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Cholesterol in T cells; homeostasis, plasma membrane organization and signaling

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Abstract

The plasma membrane of eukaryotic cells contains cholesterol and glycosphingolipids enriched nanodomains known as lipid rafts; which are believed to resemble a liquid ordered (lo) phase in model membranes. Methyl-beta-cyclodextrin (MBCD) is used to deplete cellular cholesterol and a widespread assumption is that MBCD preferentially targets cholesterol in lipid rafts. To analyze this in T cells a progressive cholesterol extraction protocols was established. At 37ºC, MBCD treatment does not lead to the preferential loss of cholesterol from TX-DRMs. At 0ºC only 35% of total cholesterol could be extracted, demonstrating that less than 35% of the cell's cholesterol is found in the plasma membrane. Moreover, incubation of cells at 0ºC causes loss of plasma membrane cholesterol and an increase in cholesteryl esters. The increase in cholesterol esters upon cold exposure is linked to the cholesterol concentration induced activation of ACAT enzyme which converts cholesterol to cholesteryl esters. Cholesterol concentration specific activation of ACAT and conversion of cholesterol to cholesteryl esters during the loading of cholesterol onto T cells by MBCD was also observed. By using MBCD for progressive cholesterol depletion from T cells at 37ºC, the effect of cholesterol depletion on T cell signaling was addressed. At 10-20% cholesterol depletion levels, tyrosine phosphorylation is increased and ERK is activated. Peripheral actin polymerization, cell spreading and membrane protrusions are also triggered by limited cholesterol depletion. Upon limited cholesterol depletion aggregation of lipid rafts in the plasma membrane was observed. The aggregation of lipid rafts upon cholesterol depletion does not dependent on the signaling proteins such as Src-kinases. Upon cholesterol depletion there is an increase in overall plasma membrane order, indicative of more ordered domains forming at the expense of disordered domains.

This thesis is based on the following papers, which are referred in the text by their respective Roman numerical.

I. **Mahammad S** and Parmryd I. (2008)
Cholesterol homeostasis in T cells. Methyl-beta-cyclodextrin treatment results in equal loss of cholesterol from Triton X-100 soluble and insoluble fractions.
Biochimica et Biophysica Acta 1778 (2008) 1251–1258

II. **Mahammad S** and Parmryd I.
Cholesterol delivery from the plasma membrane to the ER is the rate limiting factor of Acyl-Coenzyme A acyltransferase activity *in vivo*

III. **Mahammad S**, Dinic J, Adler J and Parmryd I. (2010)
Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation
Biochimica et Biophysica Acta xxx (2010) xxx–xxx

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List of abbreviations

ACAT	acyl CoA: cholesterol acyl transferase
AFM	atomic force microscope/microscopy
APC	antigen presenting cell
BCR	B cell receptor
CD	cluster of differentiation
CHAPS	3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate
Cryo-EM	cryo electron microscopy
CT-B	cholera toxin B
DAG	diacyl glycerol
DHE	dehydroergosterol
DRMs	detergent resistant membranes
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
ERM	Ezrin-Radixin-Moesin
FACS	fluorescence activated cell sorting
FCS	fluorescence correlation spectroscopy
FLIM	fluorescence lifetime imaging microscopy
FRAP	fluorescence recovery after photo bleaching
FRET	fluorescent resonance energy transfer
GFP	green fluorescent protein
GPI-anchored proteins	glycosylphosphatidylinositol-anchored proteins
HDL	high density lipoprotein
HMG-CoA	hydroxymethyl glutaryl CoA
ICS	image correlation spectroscopy
IL-2	interleukin-2
ITAMs	immunoreceptor tyrosine based activating motifs
LAT	linker for activation of T cells

Lck	lymphocyte specific protein tyrosine kinase
LDL	low density lipoprotein
Ld-phase	liquid disordered phase
Lo-phase	liquid ordered phase
MAP kinase	mitogen activated protein kinase
MBCD	methyl beta cyclodextrin
MDCK cells	Madin-Darby canine kidney cells
MHC	major histocompatibility complex
NP-40	Nonidet P-40
NPC 1/2	Niemann-Pick Type C1/2
PIP2	phosphoinositide 4, 5 bis phosphate
PIP3	phosphatidylinositol (3, 4, 5) - trisphosphate
PMA	phorbol-12-Myristate-13-Acetate
RBL cells	Rat basophil leukaemia *(*RBL*)* cells
SCAP	SREBP/cleavage activating protein
SICM	scanning ion conductance microscopy
So-phase	solid ordered phase
SREBP	sterol regulatory element-binding protein
SSD	sterol sensing domain
TCR	T cell receptor
TIRFM	total internal reflection fluorescence microscopy
TNF	tumor necrosis factor
TX-100	Triton X-100
VLDL	very low density lipoproteins
WASP	Wiskott-Aldrich syndrome family protein
WAVE	WASP family Verprolin-homologous proteins
ZAP-70	zeta associated protein

Introduction

Eukaryotic cells contain a defined boundary known as the plasma membrane, which actively restricts access to the interior of the cell by the external environment. The plasma membrane plays a crucial role in exchange of metabolites into and out of the cells, creating pH gradients, charge differences and transduction of external signals. The plasma membrane is composed of lipids and proteins associated with non-covalent interactions. 30% of the total eukaryotic genome encoded proteins are membrane proteins and an even higher percentage of proteins at least spend some time at the plasma membrane. These plasma membrane proteins can be either transmembrane or peripheral membrane proteins and can have post translational modifications such as glycosylation, acylation and myristoylation and lipid anchorage.

