conclude that cardiolipins play an essential role in metabolic flexibility and adipose thermogenic capacity [74].

Rapid advancements in both lipidomics and computational tools have enabled us to delve into the complex biology of lipidomes. Publications showing how circadian rhythms [75–77], genetics, diet and sex influence lipidomes [78], even down to the level of site specific lipid profiles (global to organelle) [79], have provided a wealth of knowledge and highlight the essential role an individual's lipidome has on health and disease [79]. A recent publication by Popovic et al,. presents compelling data from both *Drosophila* and Human showing that Tribbles are involved in regulating the balance between sleep duration, body weight, spesificaly adipose TG abundance, and lifespan [80]. In other studies Tribbles 3 has been described as a nutrient sensor [81–83] or a nutrient deficiency survival factor [84,85], in each case partioning limited resourses to ensure short term survival by modulating resourse priority. Data presented here supports this theory as we see an intergrated adipose response to reduced or ablated Tribbles 3 levels resulting in an a more beneficial acute response to high lipid environments.

With the increasing demand for personalized medical advice and treatment plans, using lipidomes to monitor health status or even targeting specific lipidomes as a therapeutic or preventive measure could offer new avenues for the treatment for many diseases linked to stress such as obesity, type 2 diabetes, neurodegenerative diseases, cardiovascular diseases, and microbial and microbiota-related conditions (reviewed [86]).

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Author Contributions

All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest

Data Availability Statement

Lipidomics, Metabolomics & Proteomics

Figure Legends:

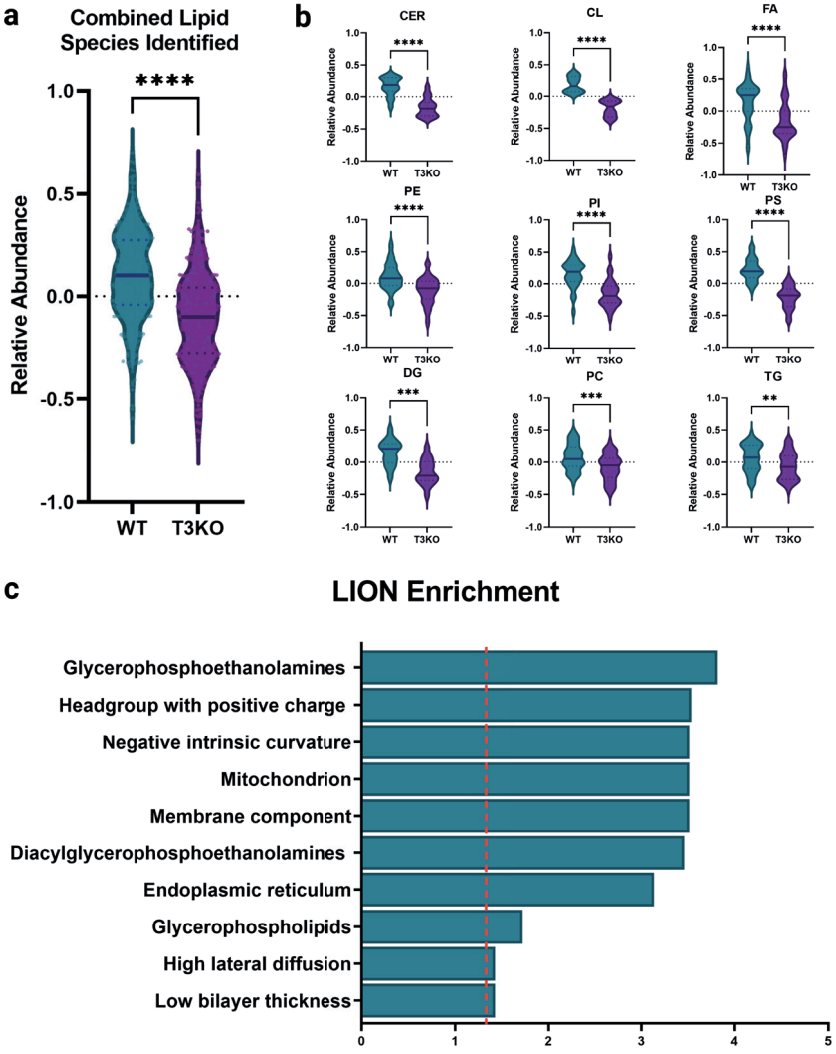

Figure 1:
(a) Untargeted lipidomic overview of vWAT wild type (WT) and Trib3$^{-/-}$ relative abundance total identified lipid species (Total #504), unpaired two-tailed t-test with welch's correction p=#16 p=#8 p=#16 p=0.0001, FA #46 p=#51 p=0.0002, PE #86 p=#20 p=#30 p=#56 p=0.002 (# number of unique species identified), complete lipid class analysis in supplemental Figure. 1. (c) LION-term enrichment analysis, red dotted line shows significance ($q < 0.05$), bar size shows scaled enrichment −LOG(FDR q- values) (n=6).

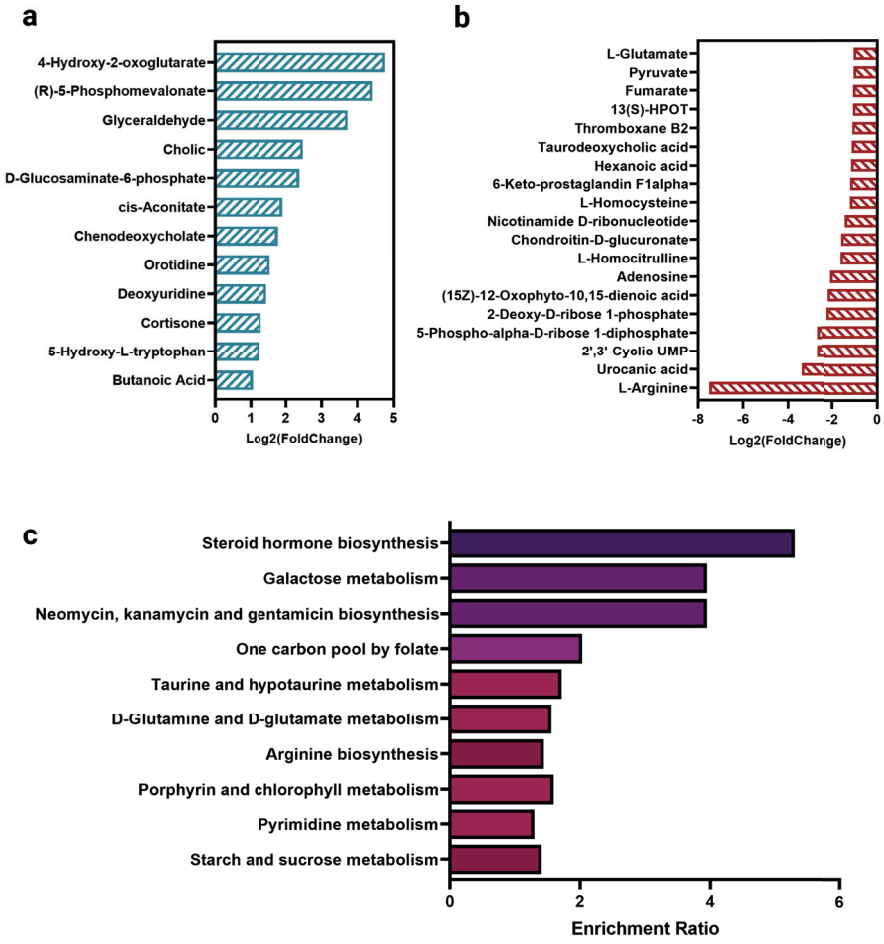

Figure 2:
Semi-targeted metabolomic overview of vWAT wild type (WT) and Trib3$^{-/-}$ relative abundance comparison. Log2(FoldChange) of individual metabolites (a) top increase (b) top decrease. (c) Fold enrichment analysis via MetaboAnalyst shows top hits of enrichened metabolic pathways (n=6).

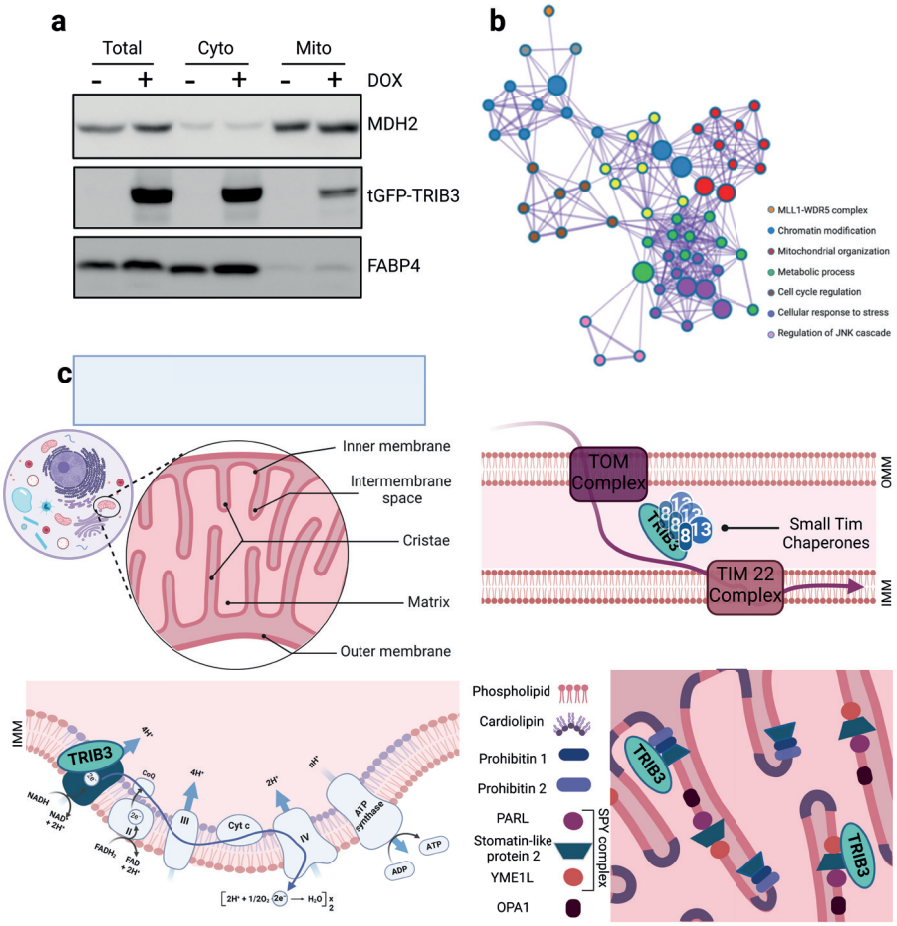

Figure 3:
Western blot of cellular fractions, total, Cyto (cytoplasmic) and Mito (mitochondria) of mature cell line IBAt-GFP-TRIB3 (-/+) DOX (Doxycycline) inducible, MDH2, tGFP-TRIB3 and FABP4 respectively. (b) Mitochondria cluster of Trib3 associated proteins. (c) Graphic of TRIB3 mitochondrial specific interactors.

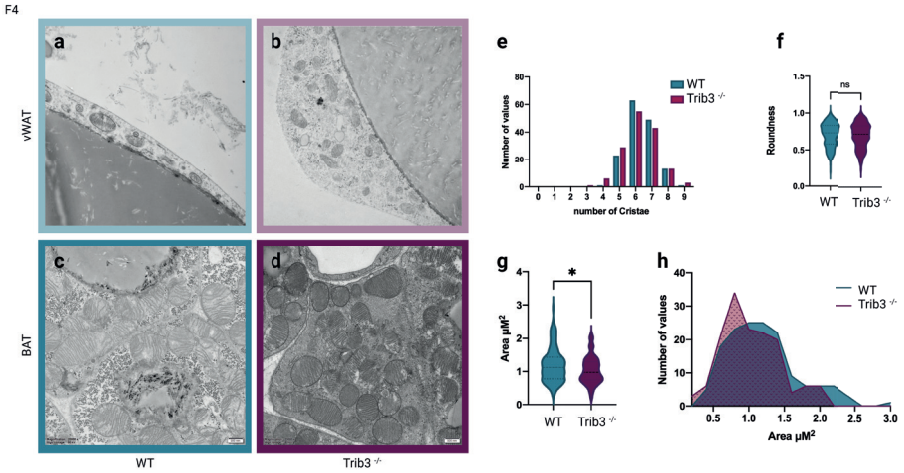

Figure 4:
Electron microscopy (EM) mitochondria analysis of (a) WT vWAT, (b) TRIB3 KO vWAT, (c) WT BAT, (d) TRIB3 KO BAT scale bar 500nm. Analysis of mitochondria ultrastructure was performed using 100 images per condition of BAT (e) Histogram of cristae per mitochondria, (b) roundness of mitochondria p=0.7599, Mann Whitney adjusted two-tailed t-test, (c) Area µM^2 of mitochondria p=0.0143, Mann Whitney adjusted two-tailed t-test (h) histogram of area µM^2 mitochondria.