The amphipathic lipid molecules due to hydrophobic forces in their fatty acid tail region, are organized in fluid bilayer to reduce the contact with water, as they contain hydrophilic head groups and hydrophobic tails. In order to reduce the exposure of their hydrophobic regions, the head groups organize very close to each other making a fluid bilayer. The lipid as well as the protein composition of the plasma membrane is different in the outer and inner leaflets of the membrane. Despite of different functions of the plasma membrane and different composition of lipids in the inner and the outer leaflet of the plasma membrane, all biological membranes have a common structure of a continuous bilayer of lipid molecules with hydrophilic external surfaces and a hydrophobic core in which membrane proteins are embedded. Over many years various studies have focused on to understanding the structure and the functional organization of the complex plasma membrane to address how cells interact with the outside environment, control molecular information and exchange it across the membrane. The first model of the phospholipid bilayer was proposed by Gorter and Grendel in 1925 (Gorter and Grendel, 1925). In 1935 Davson H and Danielli J proposed tri-laminar sandwich model for the cell membranes in which the phospholipid bilayer lay between two layers of globular proteins. The Davson and Danielli model was predominant until 1972.

The fluid mosaic model

In 1972 Singer and Nicolson proposed the concept of a fluid mosaic model for the gross organization and structure of the proteins and lipids of biological membranes (Singer and Nicolson, 1972).

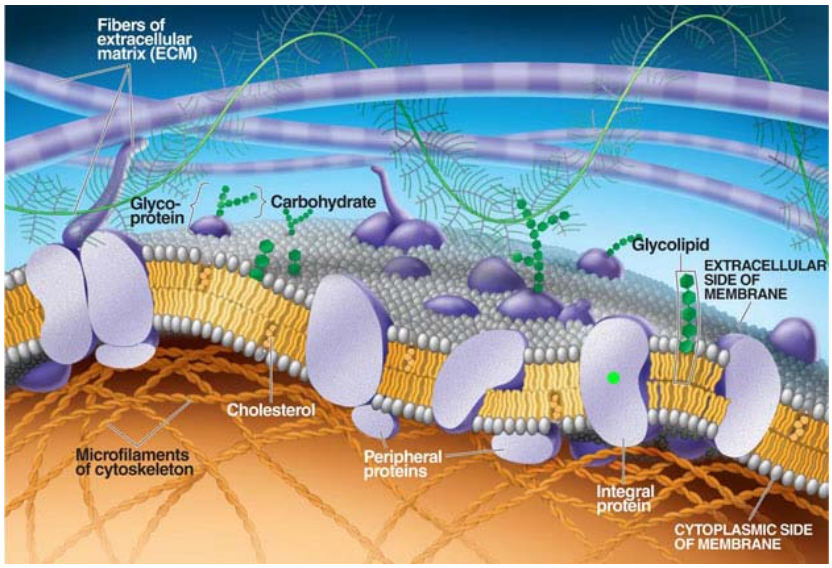


Fig 1. Fluid mosaic model of the plasma membrane

(Image from www.kentsimmons.uwinnipeg.ca/cm1504/Image127.gif)

In this model, the proteins that are integral to the membrane are a heterogeneous set of globular molecules, each arranged in an amphipathic structure, with the ionic and highly polar groups protruding from the membrane into the aqueous phase, and the nonpolar groups largely buried in the hydrophobic interior of the membrane. These globular molecules are partially embedded in a matrix of phospholipids. The bulk of the phospholipids are organized as a continuous, fluid bilayer, although a small fraction of the lipids may interact specifically with the membrane proteins. The fluid mosaic structure is therefore formally analogous to a two-dimensional oriented solution of lipids and proteins. Free diffusion of membrane constituents in the plane of phospholipid bilayer is the key concept in fluid mosaic model of the plasma membrane proposed by Singer and Nicolson.

Lipid structures in eukaryotic cells

During recent years many studies were carried out to understand the structure of the plasma membrane, with the aim to explain the complex organization of proteins and lipids. After many years in the shadow of protein and nucleic acid research, there has been a recent renewed interest in understanding the structure and dynamics of the lipids and lipid components of biological membranes (Simons and Ikonen, 1997). Mammalian membranes consist of a wide variety of lipids; it is known that these lipids are involved in an array of different functions apart from merely providing the structural matrix for embedded membrane proteins. So it is worth looking at the diverse lipid structures and their effect on membrane dynamics more closely.

Lipids in eukaryotic cells can be classified into three major classes: sterols, phospholipids/sphingolipids and glycerophospholipids.

Sterols

Sterols such as cholesterol and lanosterol and their derivatives are important components of membranes. The sterols are derived from the four-ring carbon core structures and are known to be involved in various biological functions such as hormone and cell signaling molecule.

Cholesterol

Cholesterol is an essential constituent in mammalian cells; it is the major sterol component of the plasma membrane and organelle membranes, except for the mitochondrial membranes. The levels of cholesterol vary among cellular organelles (Lange et al., 2004). Cholesterol can flip-flop between the two leaf-lets of the plasma membrane. Recent studies have shown that the half time for cholesterol flip-flop to be < 1 sec (Steck et al., 2002, Bennett et al., 2009), although earlier studies estimated longer time periods for cholesterol flip-flop movements (Leventis and Silvius, 2001, Backer and Dawidowicz, 1979). Along with the flip-flop movements' cholesterol can be rapidly exchanged among organelles by vesicular and nonvesicular transport mechanisms. The vesicular transport is mediated by membrane enclosed transport vesicles containing proteins (Maxfield and Mondal, 2006) and non vesicular transport is by direct movement of cholesterol either by membrane connections between organelles or mediated by transport proteins such as

sterol carrier proteins (SCPs). Cholesterol is very important for many biological processes; for instance it is involved in the formation of lipid rafts in cell membranes, the precursor for steroid hormones and the precursor for bile acids. Changes in cholesterol i.e., alterations in the levels of cholesterol can have profound effects on signal transduction and on membrane transport.