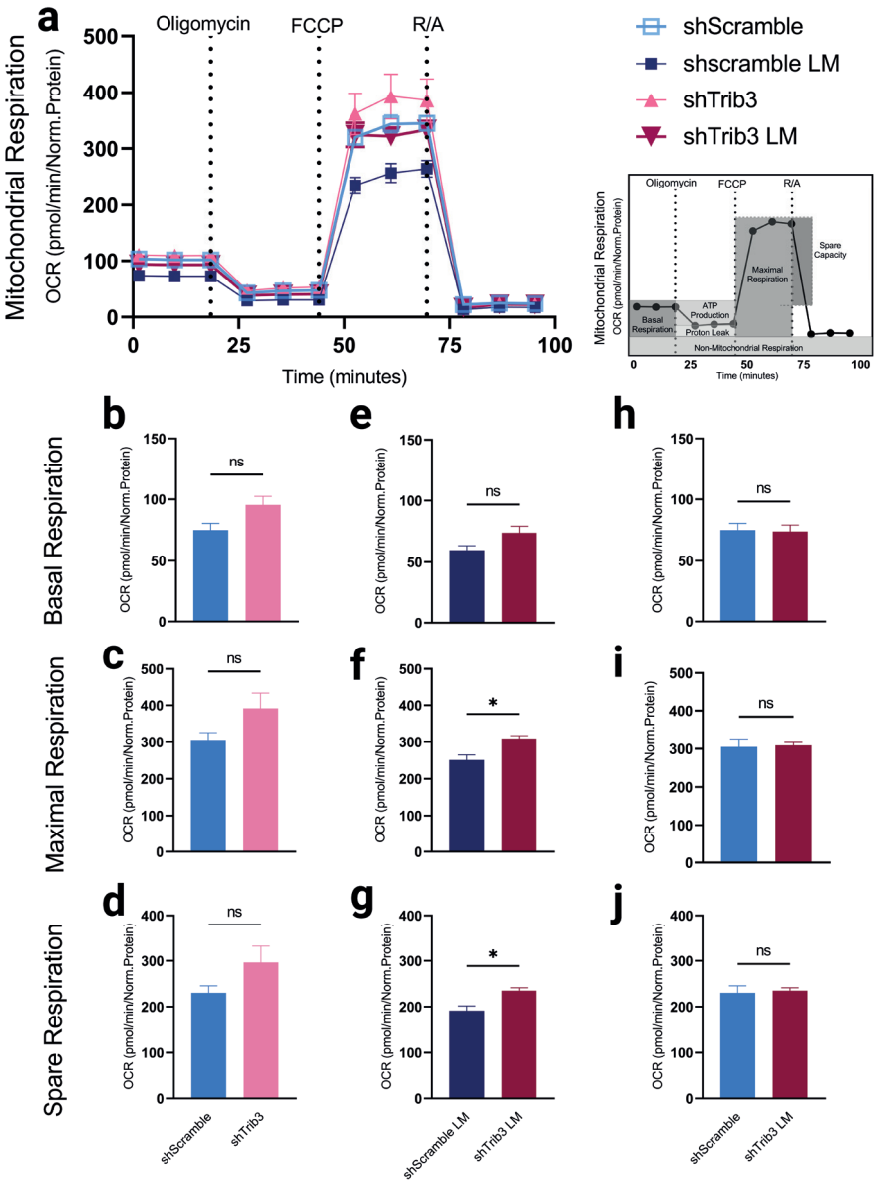

Figure 5:

Seahorse XFp Cell Mito Stress Test assay, mature shScramble and shTrib3 3T3-L1 cell lines (+/- LM). (a) OCR profile shScramble: Blue(-/+LM) and shTrib3: Pink(-/+LM), dashed lines indicate media injections of oligomycin, carbonyl cyanite-4 (trifluoromethoxy) phenylhydrazone (FCCP), and Rotenone/Antimycin A (R/A). shScramble and shShTrib3 (b) Basal Respiration p=0.0594 (c) Maximal Respiration p=0.1280 (d) Spare Respiratory Capacity p=0.1589. shScrambleLM and shShTrib3LM (e) Basal Respiration p=0.0995 (f) Maximal Respiration p=0.0329 (g) Spare Respiratory Capacity p=0.0298. shScramble and shShTrib3LM (h) Basal Respiration p=0.8610 (i) Maximal Respiration p=0.7695 (j) Spare Respiratory Capacity p=0.8811. All comparisons Mann Whitney adjusted two-tailed t-test.

Abvreviation Lipid Name	
PE	Glycerophosphoethanolamines
PG	Glycerophosphoglycerols
Cer	Ceramides
PI	Glycerophosphoinositols
TG	Triradylglycerols
PC	Glycerophosphocholines
PR	Isoprenoids
CL	Cardiolipins
ST	Sterols
Glc-cer	glycosphingolipids
Other	Other
SM	Phosphosphingolipids
PS	Glycerophosphoserines
DG	Glycosyldiradylglycerols
FA	Fatty acyls
PA	Glycerophosphates
PK	Polyketides

Materials and Methods

Lipidomics and Metabolomics:

A volume of 50 µL homogenized cells is subjected to Liquid-Liquid extraction (LLE). The sample is vortex mixed with methanol-methyl-tert-butylether (containing one internal standard per lipid class and an amount of antioxidant – BHT - to prevent lipid oxidation) after which an amount of water is added to induce phase separation. After incubation, the sample is centrifuged and the organic top layer containing all lipids is transferred to a clean sample vial. This lipid fraction is dried in a vacuum concentrator. Prior to analysis the lipid residue is dissolved in acetonitrile, thoroughly vortex mixed and transferred to an injection vial.

LC-MS/MS sample analysis is conducted on an Ultimate 3000 UHPLC with LTQ-Orbitrap XL high resolution mass spectrometry detection. For chromatographic separation an Acquity BEH C18 column (2.1x100 mm, 1.7 µm) positioned in a 60°C column oven is used. Upon injection of 5 µL sample a 10 min gradient is started (total runtime 20 min per sample). Sample analysis is conducted in both positive mode and negative mode. Generated data is submitted to MZMine for alignment and data analysis.

Lipidomic and Metabolomics data normalization and analysis:

Raw data was uploaded and normalized via Metaboanalist 5.0. Principal component analysis (PCA) analysis and heat map generation was also performed via Metaboanalist 5.0 software.

Enrichment analysis:

Metabolomics: Enrichment analysis of normalized data (MSEA)
http://www.metaboanalyst.ca

Lipidomics:Enrichment analysis of normalized lipidomic data, using 'ranking mode', performed via LIONweb:LION/web a web-based ontology enrichment tool for lipidomic data analysis. Martijn R. Molenaar, Aike Jeucken, Tsjerk A. Wassenaar, Chris H. A. van de Lest, Jos F. Brouwers, J. Bernd Helms.GigaScience, Volume 8, Issue 6, June 2019, giz061. doi: https://doi.org/10.1093/gigascience/giz061

3T3-L1 culture and differentiation

The murine 3T3-L1 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum (Invitrogen), and penicillin and streptomycin (both at a concentration of 100 µg/ml; Invitrogen). To induce differentiation of 3T3-L1 cells into adipocytes, the cells were allowed to grow until they reached confluence, and then they were stimulated with culture medium containing dexamethasone (at a concentration of 250 nM), 3-isobutyl-1-methylxanthine (at a concentration of 500 µM), and insulin (at a concentration of 170 nM) for 2 days. Subsequently, on day 2, the medium was replaced with culture medium containing insulin (at a concentration of 170 nM), and the cells were maintained in this medium for 4-6 days.

shTrib3 knockdown in 3T3-L1 cell line

SigmaAldrich MISSION shRNA clone ID: TRCN0000276731, Target Sequence: ATGTGCCTCAGGACCACAAAT, HairpinSequence:5'-CCGG-ATGTGCCTCAGGACCACAAAT-CTCGAG-ATTTGTGGTCCTGAGGCACAT-TTTTTG-3', Protocol: as per manufacturer guideline

SeaHorse MitoStress XF test assay: Agilent Seahorse XFe cell mito stress test was performed according

to the manufacturer's instructions. In short, cells we seeded and allowed to grow to confluence, cells then underwent the above 3T3-L1 differentiation protocol. Cells denoted as LipidMix were conditioned with Lipid mix 1 (sigma L0288) 1:10 ratio to medium for the final 3 days as previously described in [52].

Western: (ST1032 Sigma-Aldrich) TRB3 and (T9026 Sigma-Aldrich) Tubulin Secondary

In short, the samples were obtained using RIPA lysis buffer. Protein concentration was determined, and Laemmli sample buffer (Sigma-Aldrich, Saint Louis, MO, USA) was added to the samples before loading them onto 10% acrylamide gels. The samples were separated by SDS-PAGE and transferred onto PVDF membranes. After that, the samples were blocked using 5% milk in TBS-T for 45 minutes. The primary antibodies were incubated overnight at 4°C, followed by incubation with secondary antibodies at room temperature for 1 hour. The protein expression was visualized using an LAS4000 Image Quant (GE Healthcare, Chicago, IL, USA) with the help of ECL solution.

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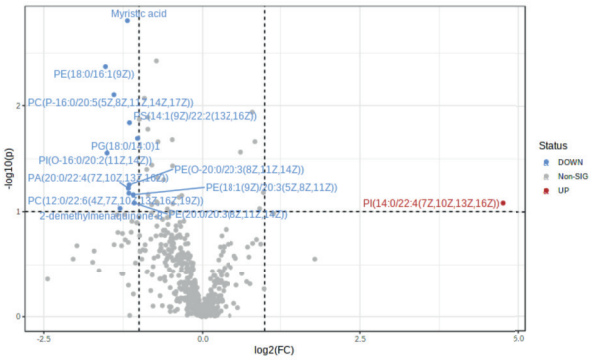
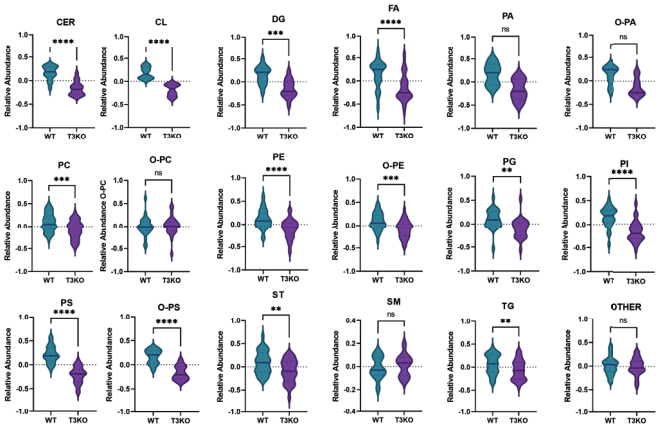
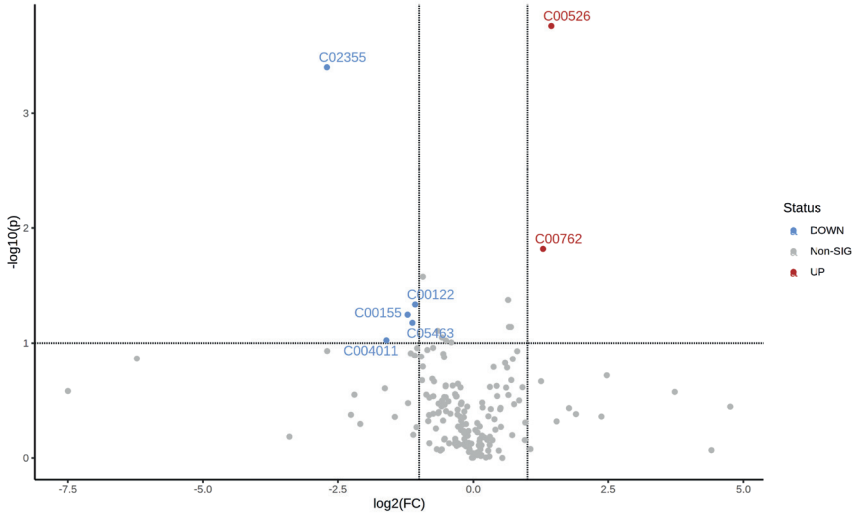


Lipid Class Iden fied unique species P Value P Value Summery
CER 16 <0,0001 ****
CL 8 <0,0001 ****
DG 16 0.0001 ***
FA 46 <0,0001 ****
PA 5 0.0555 ns
O-PA 5 0.0748 ns
PC 51 0.0002 ***
O-PC 27 0.739 ns
PE 86 <0,0001 ****
O-PE 36 0.0004 ***
PG 26 0.0018 **
PI 20 <0,0001 ****
PS 30 <0,0001 ****
O-PS 12 <0,0001 ****
SM 11 0.5916 ns
ST 30 0.0051 **
TG 56 0.002 **
Other 23 0.8237 ns
Total 504 <0,0001 ****



Supplemental Figure 1:
Untargeted lipidomic class overview of vWAT wild type (WT) and Trib3$^{-/-}$ relative abundance, unpaired two-tailed t-test with welch's correction: CER #16 p=#8 p=#16 p=0.0001, FA #46 p=#5 p=0.0748, PC #51 p=0.0002, O-PC #27 p=0.739, PE #86 p=#36,p=0.0004, PG #26 p=0.0018, PI #20 p=#30 p=#12 p=#11 p=0.5916, ST #30 p=0.0051, TG #56 p=0.002, Other #23 p=0.8237, Total #504 p=# number of unique species identified). Untargeted Lipidomics volcano plot of normalised values. Semi-targeted Metabolomics volcano plot of normalised values (n=6).