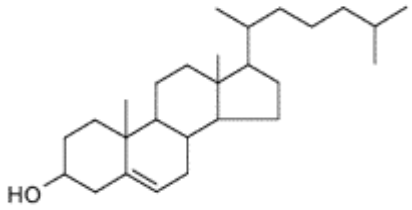


Fig: 2. Cholesterol

Phospholipids

Phospholipids are ubiquitous in nature and are the key components of the lipid bilayers in eukaryotic cells. The phospholipids are abundant in the mammalian membranes and have slow rates of spontaneous flip-flop movements ranging from 30 min to days (Tieleman and Marrink, 2006, Wimley and Thompson, 1990, De Kruijff and Van Zoelen, 1978). The phospholipids are also involved in metabolism and cell signaling.

Glycerophospholipids

Glycerophospholipids consist of diacylglycerol with typically two acyl chains of 16-18 carbon atoms, and a head group. Based on the nature of the head group glycerophospholipids are subdivided into distinct classes. Examples of glycerophosholipids found in biological membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). These phospholipids serve as primary components of the cellular membranes and phospholipids such as phosphatidylinositols (PI) and phosphatidic acids serve as membrane derived second messengers or precursors of second messengers.

Sphingolipids

Sphingolipids are a complex family of compounds that share a common structural feature known as the sphingoid base backbone. The sphingolipids are based on ceramide and the acyl chains are typically saturated with varying lengths between 16 to 26 carbon atoms. The sphingoid backbone is synthesized *de novo* from the amino acid serine and a long chain fatty acyl CoA with the help of serine palmitoyl transferase enzyme and then converted to ceramides. Ceramide is modified with phosphorylcholine to yield sphingomyelin or with sugars to yield glycosphingolipids (Futerman and Riezman, 2005, Sabourdy et al., 2008).

Glycosphingolipids display vast diversity in structure and can be classified into different series. Glycosphingolipids are essential for the development and survival of multicellular organisms. They play crucial roles in cell adhesion, regulation of membrane proteins, cell growth, survival and development (Kolter et al., 2000, Varki, 1993, Yang et al., 1996). On the cellular surface their expression varies depending on the cell ontogeny, development, viral transformation and oncogenesis (Hakomori, 1981). In the plasma membrane some of the glycosphingolipids (GM1, GM3 and GD1a) are known to reside in the lipid rafts.

Membrane phases, membrane order and fluidity

Lipids along with membrane proteins are the major structural components of the plasma membrane. Membrane lipids exist in different phases depending on their arrangements, composition and temperature, association with other lipids such as cholesterol and interactions with membrane proteins. A simple phospholipid bilayer below its melting temperature (Tm) forms a gel or solid phase (so) in which lipids are virtually immobile (diffusion coefficients ranging from 10^{-10} to 10^{-12} cm^2/s) with the fatty acid chains fully extended and packed. Above the melting temperature, the lipids are loosely packed and highly mobile in terms of translational motion as well as conformational order of the fatty acid chains (Miao et al., 2002, Vist and Davis, 1990) known as fluid phase. This fluid phase is referred as liquid disordered phase (ld).

In liquid ordered (lo) phases the lipids preferentially have saturated acyl side chains and are tightly packed with cholesterol. The acyl side chains of lipids are tightly packed and consequently there is a reduction in cross section area per lipid. In model membranes, proteins

such as GPI (glycosphingolipid)-anchored proteins and dyes such as DiI C-18 partition into the lo regions. Whereas the lipids with unsaturated acyl-side chains and most of the transmembrane proteins are excluded from lo phase (Lingwood et al., 2008). The thickness of the membrane decreases as the acyl side chains of the lipids become disordered (London and Brown, 2000).

In the ld phase, which is formed by the lipids of unsaturated acyl side chains, the molecules are less tightly packed due to the C=C double bonds in the side chains of the lipids. These liquid disordered regions of the plasma membrane are solubilized when treated with detergents. The existence of cholesterol, sphingolipid enriched lo and phospholipid liquid disordered ld has been demonstrated in ternary mixture of sphingomyelin (SM) : unsaturated PC : cholesterol with a wide range of compositions and temperature (Korlach et al., 1999, de Almeida et al., 2003).

Membrane compartmentalization of eukaryotic cells

Eukaryotic cells unlike prokaryotes are extensively subdivided into functionally distinct, membrane enclosed compartments known as organelles. These intracellular membrane systems provide functionally specialized aqueous spaces in the eukaryotic cells. This level of compartmentalization goes beyond organelle diversity, as a single organelle can be subdivided even further, for instance, early and late endosomes and the Golgi apparatus which is divided into *cis*, *trans* and medial Golgi.

Even in the same Golgi, domains form and incorporate certain molecules and exclude other molecules (Nickel et al., 2002); which is directly linked to the change in the lipid composition of the lipid bilayer, suggesting that the physical properties of lipids play a direct role in the functioning of the Golgi (Huttner and Schmidt, 2000). The lipid sorting of proteins and lipids in membrane bound organelles depends on the membrane curvature and phase separation of the membrane bilayers (Roux et al., 2005). The fluid mosaic model proposed by Singer and Nicolson could not explain the association of membrane proteins with lipids and the lateral diffusion of the membrane proteins. The fluid mosaic model of the plasma membrane did not attempt to explain the functional significance of proteins, their distribution in the membrane and the different sorts of membrane lipids, their distribution and organization.