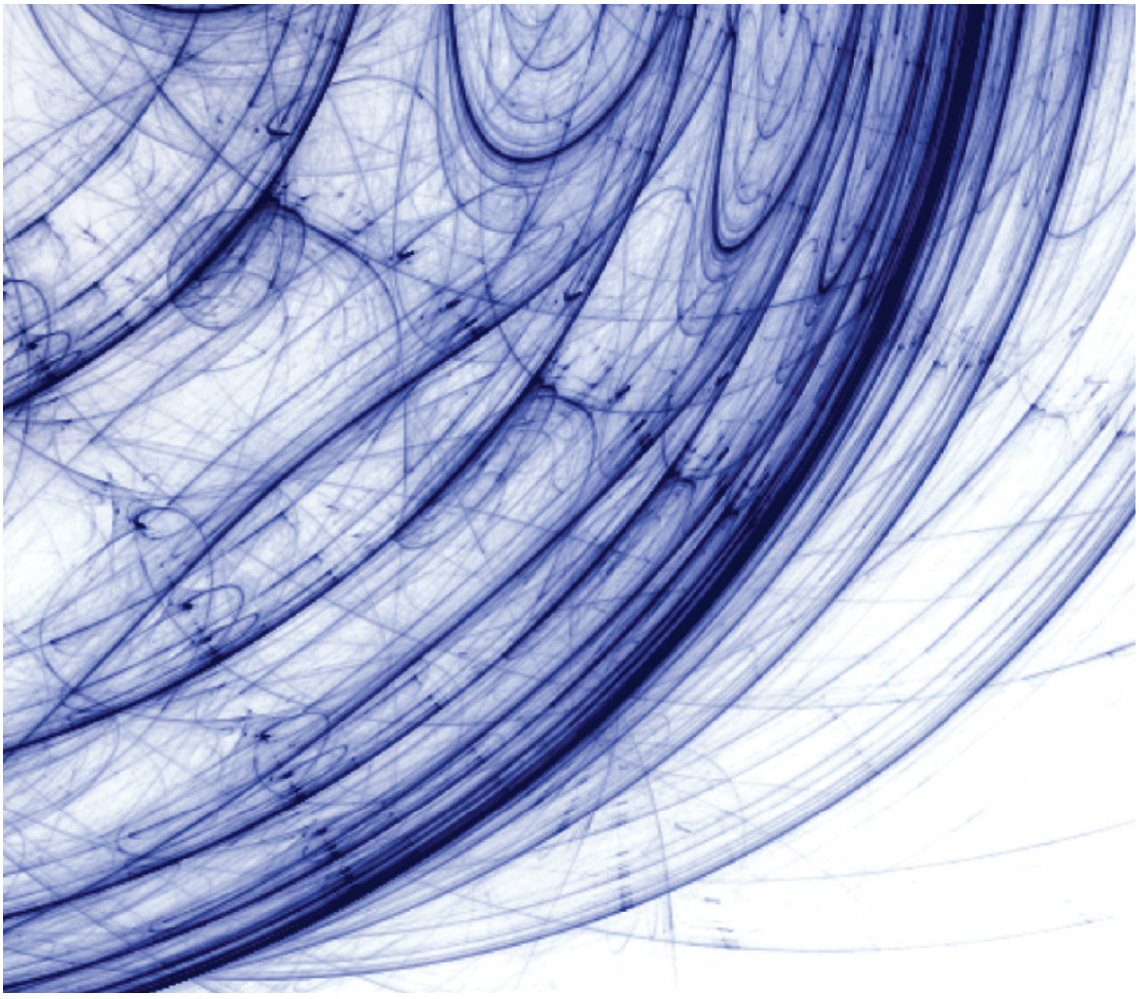


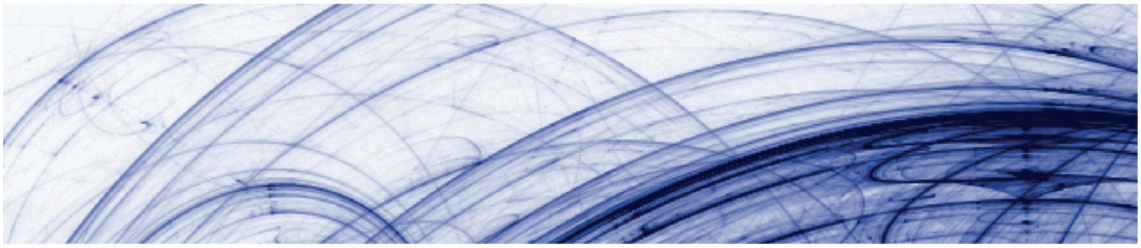
Supplemental Figure 2:
Proteomics table 1: Inhouse identified TRIBBLES 3 mitochondrial interactors from cell lines IBA, HEK293 &, MCF7. Table shows interactors with more than 3 peptides identified with a FDR cut of of 0.05

Gene Name	Cell line
TIMM13	HEK293T
TIMM8A	HEK293T
TIMM13	IBA
TIMM8B	IBA
TIMM8A1	IBA
MRPS7	IBA
TFAM	IBA
STOML2	IBA
NDUFB10	IBA
USP30	IBA
AIFM1	MCF7
MRPL12	MCF7
TECR	MCF7
SLC25A5	MCF7
UQCRFS1	MCF7
SLC25A6/A4	MCF7
UQCRC1	MCF7
ATP5L	MCF7
DNAJC19	MCF7
* More than 3 peptides were found, FDR cut off: 0.05	

Suplamental Figure 3:
Seahorse XFp Cell Mito Stress Test assays. (a) OCR profile of shScramble and shTrib3 undifferentiated 3T3-L1 cell line, dashed lines indicate media injections of oligomycin, carbonyl cyanite-4 (trifluoromethoxy) phenylhydrazone (FCCP), and Rotenone/Antimycin A (R/A) (n=3).

Chapter 6





Part a

GENERAL DISCUSSION: Developing optimal *in vitro* model systems to study lipid handling

General Discussion

Adipose tissue research has come a long way since the early belief that adipocytes merely serve as inert lipid storage centers [1]. Today, we understand that adipose tissue is a complex and dynamic tissue with multifaceted functions including its endocrine functions (secretion of adipokines and cytokines), and immune-metabolic interactions, with a remarkable capacity for plasticity [1]. This not only puts adipose tissue at the center of global energy homeostasis [1–4], but also helps to explain how it can play a pivotal role in the pathogenesis of obesity-related disorders, including insulin resistance and type 2 diabetes, cardiovascular diseases and cancer. Lipids are central to adipose tissue function and play diverse roles: they serve amongst others as a form of energy storage, as structural components of cellular membranes, as signaling intermediates, as receptor ligands and as lipid antigens. The interplay and interdependence between these functions is largely unexplored and in this thesis, we have focused on 2 particular aspects of lipid metabolism in adipose tissue function: lipid handling and lipid antigen presentation in adipocyte-immune cell communication, **Chapter 2 and Chapter 3**, and the role of the pseudokinase Trib3 in lipid handling in adipocytes**, Chapter 4 and Chapter 5**. In **Chapter 2** we explore the pro-inflammatory consequences of adipocyte insulin resistance on iNKT cell cross-talk output, followed by **Chapter 3**, where we assess the pro- inflammatory consequences of direct iNKT cell lipid loading. In both cases, we that a lipid-rich microenvironment skews the cytokine output of iNKT cells to a pro-inflammatory profile (IFNγ secretion). In addition, we show that AT-resident iNKT cells accumulate significantly more lipid than their spleen or liver counterparts. However, the many layers of this cross-talk regulation, such as co- stimulation, are not exclusively regulated by the lipid environment and lipidome. In **Chapter 6b** we take a closer look at the lipid antigens connecting adipocyte and iNKT cell cross-talk, finding a complex and layered feedback system relying on environmental resources to guide the interactions [5]. In **Chapter 4** we investigate the role of the pseudokinase Trib3 in adipose tissue biology with a combination of lipidomics, transcriptomics, interactomics and phosphoproteomics analyses to elucidate cell-intrinsic functions of TRIB3 in pre- and mature adipocytes. Our findings support a role in adipocyte lipid homeostasis and proliferation *in vitro* and *in vivo*, with Trib3 having multiple distinct regulatory roles in the cytoplasm, nucleus and mitochondria, ultimately controlling adipose tissue homeostasis, rather than affecting a single cellular pathway. In **Chapter 5** we followed up on the role of Trib3 in adipocyte lipid metabolism and describe a novel role in mitochondrial respiration.

In this chapter we will highlight the implications of the research presented, focusing on model systems to study lipid handling in adipocytes and immune cells in **Chapter 6a** and on lipid antigen presentation by adipocytes in **Chapter 6b.**

Chapter 6a

In vitro model systems have emerged as a powerful tool for targeted investigation of specific adipose tissue-related questions, devoid of interference from other mechanisms and feedback loops [8], [9]. The utility of model systems in adipose research lies in their ability to focus on specific biological processes, reducing complexity and isolating variables of interest. For instance, two-dimensional (2D) cell lines, such as immortalized (pre)adipocyte lines, provide easy maintenance and reproducibility, enabling investigation of simple phenomena. However, they may lack the complexity required to represent the multifaceted interactions between adipocytes and the many types of immune cells found in the *in vivo* situation [8]. Two-dimensional (2D) cell lines, such as immortalized (pre)adipocyte lines, provide easy maintenance and reproducibility, enabling investigation of simple phenomena. On the other hand, three-dimensional (3D) spheroid culture system have gained popularity due to their ability to recapitulate the *in vivo* environment more accurately. Researchers must, nevertheless, be cautious when interpreting results from these systems. While they currently allow precise measurements and controlled environments, many do not fully replicate *in vivo* conditions, potentially limiting their predictive capacity for outcomes within living organisms. In this section of the discussion, we will reflect on the advantages of utilizing various context-appropriate model systems in order to gain insight into the complex biology and function of adipose tissue.

The adipocyte microenvironment: lipids and beyond

An important area of metabolic research focuses on the extracellular environment, which may even include the circulation, to which adipocytes are exposed. In obese conditions, this includes amongst others various pro-inflammatory cytokines like TNF and IL-6, but also FFA as adipocyte lipolysis is more active under obesity-induced insulin resistance [9–11] Researchers have therefore often mimicked this in vivo situation by exposing adipocytes to individual cytokines or individual FFA in their cell-based approaches. In several of the chapters of this thesis we simulated a lipid rich microenvironment using a mixture of free fatty acids (FFA's) and cholesterol, **Figure 2** In previous assays we had used oleic and palmitic acid in specific ratios to lipid load adipose cells, mimicking a high fat diet environment. The rationale behind

using lipid mixture to induce insulin resistance, **Chapter 2,** to lipid load immune cells, **Chapter 3,** or to influence pseudokinase Tribbles 3 lipidome modulation, **Chapter 5,** was initially to create an environment that was more complex and therefore closer to the actual *in vivo* situation, where dyslipidemia may not be restricted to a single lipid species [12]. However, we noticed that at least in the case of lipid antigen presentation **Chapter 2**, alterations were only observed with the lipid mix but not the individual components [13]. These findings suggest that a combination of cellular pathways may have to be altered simultaneously, driven by individual lipid species or lipid classes, to result in a net alteration in adipocyte-immune cell crosstalk. These findings highlight to importance of stepwise increasing the complexity of in vitro models, to address the net effect of obesity-driven changes in cellular function. It will for example be of interest to include pro-inflammatory cytokines into the equation in future experiments or expose adipocytes to extracellular fluid from adipose tissue [14].

We have employed the lipid mix in a study spearheaded by **Ramos Pittol,** showing that FXR isoforms control various metabolic functions in liver cells as well as lipid droplet formation during NASH using mouse liver organoids, **Figure. 1,** leading to a new understanding of treatment options as well as isoform grouped subpopulations of NASH patients [15].