The lipid raft hypothesis

Many studies were carried out with the aim to explain the complex organization of proteins and lipids in the plasma membrane and proposed various models. Of those models, the lipid raft hypothesis sheds lights on the lateral organization of lipids and proteins in the plasma membrane (Simons and Ikonen, 1997, Brown and London, 1998). During the studies of lipid sorting in polarized MDCK (Madin-Darby canine kidney) cells, it was proposed that glycosphingolipid clusters were formed within the exoplasmic leaflet of the Golgi membrane (Simons and van Meer, 1988); these microdomains were considered as the sorting centers for proteins and lipids. Similarly GPI-anchored proteins use the similar mechanisms for their sorting and lipid anchoring in these domains (Brown and Rose, 1992).

These early observation led to the proposal that glycosphingolipid rich domains function in protein sorting in epithelial cells and later studies have shown that their functions extended to various cellular mechanisms such as membrane organization, protein sorting and cell signaling (Simons and Toomre, 2000, Simons and Ikonen, 1997, Brown and London, 1998, Janes et al., 2000, Pizzo et al., 2004, Maxfield, 2002). According to the lipid raft hypothesis, the lateral assembly of cholesterol and sphingolipids creates glyco-sphingolipid rich regions in the plasma membrane often associated with dually acylated proteins which include specific sets of membrane proteins and lipids (Brown and London, 1998, Simons and Ikonen, 1997, Thompson and Tillack, 1985). Such a concentration and separation of membrane proteins by the interaction and tight packing of sphingolipids and cholesterol is the fundamental principle by which the membrane domains/lipid rafts exert their function.

Proteins	**Lipids**
GPI-anchored proteins	Cholesterol
Src-family kinases	GM1 ganglioside
Transmembrane adaptor proteins	PIP2 (Phosphoinositide 4, 5 bis phosphate)
GTP binding proteins	GD1a ganglioside
Receptor/co-receptor proteins	GM3 ganglioside
Adhesion receptors	

Table 1. Proteins and lipid associated with lipid rafts.

Lipid rafts are enriched in lipids and proteins that partition into lo phase. Due to their unique lipid composition, lipid rafts are resistant to solubilization at lower temperatures by some common non-ionic detergents like Triton X-100 (TX-100) and CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate). They float to the top of sucrose density centrifugation termed as detergent resistant membranes (DRMs) (Schuck et al., 2003). This makes them separable after detergent solubilization from solubilized membrane fractions by sucrose density centrifugation. Depending on the detergent type used, concentration of the detergent to cell ratio and the cell type the DRM fraction varies; for example, in paper I, using $90x10^6$cells/ml (T cells) lysed in 0.5% TX-100 revealed that a large proportion of cholesterol partitioning into DRMs, compared with $50x10^6$cells/ml (T cells) lysed in 1% TX-100. The distribution of cholesterol in different fractions of sucrose density centrifugation depends on the type of the detergent, concentration and detergent cell ratio. In paper I we have shown that the lipid rafts cholesterol comprised of 27% of the total cellular cholesterol and the remaining 73% cholesterol was distributed between intermediate and TX-soluble fractions. The lower TX to cell ratio, results in much larger portion of total cholesterol in the TX-DRMs (Gidwani et al., 2001, Rouquette-Jazdanian et al., 2006).

The evidence for the existence of phase separation behavior and domain formation in biological membranes is reported for decades (Jain and White, 1977, Thompson and Tillack, 1985). Membrane domains in eukaryotic cells were first reported in fibroblasts as detergent insoluble glycoprotein matrix rich domains (Carter and Hakomori, 1981). A variety of microscopic and spectroscopic studies have attempted to provide direct evidence for the formation of lipid rafts and about their size. In many cases, the studies have concluded that these nanodomains are of very small size and are dynamic structures. Many studies rely on demonstrating colocalization of specific lipid raft markers such as GM1 or GPI-anchored proteins on the cell surface and use crosslinking of these lipid raft molecules to determine the size of lipid rafts. These colocalization experiments using specific antibodies and expression of fluorescent tagged GPI-anchored protein experiments provided clues about lipid raft size, ranging from several nm to 700 nm (Pralle et al., 2000, Friedrichson and Kurzchalia, 1998, Varma and Mayor, 1998, Sharma et al., 2004, Eggeling et al., 2009). Upon the cross linking of various protein receptors or lipids on T cell membrane, these lipid domains become larger in diameter, form more stable structures and often colocalize with the actin filaments underneath the membrane (Harder et al., 1998, Janes et al.,

1999, Parmryd et al., 2003). The existence of lipid rafts in intact cells has been supported by various methodologies. For example, floatation of DRMs which indicates putative raft association and possibility of raft protein (Brown and London, 1998, Simons and Ikonen, 1997, Hooper, 1999). Similarly, antibody patching of one or more components of lipid rafts and immunofluorescence studies revealed that, patching of the raft components leads to their aggregation and formation of large scale domains in the plasma membrane leading to cell signaling (Harder et al., 1998, Janes et al., 1999). Immunoelectron microscopy (Fujimoto, 1996, Wilson et al., 2000, Kurzchalia and Parton, 1999) and chemical cross linking of lipid raft components (Friedrichson and Kurzchalia, 1998) revealed the location and associated proteins of lipid rafts.