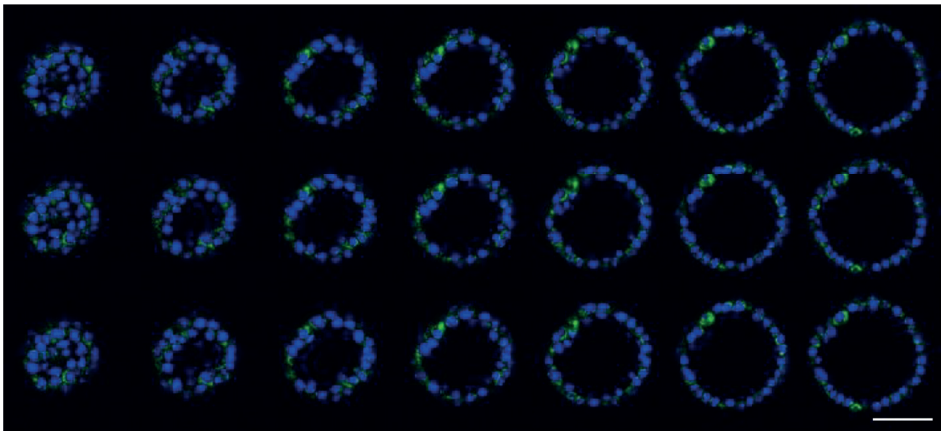


Figure 1. Lipid loaded liver organoid. Confocal image showing Z-stack serial sections of liver organoid cultured with lipid mix. Nucleus stains with DAPI (Blue) and neutral lipids stained with LipidTox (Green), white scale bar 100μM

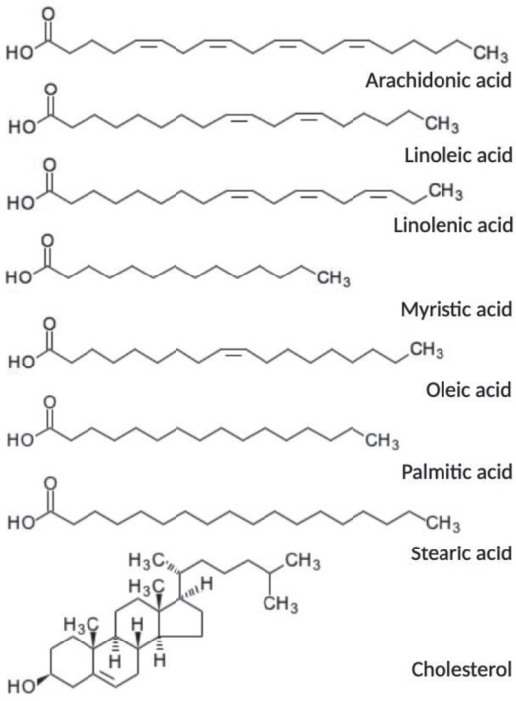


Figure 2: Chemical structure of lipids included in chemically defined lipid mix. A combination of Arachidonic acid, Linoleic acid, Linolenic acid, Myristic acid, Oleic acid, Palmitic acid Stearic acid and Cholesterol was used in this thesis to simulate a 'dysfunctional' high lipid environment during cell culture.

Adipocyte model systems

Two-dimensional (2D) cell lines, such as immortalized adipocyte lines, provide easy maintenance and reproducibility, enabling investigation of simple phenomena [6,16,17]. The best-known cellular model system is the murine 3T3-L1 (pre)adipocyte cell line, which was first described in 1974 [18]. Subsequently more 2D (pre)adipocyte cell lines were established form either murine or human origin**, Table 1.** While presenting robust model for adipogenesis and adipocyte function in many respects, it should be noted that all of these models have their limitations, **Table 1**. Even the widely used 3T3-L1 model system has its drawbacks: 3T3-L1 cells fail to produce leptin [19], one of the best known adipokines that is produced by murine and human AT and increased with obesity [20,21]. Another widely used approach is the use of primary pre-adipocytes isolated from the so-called stromal- vascular fraction (SVF) of AT, which are then differentiated *ex vivo*, as also described in **Chapter 4** of this thesis [22]. Whilst this technique is widely used to study adipose specific mechanisms the *ex-vivo* cells do not survive a high passage number and genetic manipulation is complex.

More recently, three-dimensional (3D) spheroid culture systems have gained popularity due to their ability to recapitulate the in vivo environment more accurately [23,24]. In this thesis co-culture experiments are described in 2D *in-vitro* or *ex-vivo* settings **Chapter 2**. However, we also wished to develop a 3D adipose model system that we could expose to various environments before co-culture with iNKT cells in the hope of creating a more adipose tissue-like system. 3D cultures of adipocytes, termed spheroids, were formed by suspending cells in an inverted droplet, **Figure 3 a-c.** The main advantage we found in this technique was that due to the tight packing of cells during spheroidization, the confluence reached before differentiation began was so high that many cells began forming lipid droplets before differentiation mix one was applied making differentiation consistently highly successful. The size of the spheroid could also be adjusted easily whilst maintaining a 20ul media: 20k cells, within reason of gravity. The spheroids could also fuse if left in close proximity, merging into larger constructs, **Figure 3d**. We also found that culturing the same precursor 3T3-L1 cells in 2D vs 3D spheroid could have markedly different lipid droplet formation, **Figure 3 e-h.** Finally, we examined the secretion of adiponectin, an adipokine that decreases in circulation with weight gain and obesity [25]. Unfortunately, we found that fusing multiple spheroids did not result in this obesogenic adiponectin phenotype after differentiation, **Figure. 3 i-j.** It was also observed that these spheroids often had a necrotic core, making phenotypic observations complex. Never the less, we and others, speculate that 3D adipocyte culture will provide an important platform for drug development, bioaccumulation and pharmacokinetics, not to mention the increasing emphasis on obesity research [6,24].

Our attempts at creating a 3D model serve to illustrate that each *in vitro* model has its unique strengths and limitations**, Table 1**, and that the choice depends on the specific research questions and goals of the study. It is therefore recommended to combine multiple in vitro and in vivo model systems to gain a more comprehensive understanding of adipose tissue biology and function, as also illustrated by our studies on Trib3 in **Chapter 4** [22].

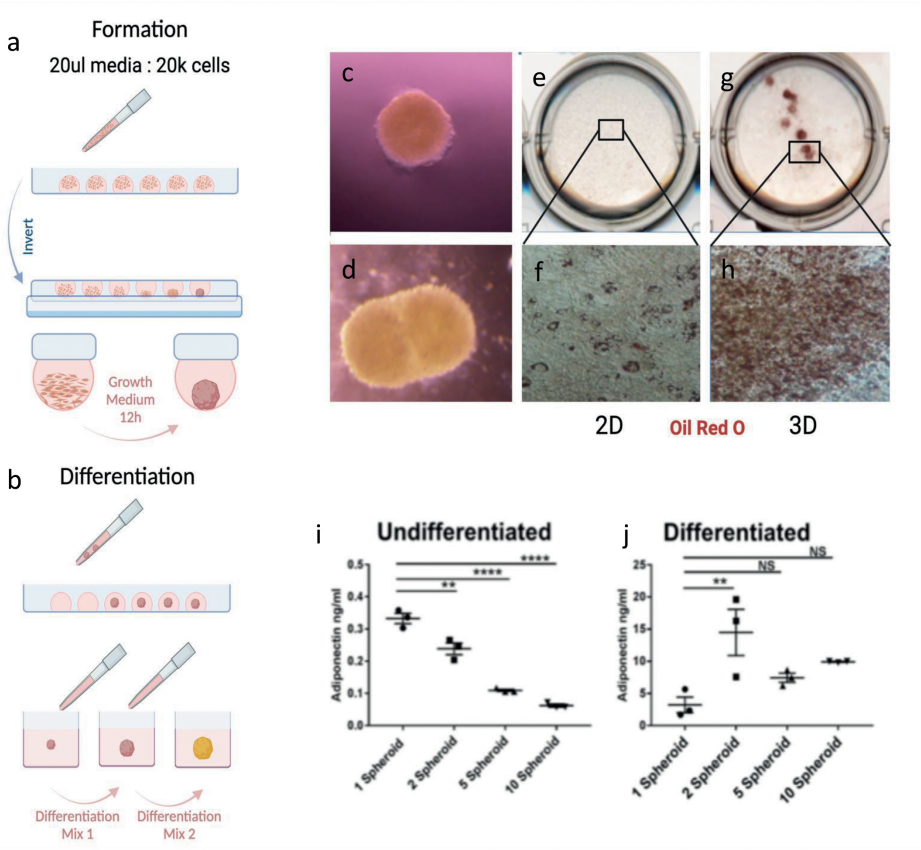


Figure 3: Spheroid culture of 3T3-L1 adipocytes. A) spheroid formation using a 20ul-media-to-20k- cell ratio, droplets were inverted in a humid environment overnight. After 12 hours hanging in suspension the spheroids form. B) After spheroid formation the plate was flipped, and spheroids were retrieved and differentiated. C) Spheroid differentiation at day 8 D) Fused spheroid at day 8. E) Lipid accumulation in 3T3-L1 adipocytes (day 8 of differentiation) in 2D culture, as visualised by oil red O stain, 2x magnification. F) Lipid accumulation as in panel E, 10X magnification G) Lipid accumulation in 3T3-L1 adipocytes (day 8 of differentiation) in 3D spheroids, as visualised by oil red O stain, 2x magnification. H) Lipid accumulation as in panel G, 10X magnification I) Adiponectin secretion by undifferentiated single or fused 3T3-L1 spheroid cultures, assessed by ELISA of culture media. J) Adiponectin secretion as in panel I, by differentiated (day 8) single or fused 3T3-L1 spheroids.

Invariant natural killer T cells sense and respond to lipid environments

Invariant NKT cells play a role in immunometabolism, acting as lipid sensors and modulating their surroundings accordingly by secreting a wide range of both pro- and anti-inflammatory cytokines [13,26,27]. Deconstructing iNKT cell cross-talk with adipocytes by simulating a lipid-rich microenvironment *in vitro* has given us insights into this mechanism, which were previously inaccessible due to technical limitations.

In **Chapter 3,** we show that AT- resident iNKTs can accumulate lipids and do so significantly more than their spleen or liver counterparts. Considering the conserved nature of the LD's structure as well as their many functions, it is perhaps not such a shock that we find them present in control culture conditions (no lipid mix addition). We speculate two reasons for this; first, tissue culture media are very rich in nutrients and does not experience the same feed-fast cycle as an organism eating ad libitum. Second, as previously discussed, the LD is a highly conserved structure found in the majority of cell types, including various immune cells [28,29]; perhaps it would have been unexpected not to find them in iNKT cells [30]. Several recent publications highlight the plethora of lipid antigens which can be loaded into CD1(a-e) [31,32]. Considering the multilevel regulation we have already uncovered, **Chapter 6b** [5], we speculate there is still much more to be uncovered about the relationship between lipid droplet accumulation and iNKT cell response to presented lipid antigens.

Tribbles 3 and mitochondria: avoiding stress is the answer?

While the pseudokinase Trib3 had previously been linked to cytoplasmic and nuclear processes via its interactions with various proteins, we detected in addition to these various mitochondrial proteins in the Trib3 interactome and a clear impact on the adipocyte lipidome, **Chapter 4 and 5**. Our data further indicated a role for Trib3 in mitochondrial respiration, especially when cells were challenged with excess exogenous lipids. A lipid-rich microenvironment can be viewed as a stressed condition [30,33,34], and some reports already hinted at a special role for Trib3 in stressed conditions, even providing a link to changes in cardiolipins that we observed in **Chapter 5**. Trib3 has previously been associated with ER stress, alterations in cell membrane structure and adaption to mitochondrial stress via CHOP-10 [35]. Sustarisc *et al.* has shown that depletion of Cardiolipin (CL) in adipose promoted whole body metabolic inflexibility and therefore insulin resistance via ER stress response factors CHOP-10's repression of UCP1 [36]. Confirming this mechanism reduced CL mouse model (AdCKO) found that reduced CL caused ER stress and elevated Ero1l activated by CHOP-10, which in turn significantly upregulates *Trib3*. This phenotype only exacerbated when exposed to a 60% HFD leading the researchers to conclude that cardiolipins play an essential role in metabolic flexibility and adipose thermogenic capacity [36].