At the same time, using new techniques such as single fluorophore tracking microscopy (Schutz et al., 2000), photonic force microscopy (Pralle et al., 2000), fluorescent resonance energy transfer (FRET) which monitor the diffusion and dynamics of individual raft proteins or lipids, determines the diffusion constant, size and dynamics of individual raft entities and detects whether two raft components are spatially close enough (less than 10nm) (Varma and Mayor, 1998, Kenworthy et al., 2000). Another useful approach is to manipulate raft constituents; for example cholesterol depletion or sphingolipid depletion. In paper III, we reported that moderate cholesterol depletion leads to the aggregation of lipid rafts in the plasma membrane. Signaling molecules concentrate in the aggregated domains leading to T cell activation upon cholesterol depletion.

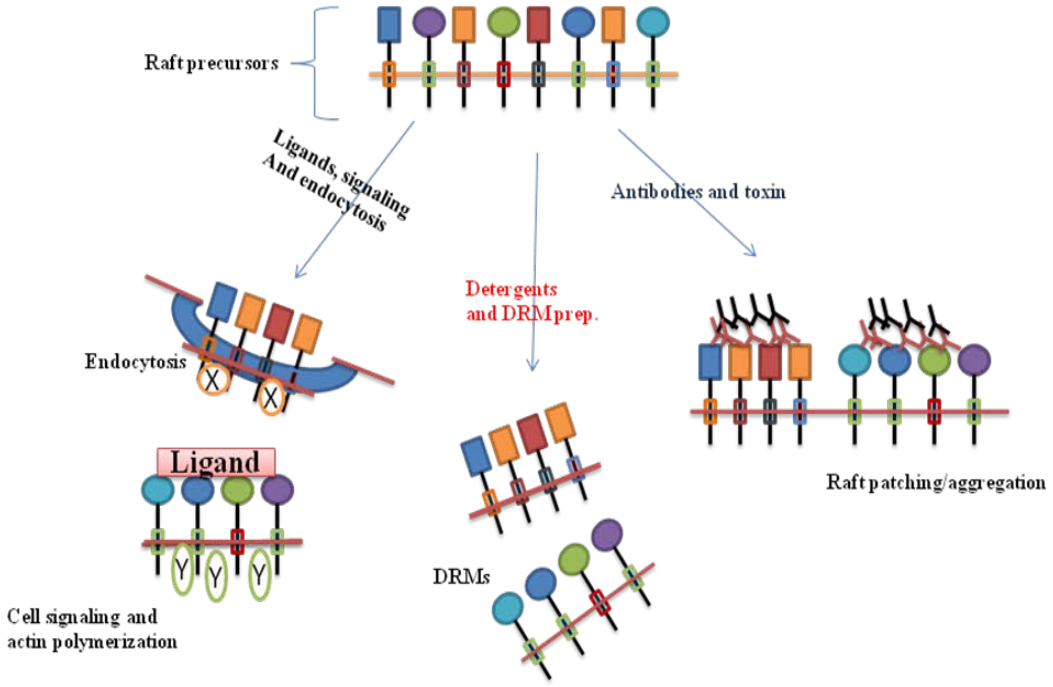


Fig. 3 Schematic diagram showing the raft precursors in the plasma membrane and different methods to study and purify lipid rafts.

Plasma membrane/lipid raft connections with actin cytoskeleton

One of the early descriptions of the lipid raft model is that the interactions between cholesterol and the sphingolipids generate a lipid platform within which specific proteins associate (Brown and London, 2000, Simons and Toomre, 2000, Simons and Ikonen, 1997). More and more studies suggest that the plasma membrane domains form through interaction and stabilization of the membrane lipids with the underlying actin cytoskeleton (Plowman et al., 2005, Plowman and Hancock, 2005, Tian et al., 2007). These findings suggest that the ordering of lipid rafts arises from ordering of lipids by lipid interacting proteins in the membrane rather than rafts occurring as preformed lipid complexes with which specific proteins interact.

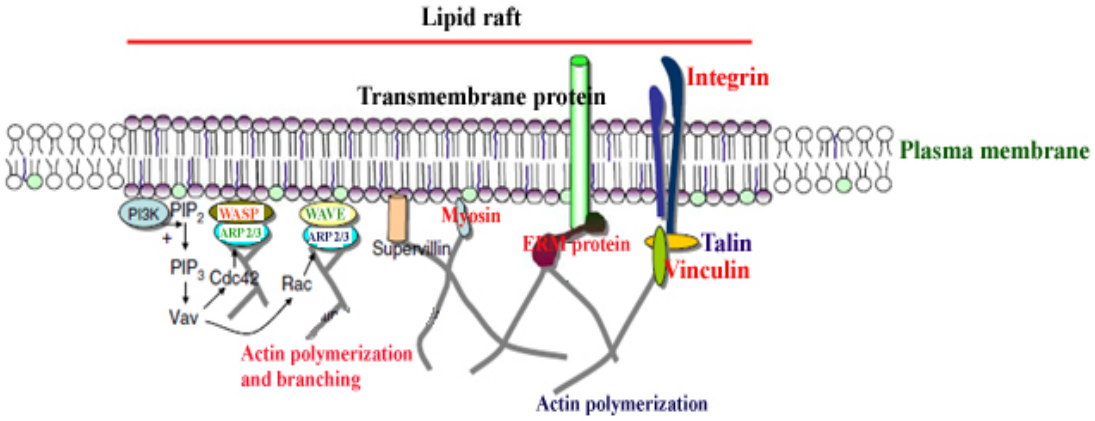
The protein actin, forms filaments that provide mechanical, structural support for the cell (Pfaendtner et al., 2010, Pollard and Cooper, 2009) and the actin cytoskeleton is a dynamic structure that plays a fundamental role in diverse processes in all eukaryotic cells. The structure and dynamics of the actin cytoskeleton are regulated by actin associated proteins (Saarikangas et al., 2010). Many studies have reported that plasma membrane rafts colocalize with actin

filaments, shown by cross-linking lipid raft associated proteins and co-staining for actin (Holowka et al., 2000, Rodgers and Zavzavadjian, 2001, Oliferenko et al., 1999). The plasma membrane with its inner leaflet proteins such as ezrin–radixin–moesin (ERM), myosin and talin (Hirao et al., 1996, Heiska et al., 1998, Blin et al., 2008) that interact with the actin filament system and cytosolic signaling proteins or downstream molecules. In RBL cells, cross linked IgE-FCεRI is associated with lipid rafts that recruit Src family kinases and these interactions are regulated by the actin filament system (Holowka et al., 2000). A link between raft associated proteins and the actin cytoskeleton could be explained by the studies on CD44, a transmembrane protein, which prefers lipid rafts, and interacts with actin cytoskeleton through its cytoplasmic domain. The cytoplasmic domain of CD44 interacts with ERM proteins which interacts with the actin cytoskeleton (Oliferenko et al., 1999).

Interactions between plasma membrane and the actin cytoskeleton suggest that the proteins and lipids that connect the actin filament system to the cell membrane are also important in the formation and stabilization of the membrane rafts. One of the important regulators of the membrane-cytoskeleton interactions is the PIP2 (Czech, 2000, Yin and Janmey, 2003, Varnai and Balla, 1998, Harlan et al., 1994, Lemon et al., 2003), which acts as the cofactor for many proteins that anchor actin filaments to the plasma membrane (Czech, 2000, Yin and Janmey, 2003). PIP2 is an important signal generating lipid that gives rise to inositols 1, 4, 5 triphosphate (IP3) and diacylglycerol (DAG) in response to agonist-dependent activation of PIP2 specific phospholipase C (PLC). The PIP2, IP3 and DAG are known to reside in lipid rafts moreover, PIP2 metabolism occurs in lipid rafts (Parmryd et al., 2003). PIP2 also can regulate the activity of numerous enzymes and a growing number of cytoskeletal proteins through direct interactions with pleckstrin homology domains and other PIP2 binding motif (Varnai and Balla, 1998, Lemon et al., 2003). Furthermore, ERM proteins are known to be regulated by PIP2. The ERM proteins anchor F actin to the membrane proteins which contain FERM-binding sequence (Hirao et al., 1996, Heiska et al., 1998, Blin et al., 2008, Zhang et al., 2005).

Along with PIP2, other molecules such as PIP3 (Phosphatidylinositol 3, 4, 5-trisphosphate) are also known to regulate the interactions between the plasma membrane and actin cytoskeleton. PIP3 is required for the activation of Rho family guanidine exchange factor Vav. The Rho GTPases activates actin polymerization via Cdc42 and Rac and in turn activating WASP (Wiskott-Aldrich syndrome family protein) and WASP family Verprolin-homologous proteins WAVE (Inabe et al., 2002, Bustelo, 2000, Higgs and Pollard, 2000, Rozelle et al., 2000, Lai et

al., 2008). These in turn activate the Arp 2/3 complex which induces F actin and bring about further actin polymerization and branching of actin filaments (Goley and Welch, 2006, Tseng and Wirtz, 2004).



Analysis of DRMs shows that lipid rafts are enriched with proteins and lipids that function in connecting the plasma membrane to the actin filament system or *vice versa*. One important example is PIP_2 which resides in the cytoplasmic leaflet of the plasma membrane. Another important lipid effector molecule is PIP3, which is involved in the activation of Rho-family GEF Vav. The Rho GTPases activate actin polymerization through WASP and WAVE. Other raft associated proteins that bind actin filaments are myosin-IIA and myosin IG. ERM proteins link transmembrane proteins such as adhesion receptor to the actin cytoskeleton.

Fig.4 The mechanisms of lipid raft association with the actin cytoskeleton. Fig adapted from (Chichili and Rodgers, 2009)

In summary, PIP2 act as second messenger that control cytoskeleton-membrane adhesion. Lipid rafts in most cell types are enriched with signaling proteins and function as specialized signaling platforms in the cell membranes. Recent data suggests that the actin cytoskeleton participates in regulating and activating the lipid raft associated signaling events; for example, the activity of G-protein coupled receptors, ERK and Src family kinases is tightly regulated by an intact actin cytoskeleton (Chichili and Rodgers, 2007, Sah et al., 2000, Pullikuth and Catling, 2007). In the similar way, crosslinking of lipid raft associated protein in T cell leads to the polymerization of actin filaments underneath the plasma membrane thus leading to increased plasma membrane order, large scale domain formation of lipid rafts in the plasma membrane associated with signaling proteins (Jelena D and Parmryd I; Manuscript).

During cell activation, lipid rafts in the plasma membrane forms aggregated domains and these domains segregate specific set of proteins. The formation of domains in the plasma membrane occurs before the signaling events pass from the cell membrane to the interior (paper III) but for the large scale aggregation actin polymerization underneath the plasma membrane is required. During this, the phosphatases such as CD45 will be excluded from the lipid rafts leading to the activation of signaling proteins and transduction of these signals into the cytoplasm leading to actin polymerization and then large scale domain formation. The interaction between the raft associated protein LAT and the actin cytoskeleton helps in the distribution of proteins during cell activation. i.e., actin filament bound signaling proteins helps in bringing these signaling proteins together. This leads to the signaling events pass on from cell membrane to the interior of the cell.