Another link between Trib3, CL and cellular stress is provided by its interaction with SLP-2, **Chapter 5,** a protein known to regulate biogenesis of mitochondria and responding to stress by forming and interacting with various complexes and proteins within the IMM [37–39]. SLP-2 is required to stabilize complex PHB1 and PHB2, enabling them bind to, mature and stabilize Cardiolipin rich membrane domains [40]

as well as complex I and IV of the electron transport chain [40]. Upregulation of SLP-2 increases mitochondrial synthesis of CL as well as mitochondria biogenesis [37,41]. This increased membrane stability in turn stabilizes OPA1, a GTPase involved in sequestering cytochrome C and respiratory chain super-complex formation. SLP-2 also interacts with the i-AAA protease YME1L and PARL to for the SPY complex, this complex activates both OPA1 and Cardiolipin synthase[42–44]. These interactions within the IMM facilitate respiratory super-complex formation within the CL rich membrane domain bringing the complex subunits closer together [45], essentially becoming a proton trap and maintaining the pH gradient of the IMM essential for oxidative phosphorylation [46]. We also show evidence of TRIB3 interacting with Ndufb10 of Complex I in the electron transport chain, which is comprised of several complexes with optimal function relying on close proximal interactions ultimately facilitated by Cardiolipin-rich membrane domains of the IMM [47]. Specifically, cardiolipin interacts with Complex I inducing conformational changes, modulating the accessibility of quinone and the coupling mechanism of complex I [48], **Chapter 5 Figure. 3c**.

The Tribbles 3 adipose ineractomes identified here and previously in other cell types, **Chapter 5 Supplemental. 3**, reveals that it interacts with several key mitochondrial proteins and complex's, many of which rely on CL distribution throughout the mitochondrial membranes to function optimally [49]. Strikingly, TRIB3 interacts specifically with mitochondrial proteins which either modulate CL content or rely on optimal cardiolipin distribution for correct function, reflected in our lipidomic data and enrichment analysis. Moreover, these dynamically interacting lipid classes provide information on the nutrient and metabolic status of the cell (reviewed [50]). With more refined lipidomic techniques the many roles of cardiolipins are now being explored. These roles include, but are not limited to (i) triggering apoptosis once oxidized (ii) acting as a substrate carrier, (iii) mitochondrial import, and (iv) stimulating TLR4 and activating pro-inflammatory signaling once oxidized [51,52]. Cardiolipin abundance in humans, animal and cell models of obesity is consistently reduced, resulting in a decreased overall mitochondrial mass (reviewed [53]) whereas, weight loss and exercise have been shown to restore CL abundance along with glucose control [54]. We can see that in lean mice the cardiolipin content is significantly altered, however it is only during stress we observe the full implications of its action, **Chapter 5 Figure. 5**. Taken together, our experiments point to a possible Trib3-CL-stress induced pathway but additional experiments are required to substantiate this view.

Tribbles 3, a drug target in immunometabolism?

Research into the roles of the pseudokinase of Tribbles 3 has often proved confusing and even contradictory [55]. Logic would dictate that the high conservation, extensive expression and multiple interaction partners would lead to strong phenotypes when this pseudokinase is knocked out. Tribbles 3 does not follow logic and has provided inspiration for many highly speculative debates.

Liu et al,. 2012 suggests opposing short term opposing roles for Tribbles activities in muscles vs adipose cells [56], allocating resources between these tissues during times of either fasting of overnutrition. They show that short-term fasting in muscle cell lines decreased TRIB3 protein and mRNA levels in muscle cells but enhanced them in adipose, leading authors to speculate this causes reallocation of nutrient uptake as the adipocytes reduce their fat uptake whereas the muscle cells increase their glucose uptake capacity leading to an overall reallocation of available recourses, with the opposite taking place during nutrient excess. The link between adipose cells as fuel storage and muscle cells as fuel burners is well studied [56,57]. Here we show in **Chapter 5** further evidence supporting this theory from the adipose side, as knockdown of Tribbles3 enhances the adipocytes ability to respond to mitochondrial stress during acute periods of excess nutrition.

Pseudokinases, such as TRIB3, have become an exciting area for drug prediction and development as well as a multitude of biomarkers [58]. The TRIBBLES pseudokinase family has been shown to be highly conserved though out evolution, with further studies detecting these proteins in the majority of cell types. Our data suggests TRIB3's link to optimal mitochondria structure and function in a dysfunctional lipid rich environment makes it a viable drug target. Our lipid enrichment shows and acute response, but due to technical limitations of the 3T3-L1 cell line we were not able to study the chronic effect on mitochondrial stress response. We speculate that our data therefore reflect the initial stages of adipocyte dysfunction in a high lipid environment. Therefore, site specific dosing or tracking distribution of a treatment agents can further aid our knowledge of how TRIB3 behaves over time as well as providing essential data on which disease subgroups would benefit, or not, from modulating TRIB3 [58–60]. Modern machine learning techniques are now being deployed to identify easily druggable targets, with several studies identifying pseudokinases as an interesting avenue (reviewed [61]). Others also hypothesize that specific environments or genetic variations in highly conserved genes such as Trib3 alters its role as a coordinator for stress-adaptive mechanisms [62–64]. This ability to function in a general yet cell specific manner allows fine tuning of cell type specific functionality depending on its specific environment, interacting with pathways regulating differentiation proliferation, and

metabolic health and cancer [62]. Our study is in line with these findings, and we agree that Trib3 is a useful biomarker or drug target because its expression and effect appear to be intertwined with the length and intensity of a cellular or global stressor.

A recent publication, using *Drosophila melanogaster* coupled with human Biobank data, sheds light on Tribbles overall mechanism of action and highlights its role in how its expression levels impact recourse allocation which in turn dictate life span via regulation of body weight and sleep duration. Popovic et al,. present findings where elevated Tribbles expression decreases overall body weight, irrespective of food intake, whilst increasing the duration of sleep time, importantly the researchers found that this has a negative impact on lifespan, highlighting the longitudinal role of Tribbles in resource allocation [65]. This research along with our data sets generated in **Chapter 4** [22] **and 5** shows Tribbles3 extensive interaction partners and thus mechanism modulation capabilities highlight the complexity of this pseudokinases finetuning abilities. Combining these complimentary data show the intricate regulation of lipidome components, their storage and utilization enabling inter-organ communication and complex mechanisms.

Conclusions and future directions

One of the primary issues in adipose tissue models centers around the translational relevance of in vitro and animal models to human physiology [66,67]. While current models provide valuable insights, their limitations in replicating the full complexity of human adipose tissue has meant many important discoveries, such as human brown, beige and bright adipose tissue have only recently come to the fore [68]. As we move forward, the incorporation of single-cell analysis, patient-specific models, and integrated-multi-omics approaches will provide a more holistic view of adipose tissue biology. Longitudinal studies in both animal models and human subjects will capture the dynamic changes in adipose tissue under different conditions, offering valuable insights into disease progression and therapeutic interventions. An impressive example of this is a recent longitudinal study which changed the long-held dogma which held that metabolism is highly individually flexible over the course of a lifetime, instead researchers robustly showed that between 20 and 60 years of age metabolism remains consistent [69]. Lipidomic and metabolic studies are heading into a new age where integrated-omics studies can provide important insights into resource allocation as well as insights into the long term effects of altered or dysfunctional lipidomes on the global organism instead of a snapshot of a localized system [5,31,32,70]. By delving into this research, we emphasize the need to bridge the knowledge gap in our current

understanding of adipose tissue and the treatment options for the many kinds of co-morbidities caused by adipose dysfunction.

Our work underscores the importance of continually evolving and refining adipose tissue models to align with our ever-growing appreciation of this vital tissue's multifaceted functions and contributions to overall health.

Table 1: Adipocyte model systems

Adipose Cell Line Model	Advantages	Disadvantages
3T3-L1 Cells [71,72]	• Well-established and easy to culture. • Widely used in adipose tissue research.	• Limited representation of the complexity of adipose tissue compared to in vivo models. • Limited capacity to fully mimic the diversity of adipose tissue and its cellular interactions. E.g. low leptin capacity [19] • Difficult to genetically manipulate
C3H/10T1/2 Cells [73,74]	• Suitable for studying adipogenesis and lipid metabolism.	• May require additional modifications or treatments for complete differentiation. • Lack some characteristics of mature adipocytes.
3T3-F442A Cells [71,75]	• Can differentiate into adipocytes with a distinct morphology. • Useful for investigating adipocyte differentiation and lipid metabolism. • Provides an alternative to 3T3-L1 cells.	• Limited information available compared to more well-established cell lines like 3T3-L1. • May have specific limitations for certain research questions.
OP9 Cells [76,77]	• Differentiates into adipocytes and can be used for studying adipogenesis. • Offers a versatile model system for various applications including high throughput.	• May require specific culture conditions and expertise. • Not as commonly used as some other adipose cell lines.
Simpson Golabi Behmel Cells (SGBS) [78]	• Capable of differentiating into metabolically active adipocytes. • Retain characteristics of human adipose tissue. • Suitable for studying obesity-related factors.	• May have more specialized requirements compared to widely used murine cell lines. Not as well-known in the research community.
hMADS Cells [79,80]	• Derived from human adipose tissue, providing human-specific insights. • Maintain the ability to differentiate into multiple lineages. • Useful for studying adipogenesis and lineage differentiation.	• Limited availability and variability in response between cell lines. • Require specialized culture conditions.

Adipose Cell Line Model	Advantages	Disadvantages
SW872 Cells [81]	• Derived from human liposarcoma, offering an alternative to human adipose cell lines. • Suitable for studying adipocyte differentiation and lipid metabolism.	• Considered cancer-derived, which may not be ideal for certain research questions.
IM-BAT Cells [82]	• Immortalized brown adipose tissue-derived cells, ideal for studying brown adipocyte biology. • Useful for investigating thermogenesis and energy expenditure.	• Limited availability and may require specific expertise for handling.
A31 Cells [83]	• Preadipocyte cells derived from rat adipose tissue, capable of differentiation into mature adipocytes.	• Limited information on these cells compared to more commonly used cell lines.
HIB-1B Cells [84]	• Brown adipocyte precursor cells that differentiate into brown adipocytes, suitable for brown adipose tissue and thermogenesis studies.	• Limited availability and may require specific expertise for handling.
LS14 Cells [85]	• Human subcutaneous preadipocyte cells, can differentiate into mature adipocytes. • Useful for studying adipogenesis and lipid metabolism.	• Limited availability and may require specific culture conditions.
IBA [86]	• Capacity to differentiate to either white or brown adipocytes making them suitable for a wide range of studies • Tolerate genetic manipulations	• Limited information on these cells compared to more commonly used cell lines.

Table 2: Adding complexity to culture models
Improving adipose tissue models to address the limitations and enhance their relevance to physiological and clinical contexts is an ongoing challenge in research. Below are some strategies and approaches to improve adipose models. These strategies offer ways to enhance the relevance and complexity of adipose tissue models but come with their own sets of advantages and challenges that researchers need to consider when selecting the most appropriate approach for their specific research goals.

Strategy	Advantages	Drawbacks
Incorporate 3D Culture Models	• Better mimic the 3D architecture of adipose tissue. • Allow for cell-cell interactions and tissue-like structures.	• May require specialized culture techniques and equipment. • Increased complexity may make experiments more challenging to conduct and interpret.
Multicellular Models	• Replicate cellular diversity and interactions within adipose tissue. • More physiologically relevant.	• Complex and may require optimization of culture conditions. • Increased cost and potential for donor-to-donor variability in primary cell cultures.
Use Primary Human Cells	• Ensure human-specific relevance. • Reflect individual variations in adipose tissue biology.	• Limited availability of primary human cells, especially for specific patient populations. • May require ethical approvals and access to human samples.
Advanced Differentiation Protocols	• Generate more mature and functional adipocytes. • Improve representation of adipose tissue.	• Development and optimization of protocols can be time-consuming. • Variability in differentiation efficiency and outcomes.
Tissue Engineering	• Create physiologically relevant adipose tissue constructs. • Enable controlled and customizable models.	• Requires advanced tissue engineering techniques and materials. • May be resource-intensive. • Challenges in mimicking tissue vasculature.
Microfluidic Devices	• Simulate dynamic aspects of adipose tissue, including nutrient flow. • Enable real-time monitoring.	• Requires expertise in microfluidics and device fabrication. • May have limitations in replicating tissue-scale complexity.
Bioreactors	• Mimic physiological conditions, including mechanical forces. • Support long-term culture.	• Complexity in setting up and maintaining bioreactor systems. • Potential challenges in replicating all aspects of tissue physiology.
Advanced Imaging Techniques	• Visualize cellular dynamics and interactions in real-time. • Provide high-resolution data.	• Requires specialized imaging equipment and expertise. • May not capture all cellular processes or interactions.

Strategy	Advantages	Drawbacks
Single-Cell Analysis	• Dissect cellular heterogeneity and functions at the single-cell level. • Provide insights into rare cell populations.	• Complex data analysis and interpretation. • May require high-throughput sequencing and additional resources.
In Vivo Imaging and Monitoring	• Study adipose tissue physiology in living animals over time. • Non-invasive and avoids sacrificing animals.	• Limited to animal models. Imaging techniques may have limited resolution or specificity. • Ethical considerations in animal research.
Patient-Specific Models	• Enable personalized medicine approaches. • Study disease mechanisms using patient-derived cells.	• Limited availability of patient samples and potential difficulties in obtaining specific patient cells. • Variability in genetic and disease backgrounds among patients.
Lipidomic and Metabolomic Profiling	• Comprehensive characterization of the adipose tissue lipidome and metabolism. • High-resolution data.	• Relies on sophisticated analytical techniques and equipment. • Requires extensive data analysis and interpretation. • May not capture dynamic changes in real-time.
Data Integration and Systems Biology	• Gain a systems-level understanding of adipose tissue biology. • Combine multi-omics data for comprehensive insights.	• Requires advanced computational resources and expertise. • Assumptions and simplifications in modeling may introduce limitations.
Longitudinal Studies	• Capture dynamic changes in adipose tissue in response to stimuli over time. • Provide insights into disease progression.	• May require extended study periods. • Challenges in maintaining consistent conditions over time.
Cross-Disciplinary Collaboration	• Encourage diverse expertise to address complex research questions. • Foster innovation and holistic approaches.	• Coordination and communication challenges between disciplines. • Requires interdisciplinary training and collaboration.

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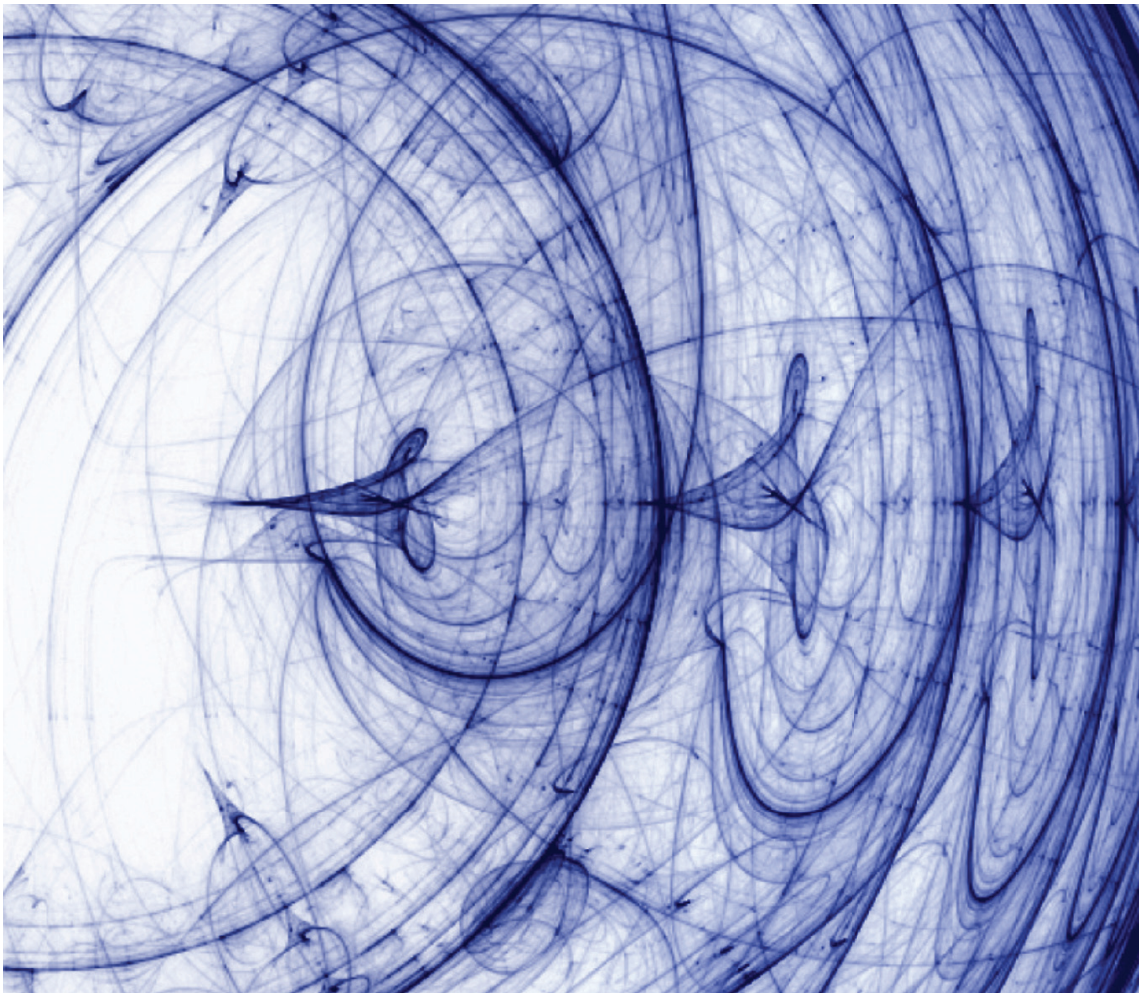
Chapter 6



Part b

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

Morris, I*., Croes, C.-A., Boes, M., and Kalkhoven, E.

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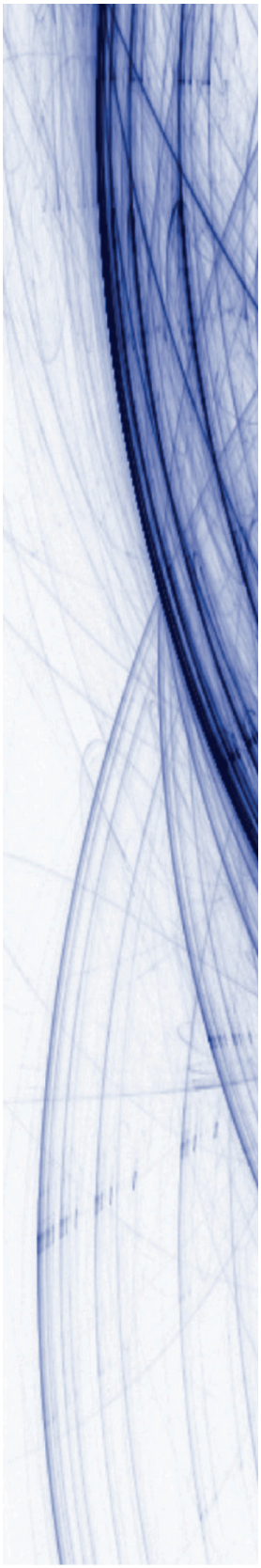

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Review

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

Imogen Morris [a], Cresci-Anne Croes [b], Marianne Boes [c,d,1], Eric Kalkhoven [a,*,1]

[a] Center for Molecular Medicine, University Medical Center Utrecht, Utrecht University, Universiteitsweg 100, 3584, CG, Utrecht, the Netherlands
[b] Nutrition, Metabolism and Genomics Group, Division of Human Nutrition and Health, Wageningen University, 6708WE Wageningen, the Netherlands
[c] Center for Translational Immunology, University Medical Centre Utrecht, Utrecht University, Lundlaan 6, 3584, EA, Utrecht, the Netherlands
[d] Department of Paediatric Immunology, University Medical Center Utrecht, Utrecht University, Lundlaan 6, 3584, EA, Utrecht, the Netherlands

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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.

* Corresponding author at: Center for Molecular Medicine, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands.
E-mail address: e.kalkhoven@umcutrecht.nl (E. Kalkhoven).
[1] These authors contributed equally.

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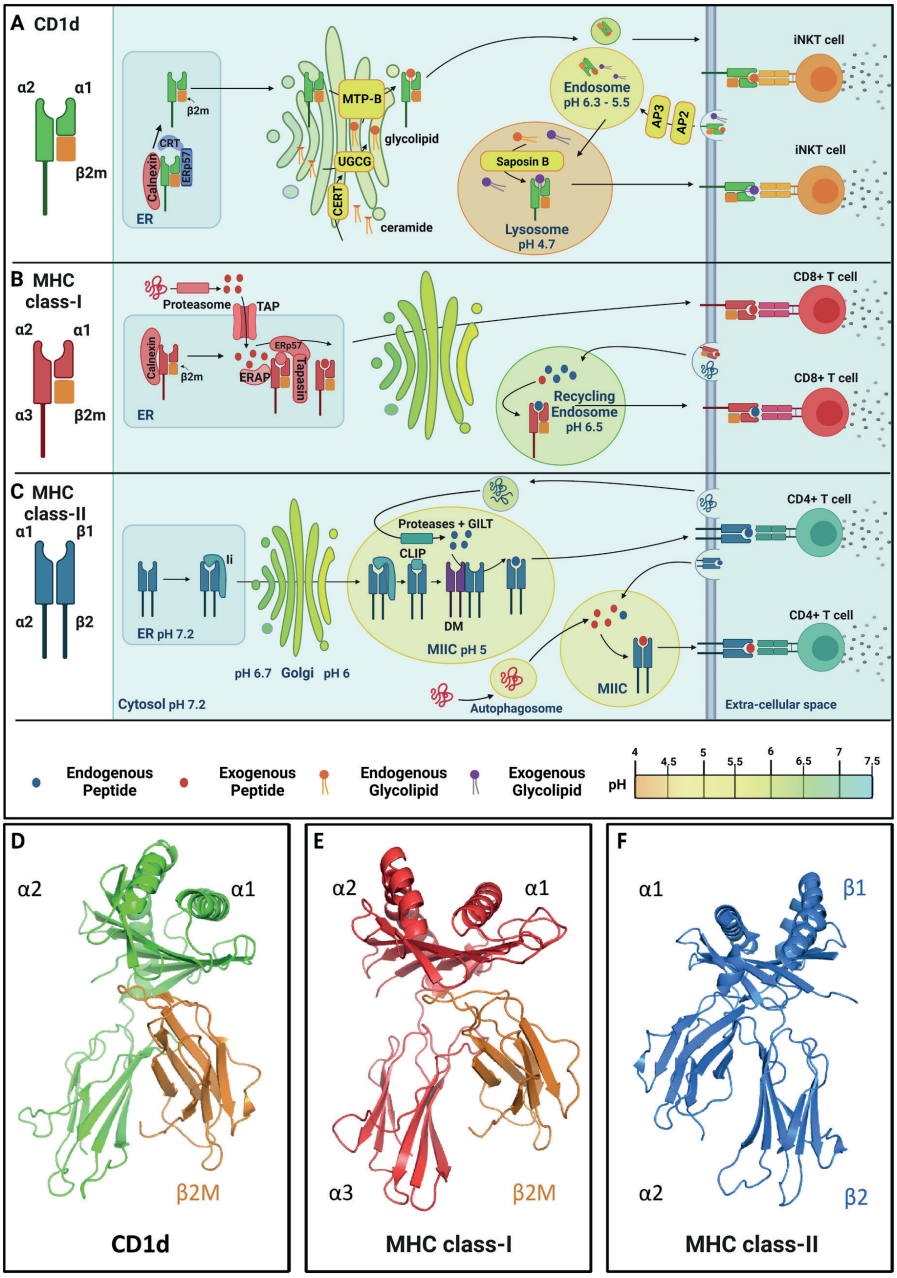

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Fig. 1. MHC class-I, MHC class-II, and CD1d loading and presentation pathways overview and comparison: A) CD1d is produced in the ER (pH 7.2), where Calnexin, CRT, and ERp57 enable b2M to stabilize the complex. CD1d then moves through the Golgi (pH 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