Lipid rafts, protein secretion and targeting

Early identification of lipid rafts using Triton X-100 revealed that the cholesterol/sphingolipid enriched structures of secretory pathway such as the ER, the *cis*-Golgi and in the *trans*-Golgi network purified TX-DRMs. This provided the earliest evidence that the membranes of these compartments have different lipid composition that would also influence the biochemical properties of lipid rafts occurring at these stages of early protein/lipid sorting (Brown and Rose, 1992, Mayor and Maxfield, 1995, Simons and Ikonen, 1997). In yeast, it has been shown that GPI anchored proteins that are selectively included in distinct vesicles budding from the ER, suggesting that raft associated sorting of proteins occurs at the ER (Muniz et al., 2001).

The mechanism of selective inclusion or transmembrane or apical or GPI anchored proteins into detergent resistant membrane domains during early steps of protein transport facilitates enhanced anterograde trafficking through secretory pathway. The association is required for an efficient exit of the proteins from ER or ER-Golgi compartments and anchoring domain mutants of GPI-anchored proteins associate loosely with lipid rafts are retained in ER for prolonged period of time reassuring the role of lipid rafts in protein secretary pathway (Hein et al., 2009).

Caveolae

Caveolae are flask shaped invaginations in the plasma membrane that were first observed by electron microscopy (Yamada, 1955). Ever since their discovery there were many studies to understand their structure, molecular markers, tissue distribution and their functional aspects. The cup shaped caveolae invaginations are rich in cholesterol and marker proteins called caveolins (caveolins 1-3) (Okamoto et al., 1998). These membrane structures are known to be involved in a variety of cellular processes which includes signal transduction pathways, membrane organization, endocytosis, protein trafficking and cholesterol homeostasis (Stan, 2002, Tuma and Hubbard, 2003). Caveolae are abundant in adipocytes, endothelial cell, fibroblasts and muscle cells. On the other hand neuronal cells and lymphocytes lack caveolae (Liu et al., 2002, Razani et al., 2002). The distribution of caveolins and GPI-anchored proteins is different and they reside in different domains (Fielding and Fielding, 2003) so there is no similarity between caveolae and lipid rafts other than the enriched cholesterol and appearing in the DRM fraction of the sucrose density centrifugation.

T cells do not express caveolins 1-3; the structural proteins required for the formation of caveolae. Absence of caveolae in T cells makes it possible to study the role of cholesterol in T cell signaling and cholesterol homeostasis. Any changes in the membrane organization and cell signaling upon cholesterol depletion will be related to the involvement of lipid rafts. Most importantly to understand their role in cell signaling, T cells are ideal model to study as most of the signaling proteins in T cells are associated with lipid rafts and during activation T cells form large scale membrane regions with aggregated signaling proteins.

Isolation and purification of detergent resistant membrane domains

One operational definition of lipid rafts is their insolubility by non-ionic detergent at low temperatures resulting in their enrichment in DRMs. Due to their high lipid content, the DRMs float on sucrose density gradients. The resulting low density fraction is not only enriched in cholesterol but also many signaling proteins (Chang et al., 1994, Lisanti et al., 1994). A variety of detergents are, used to isolate these domains like TX-100 (Chung et al., 2000) and CHAPS (Ilangumaran et al., 1999) which are non-ionic detergents, Brij-98 (Drevot et al., 2002) and Brij -

58 (Bohuslav et al., 1993) which are non-ionic surfactants. However these DRMs may or may not represent lipid rafts *in vivo* and the content of DRMs differs depending on the detergent type, its concentration and the detergent to cell ratio (Schuck et al., 2003, Madore et al., 1999, Drevot et al., 2002, Ilangumaran et al., 1999). Purified DRMs do not exactly represent lipid rafts as DRMs are the result of detergent resistant regions of all membrane structures of the cell, not just the plasma membrane (Lichtenberg et al., 2005).

Out of the non-ionic detergents, only TX-100 was shown to produce TX-DRMs enriched in lipid raft markers and devoid of non raft markers (Brown and Rose, 1992, Hope and Pike, 1996). Milder detergents such as Brij-58 and Brij-98 produce not only a higher yield of buoyant DRMs, containing a majority of cellular GPI-anchored proteins, transmembrane adaptors (for example. LAT) and Src-family kinases, but also yield several atypical proteins. At the same time, using milder detergent such as Brij-58 and Brij-98 or using lower concentrations of TX-100 to high cell number, leads to higher accumulation of cholesterol in DRMs compared to 1% TX-100 treatments. In paper I we have reported that using $90x10^6$cells/ml (T cells) lysed in 0.5% TX-100 results a large proportion of cholesterol partitioning into DRMs compared $50x10^6$cells/ml (T cells) lysed in 1% TX-100.

Many researchers studying lipid rafts, after sucrose density centrifugation, collect only the TX-DRMs and the TX-soluble fraction, but not many studies have carefully analyzed each and every fraction of sucrose density centrifugation. We have analyzed the TX-DRM upper layer, the TX-DRM fraction, the Intermediate fraction, the TX-soluble fraction and the pellet fraction for the quantification of cholesterol and for the protein content (Delaguillaumie et al., 2004) (Paper I and paper III). To understand the role of proteins or lipids associated with lipid rafts it is important to use appropriate solubilization conditions and analyze each fraction of the sucrose density centrifugation.