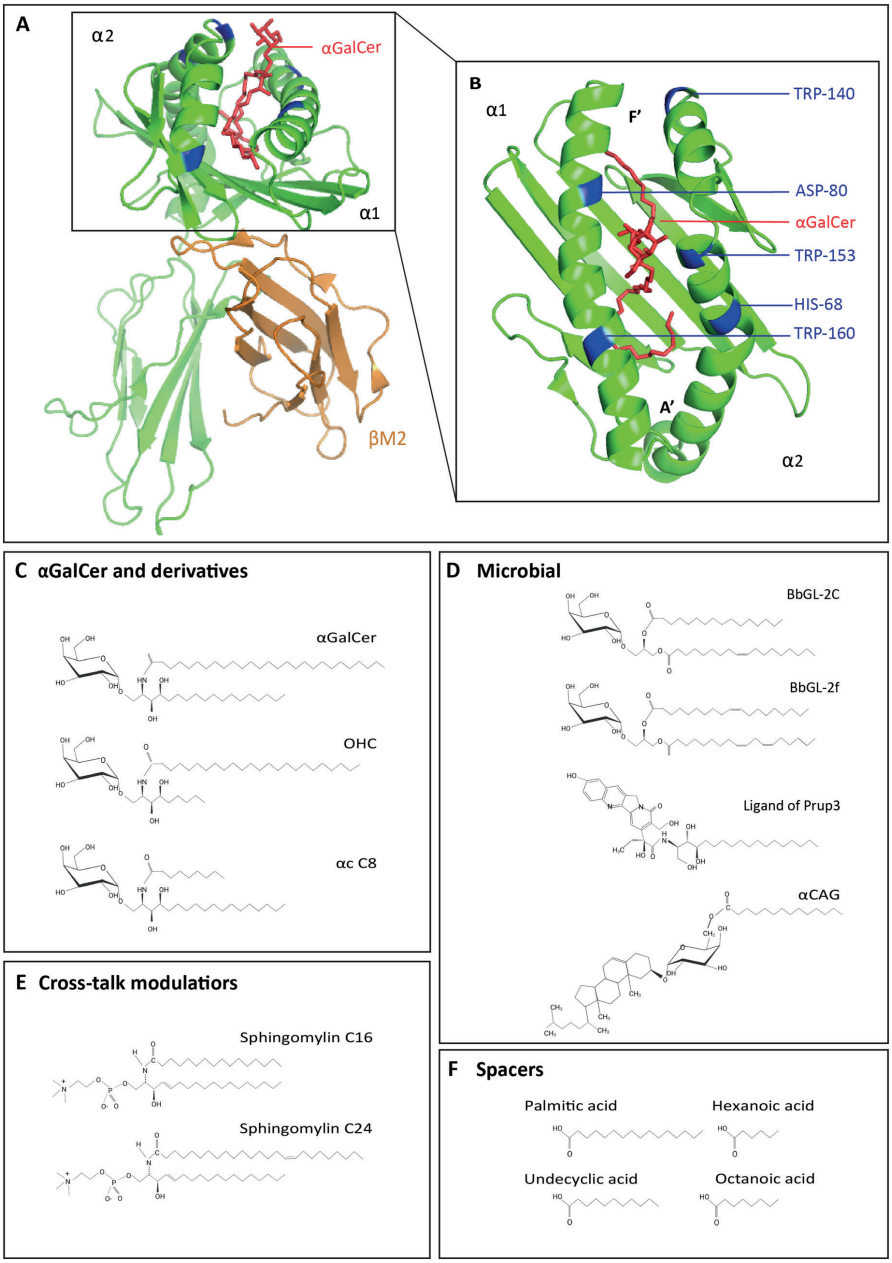


Fig. 2. Variations of CD1d antigens: A) Protein structure of CD1d loaded with aGalCer (red) B) Enlargement and top view of CD1d antigen portal pockets A' and F' loaded with aGalCer (red). Highlighted in blue are the residues, which play a role in the pH regulated loading of lipid antigens. Trp-140, Asp-80, 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

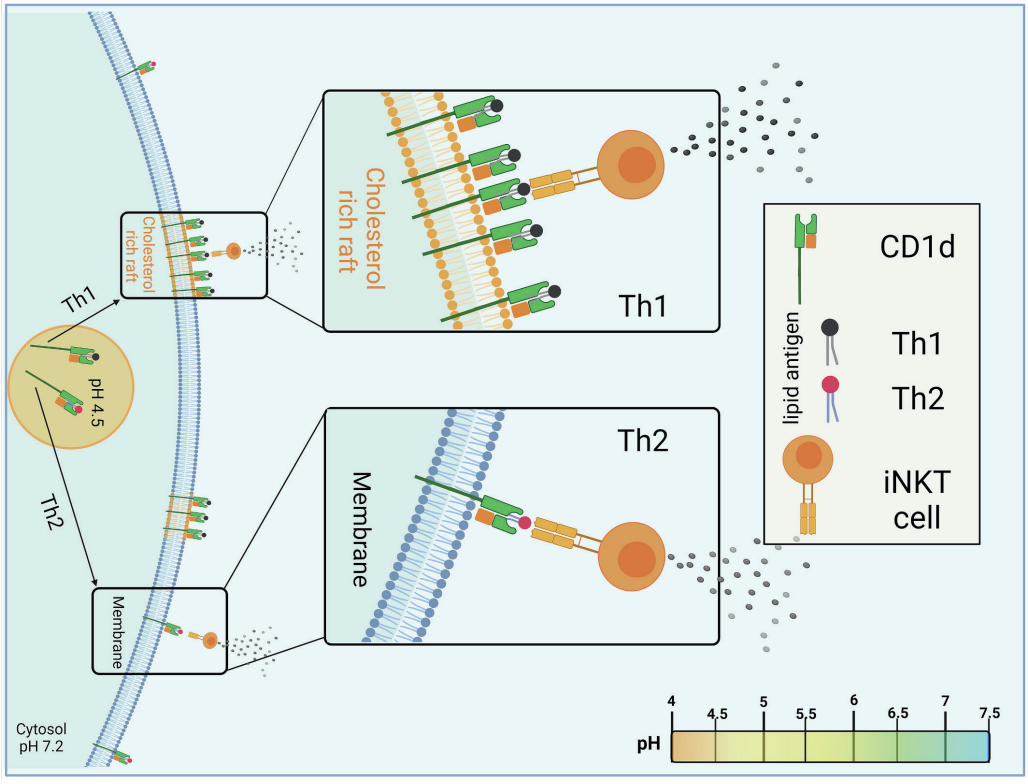


Fig. 3. pH regulated lipid antigen membrane distribution CD1d: The pH of the lysosome ensures that antigens associated with Th1 response following cross-talk with iNKT cells are transported to and presented at cholesterol rich membrane rafts clustering them together. Whereas antigens associated with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

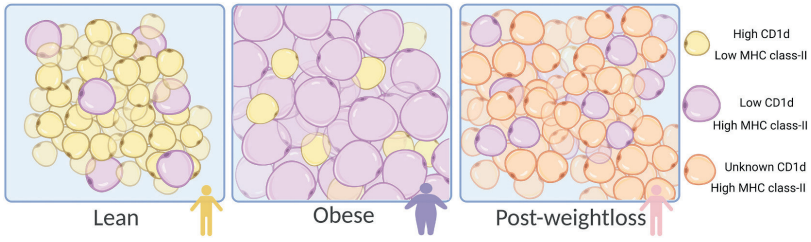
7. Concluding remarks

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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English Summary
Dutch Summary

English Summary

The intricate interplay between adipocytes and iNKT cells within the lipid-rich milieu of the adipose tissue affects the dynamics of cytokine production with high lipid environments contributing to pro-inflammation. Lipid droplets are among some of the most evolutionary conserved organelles, comprised of a monolayer of phospholipids containing a core of lipids, recent research has shown these humble droplets play many other essential roles aside from lipid storage. Lipid accumulation within lipid droplets shapes the inflammatory phenotype of adipose tissue-resident iNKT cells. Furthermore, regulatory mechanisms of lipidomes are modulated in part by the highly conserved pseudokinase Tribbles 3. Where it acts as a nutrient sensor to influence homeostasis on many levels, including; adipocyte lipid homeostasis, proliferation, and mitochondrial function, thereby shedding light on their interconnected response to environmental changes with available resources. The complex and often nuanced network of Tribbles 3 action is yet to be fully understood, its potential link to lipid metabolism and regulation of resource allocation provides an as of yet unexplored avenue into lipidomes highlighting it as a multipurpose biomarker. Within this thesis we utilized and developed several model systems allowing us to strategically address the different levels of complex interactions and mechanisms. In chapter 2 we use a mixture of FFA's mimicking the lipid-rich microenvironment of obesity in vitro, co-culturing these now insulin resistant (IR) adipocytes with iNKT cells demonstrated that the adipocyte IR phenotype causes a pro-inflammatory skew (IFNγ) in iNKT cell cytokine output, suggesting that cross-talk is an important target for further research. Co-culture assays with a iNKT hybridoma cell line (DN32.D3) skewed the cytokine output toward reduced IL-4 secretion and increased IFNg secretion. Importantly, co-culture assays of mature 3T3-L1 adipocytes with primary iNKT cells isolated from visceral AT showed a similar shift in cytokine output. Collectively, these data indicate that iNKT cells display considerable plasticity with respect to their cytokine output, which can be skewed toward a more pro-inflammatory profile in vitro by microenvironmental factors like fatty acids. Following this, in chapter 3, we hypothesized that AT-resident immune cells may also be detrimentally affected when exposed to a lipid-rich microenvironment. As Lipid droplets (LD's) are found in many immune cells perhaps the elevated lipid environment would cause dysfunction and pro-inflammatory cytokine secretion, in iNKT cells as well, creating a compound effect of dysfunctional communication between adipocytes and iNKT cells. We found iNKT cells are capable of taking up and storing lipids in droplets, even in "lean" conditions making them highly sensitive to their lipid environment as well as their lipid content. These findings highlight the importance of distinguishing between resident immune populations, as AT-resident iNKT cells respond differently to lipid environments compared to other

iNKT cells isolated from both liver and spleen. A lipid-rich microenvironment, as observed in obesity, skews this adipocyte-driven cytokine output towards a more inflammatory output. Whether a lipid rich microenvironment also affects iNKT cells directly, however, is unknown. Here, we show that primary mouse iNKT cells isolated from AT can accumulate lipids in lipid droplets (LDs), more so than liver- and spleen-resident iNKT cells. Furthermore, a lipid-rich microenvironment increased production of the proinflammatory cytokine IFNγ. Next to an indirect, adipocyte-mediated cue, iNKT cells can directly respond to environmental lipid changes, supporting a potential role as nutrient sensors. In chapter 4 and chapter 5 we explore the highly conserved pseudokinase Tribbles 3, showing the nuanced nature of Tribbles 3 interactions with the available local partners to deliver site specific action. Chapter 4 addresses the consequences of a global Tribbles 3 knock out, focusing on alterations –including the lipidome– in adipose tissue and adipocytes. Trib3KO mice display increased adiposity, but their insulin sensitivity remains unaltered. Trib3KO adipocytes are smaller and display higher Proliferating Cell Nuclear Antigen (PCNA) levels, indicating potential alterations in either i) proliferation-differentiation balance, ii) impaired expansion after cell division, or iii) an altered balance between lipid storage and release, or a combination thereof. Lipidome analyses suggest TRIB3 involvement in the latter two processes, as triglyceride storage is reduced and membrane composition, which can restrain cellular expansion, is altered. Integrated interactome, phosphoproteome and transcriptome analyses support a role for TRIB3 in all three cellular processes through multiple cellular pathways, including Mitogen Activated Protein Kinase-(MAPK/ERK), Protein Kinase A (PKA)-mediated signaling and Transcription Factor 7 like 2 (TCF7L2) and Beta Catenin-mediated gene expression. Our findings support TRIB3 playing multiple distinct regulatory roles in the cytoplasm, nucleus and mitochondria, ultimately controlling adipose tissue homeostasis, rather than affecting a single cellular pathway. We further investigated this in chapter 5 focusing on the change in abundance of mitochondrial specific lipid species Cardiolipins. Here we find that changes in the ultrastructure of mitochondria result in better stress tolerance under acute stress. Given its diverse interactions and implications in various diseases, understanding the interactions and functions in specific cellular contexts, such as Tribbles 3's role in adipose tissue lipidome modulation, offers potential avenues for targeted therapeutic interventions. The intricate interplay between local and global lipidomes and regulators or disruptors is an area of growing interest, as these conserved mechanisms appear to converge on crucial cellular processes. In chapter 5 we present our data which suggests that pseudokinase Tribbles 3 may participate in location specific lipid metabolism and lipid signaling pathways, modulating the lipidome and thereby influencing responses to external cues. We focused on Tribbles 3 action in the mitochondria and found a multifaceted regulation as it was found

to interact with membrane transport complexes TIMM8A and TIMM 13, complex 1 of the respiratory chain as well as having significant influence on the abundance of mitochondrial specific species cardiolipins, resulting in smaller overall mitochondria enhancing their to respond to acute stress. In chapter 6 part a we reflect on the available adipose tissue model systems and the limitations they pose, showing their usefulness in specific contexts depending on the research question. Finishing with chapter 6 part b, we review how lipidome species ratios may impact the local and global action of adipose resident iNKT cells. 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.

Dutch Summary

Lipiden zijn belangrijke biomoleculen die niet alleen een essentiele energiebron zijn en een essentieel onderdeel vormen van cellulaire structuren zoals celmembranen, maar ook van signaalroutes en voor de regulering van energieopslag en -gebruik, en zelfs dienen als antigenen. De opname, opslag en afgifte van lipiden, maar uiteraard ook hun biosynthese, onderlinge omzetting en afbraak, processen die samen kunnen worden aangeduid als 'lipid handling', zijn daarom allemaal sleutelelementen bij het reguleren van de dynamische wisselwerking tussen de verschillende biologische structuren en processen in het lichaam waarin lipiden een sleutelrol spelen. De term 'lipidoom' verwijst naar de volledige reeks lipiden die aanwezig is in een biologisch systeem, zoals een cel, weefsel of organisme, onder specifieke fysiologische of pathologische omstandigheden. Een celtype dat nauw verbonden is met lipiden is de vetcel of adipocyt, een gespecialiseerd celtype dat zich toelegt op de opslag en afgifte van lipiden. Adipocyten slaan lipiden op in zogenaamde 'lipidendruppeltjes' (LD's), evolutionair geconserveerde structuren bestaande uit een monolaag van fosfolipiden die een kern van lipiden bevatten. De optimale werking van adipocyten en vetweefsel draagt in belangrijke mate bij aan een optimale homeostase van de energie van het hele lichaam, en omgekeerd ligt disfunctie van de adipocyten en vetweefsel aan de basis van de met obesitas-geassocieerde ontregeling van de homeostase van de energie van het hele lichaam. De (dys)functie van adipocyten is gekoppeld aan de 'lipid handling' en het adipocyten lipidoom, maar ook aan de endocriene output van deze cellen via zogenaamde adipokines, hun wisselwerking met immuun cellen die zich in vetweefsel bevinden (vetweefsel-residente immuuncellen) en de combinatie daarvan.

Het samenspel tussen adipocyten en immuun cellen valt onder een relatief nieuw vakgebied genaamd 'Immunometabolisme'. Het samenspel tussen adipocyten en een bepaald type immuun cel, de 'invariant Natural Killer T cel' (iNKT cel) is een goed voorbeeld van wat 'Immunometabolisme' in kan houden. Eerder onderzoek heeft aangetoond dat deze iNKT cellen aanwezig zijn in vetweefsel en daar samenspelen met adipocyten om vetweefsel optimaal te laten functioneren en type 2 diabetes te voorkomen. Bovendien kunnen iNKT cellen direct communiceren met adipocyten via lipiden antigenen op het oppervalkte van de adipocyten; iNKT cellen kunnen als gevolg hiervan anti-inflammatoire of pro-inflammatoire cytokines uitscheiden. Of veranderingen in 'lipid handling' door adipocyten en het lipiden milieu buiten de cel–zoals beiden gezien worden in obees vetweefsel– resulteren in veranderingen in adipocyte-iNKT cell communicatie, was onbekend bij de start van dit onderzoek. Daarnaast was in eerder onderzoek een belangrijke rol gesuggereeerd voor een specifiek eiwit, het pseudokinase Tribbles 3, in 'lipid handling' door de adipocyt, maar waren de onderliggende mechanismes onbekend.

Binnen dit proefschrift hebben we verschillende modelsystemen gebruikt en ontwikkeld die ons in staat stellen om de verschillende niveaus van complexe interacties en mechanismen die samenhangen met bovenstaande vragen te onderzoeken. In hoofdstuk 2 gebruiken we een mengsel van vrije vetzuren die de lipidenrijke micro-omgeving van obesitas in vitro nabootsen. Door deze nu insuline-resistente (IR) adipocyten samen te kweken met iNKT cellen werd onderzocht of en hoe dit de communicatie en daarmee de cytokine output van iNKT cellen beïnvloedde. Co-kweektesten van 3T3-L1 adipocyten met een iNKT-hybridomacellijn (DN32.D3) lieten zien dat de cytokine output verschoof in de richting van verminderde IL-4 secretie en verhoogde IFNγ secretie, wat netto een meer inflammatoire output geeft. Belangrijk is dat co-kweektesten met primaire iNKT-cellen geïsoleerd uit visceraal vetweefsel (zogenaamde 'buikvet') een vergelijkbare verschuiving in cytokine output vertoonden. Gezamenlijk geven deze gegevens aan dat iNKT-cellen aanzienlijke plasticiteit vertonen met betrekking tot hun cytokine-output, dat in vitro kan worden verschoven naar een meer pro-inflammatoir profiel door micro-omgevingsfactoren zoals vetzuren, via een indirect, adipocyt-gemedieerd mechanisme. Hierna hebben we in hoofdstuk 3 de hypothese opgesteld dat AT-residente immuuncellen ook direct kunnen worden beïnvloed wanneer ze worden blootgesteld aan micro-omgevingsfactoren zoals vetzuren: omdat LD's in verschillende typen immuuncellen worden aangetroffen, zou de verhoogde lipiden-omgeving ook in iNKT-cellen mogelijk direct dysfunctie en pro-inflammatoire cytokinesecretie veroorzaken. We ontdekten dat iNKT-cellen in staat zijn lipiden in LD's op te slaan en dit zelfs doen zonder dat extra lipiden worden aangeboden, wat de suggestie wekt dat deze cellen zeer gevoelig zijn voor hun lipidenomgeving en hun lipidengehalte. Gezien werd dat primaire iNKT-cellen van muizen geïsoleerd uit vetweefsel beter lipiden kunnen accumuleren in LD's dan iNKT-cellen die in de lever en de milt verblijven, wat het belang benadrukt van het onderscheid maken tussen verschillende residente immuunpopulaties. Een lipiden-rijke micro-omgeving verhoogde direct de productie van het pro-inflammatoire cytokine IFNγ door iNKT cellen. Deze bevindingen laten zien dat naast een indirect, door adipocyten gemedieerd signaal (beschreven in hoofdstuk 2), iNKT-cellen ook direct kunnen reageren op lipiden veranderingen in hun omgeving, wat een potentiële rol voor deze cellen als voedingssensoren ondersteunt.

In hoofdstuk 4 en hoofdstuk 5 onderzoeken we het sterk geconserveerde pseudokinase Tribbles 3 (Trib3), waarbij we de interacties van Trib3 met andere eiwitten en de gevolgen daarvan op het metabolisme op cel- en organisme-niveau bestuderen. Hoofdstuk 4 behandelt de gevolgen van het uitschakelen van Tribbles 3 (Trib3KO) in de muis, waarbij de nadruk ligt op veranderingen – inclusief het lipidoom – in vetweefsel en adipocyten. Trib3KO-muizen hebben meer vetweefsel, maar hun insuline gevoeligheid

blijft onveranderd. Trib3KO-adipocyten zijn kleiner en vertonen hogere Proliferating Cell Nuclear Antigen (PCNA) niveaus, wat wijst op mogelijke veranderingen in ofwel i) proliferatie-differentiatie-evenwicht, ii) verminderde expansie na celdeling, of iii) een veranderd evenwicht tussen lipidenopslag en -afgifte, of een combinatie daarvan. Lipidoomanalyses suggereren dat Trib3 betrokken is bij de laatste twee processen, omdat de opslag van triglyceriden wordt verminderd en de membraansamenstelling, die de cellulaire expansie kan tegenhouden, wordt gewijzigd. Geïntegreerde interactoom-, fosfoproteoom- en transcriptoom-analyses ondersteunen een rol voor Trib3 in alle drie de cellulaire processen via meerdere cellulaire routes, waaronder door mitogeen geactiveerde proteïnekinase- (MAPK/ERK), proteïnekinase A (PKA)-gemedieerde signalering en transcriptiefactor 7 zoals 2 (TCF7L2) en door bèta-catenine gemedieerde genexpressie. Onze bevindingen ondersteunen dat Trib3 meerdere verschillende regulerende rollen speelt in het cytoplasma, de kern en de mitochondriën, waardoor uiteindelijk de homeostase van het vetweefsel wordt gecontroleerd, in plaats van dat Trib3 een enkele cellulaire route te beïnvloeden. We hebben dit verder onderzocht in hoofdstuk 5, waarbij we ons concentreerden op de veranderingen in mitochondriën ten gevolge van het verlies van Trib3. We zagen opvallende veranderingen in het lipidoom, inclusief mitochondrion-specifieke lipidensoorten zoals Cardiolipines, en kleinere mitochondriën met een verbeterd reactievermogen op acute stress. Bovendien vonden we interacties van Trib3 met verschillende mitochondriële eiwitten zoals membraantransport complexen TIMM8A en TIMM 13 en complex 1 van de ademhalingsketen. Onze data suggereren dat het pseudokinase Trib3 mogelijk betrokken is bij het locatie-specifieke lipidenmetabolisme –in dit geval de mitochondriën– en de lipiden-signaleringsroutes, waardoor het lipidoom wordt gemoduleerd en daardoor de reacties op externe signalen worden beïnvloed.

In hoofdstuk 6–deel a reflecteren we op de beschikbare vetweefsel modelsystemen en de beperkingen die deze met zich meebrengen, waarbij we hun bruikbaarheid aantonen in specifieke contexten, afhankelijk van de onderzoeksvraag. We eindigen met hoofdstuk 6–deel b, waarin we bekijken hoe de lipidoomsamenstelling de lokale en mondiale werking van iNKT-cellen in vetweefsel kan beïnvloeden. Door de reis van een lipide-antigeen te volgen, identificeren we vijf sleutelgebieden die het cytokine profiel kunnen dicteren: co-stimulatie, structurele eigenschappen van het lipide-antigeen, stabiliteit van lipide-antigeen/CD1d-complexen, intracellulaire en extracellulaire pH, en intracellulaire en extracellulaire lipiden omgeving. Recente publicaties geven aan dat de combinatie van geavanceerde omics-achtige benaderingen en machine learning een vruchtbare manier kan zijn om deze vijf gebieden met elkaar te verbinden, met als uiteindelijk doel nieuwe therapeutische routes te identificeren.

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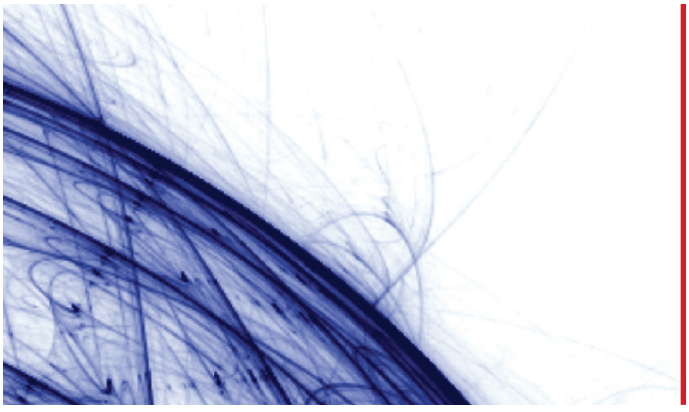
This thesis has been a long time in the making, and an even longer pipedream of mine. Inspiration can come in many forms, if you're reading this then you have been a lovely part of that for me. I would like to end on a quote I heard years ago, that still drives me now:
Mulder: When convention and science offer us no answers, might we not finally turn to the fantastic as a plausibility?
Scully: What I find fantastic is any notion that there are answers beyond the realm of science. The answers are there. You just have to know where to look.

p.s. if I have missed you here, come and find me, I owe you a drink!

Short Biography



Biography

Imogen Morris is a dedicated and inquisitive scientist known for her innovative and interdisciplinary research in obesity, metabolism, and immunology. Her academic and professional journey exemplifies thoughtful exploration and substantial contributions to her field.

Imogen began her academic career at the University of Manchester, earning a BSc in Developmental and Evolutionary Biology. Her undergraduate thesis, "Localisation of Laminin and GPSM2 in Pre-implantation Mouse Embryo," laid a strong foundation for her interest in cellular and molecular biology. She then pursued an MSc in Molecular Life Sciences at Wageningen University, focusing on ground tissue specification in Arabidopsis thaliana.

Following her MSc, Imogen held a fellowship at the Instituto Gulbenkian de Ciência, where she managed the Obesity Lab and animal colony. During this time, she developed advanced imaging techniques, including two-photon live intra-vital imaging and optoacoustic imaging, and optimized long-term culture methods for neuronal and immune cells. Her research on the peripheral sympathetic nervous system and blood-brain barrier integrity in obesity significantly advanced drug development and delivery.

As a PhD candidate at UMC Utrecht, Imogen worked in the Kalkhoven Lab as part of the Marie Curie ITN TRAIN consortium. Her research focused on the complex interplay between adipocytes and immune cells (invariant natural killer T cells) within lipid-rich environments, uncovering novel pathways essential for understanding the impact of adipose tissue physiology on immunometabolism. She specifically examined the highly conserved pseudokinase Tribbles 3 and its role in resource allocation within the adipose tissue lipidome.

Imogen's work has been widely recognized, with publications in prestigious journals such as Nature Medicine, Cell Metabolism, and Frontiers in Endocrinology. She has received numerous accolades, including the Marie Skłodowska-Curie Actions Fellow of the Week award. Her dedication to scientific excellence is matched by her personal interests in art, music, and travel, reflecting her creative and inquisitive nature.

Imogen Morris's scientific career is marked by her relentless pursuit of knowledge, innovative research approaches, and commitment to advancing our understanding of complex biological systems.