The amount of cholesterol in different fractions of sucrose density centrifugation varies depending on both detergent and detergent to cell ratio. Therefore, a conventional and acceptable method to prepare TX-DRMs is lysing the cells in a buffer containing 1% TX-100 and purifying DRMs (Heerklotz, 2002, Heerklotz et al., 2003). These TX-DRMs can be isolated by centrifuging for 17-18 hrs at 100000g in a 5-40% discontinuous sucrose density gradient. Then the TX-DRMs are collected between the sucrose concentration 5-30% and are enriched in cholesterol, glycosphingolipids (example GM1) and GPI-anchored proteins (Simons and Ikonen, 1997, Brown and Rose, 1992). The analysis of cholesterol distribution in various fractions of

sucrose density centrifugation after lysing the cells in a buffer containing 1% TX-100 revealed that lipid raft cholesterol comprised 27% of the total cellular cholesterol with the remaining 73% cholesterol distributed between the intermediate and TX-soluble fractions (Paper I).

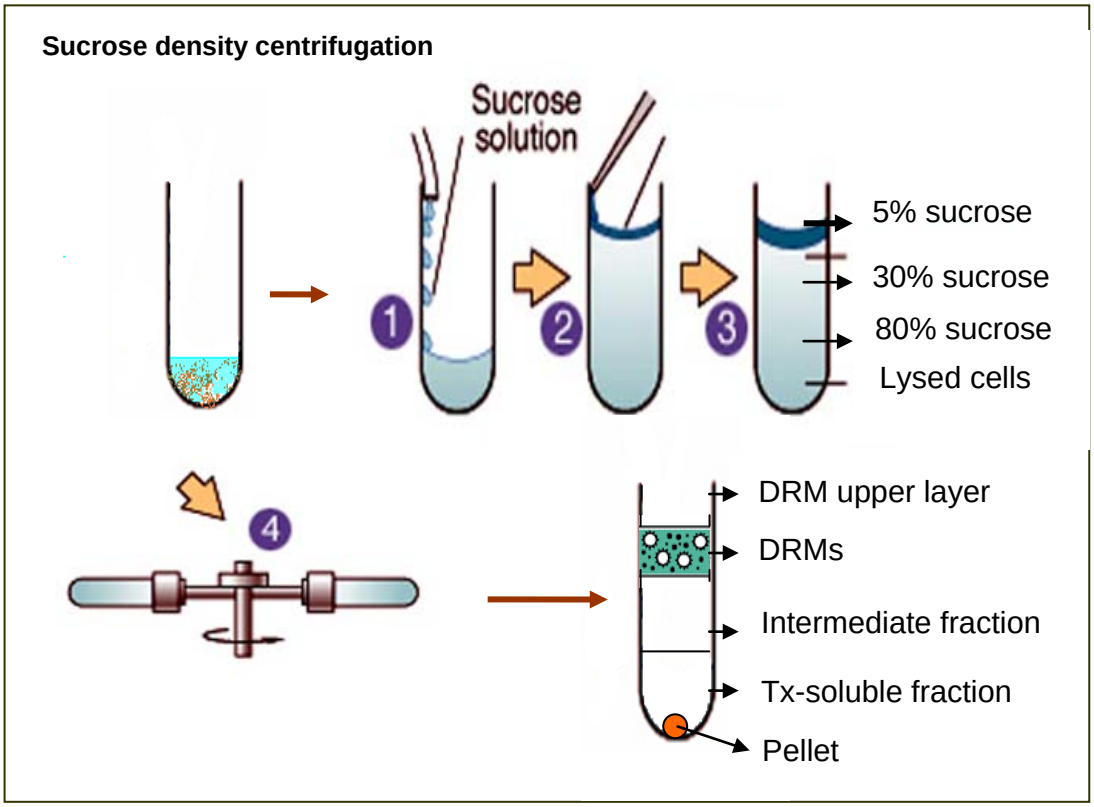


Fig.5 Sucrose density centrifugation

Studying lipid rafts and associated proteins by microscopy

Lipid rafts, of nanoscale range *in vivo* can be studied using fluorescence microscopy where these domains are aggregated after cross-linking of raft associated proteins or lipids, using fluorescent labeled antibodies and using order sensitive probes such as laurdan. Some common lipid-raft markers and microscopic methods used to study lipid domains/lipid rafts in live or fixed cells are summarized in the tables below.

Probe	Description
Antibodies	Fluorescent labeled antibodies against specific proteins (frequently indirect method)
CTB	GM1 binding fluorescent probe
DHE	cholesterol analogue (fluorescent probe)
Filipin	Cholesterol binding fluorescent probe
GPI-GFP	GPI anchored protein fluorescent construct
Laurdan	Order sensitive fluorescent probe
Lipophilic dyes	Fluorescent dyes which prefer either lo or ld phase

Table 2. Probes used to study lipid rafts

Microscopic method	**Primary observations**	**Comments**
AFM, atomic force microscopy	Membrane topography	Primarily for model systems, small height differences readily detected
Cryo-EM, cryo electron microscopy	Electron dense regions in the cellular sections	Sample preservation in its near-native hydrated state and high resolution information
FCS, fluorescence correlation spectroscopy	Fluorophore translational mobility& clustering	Highly sensitive to clustering; single and multiple colors
FLIM, fluorescence lifetime imaging microscopy	Spatial dependence of fluorophore lifetime	Relatively independent of probe density
FRAP, fluorescence recovery after photo bleaching	Fluorophore translational mobility	Well-established, ensemble-average method;
FRET, fluorescence resonance energy transfer	Donor-acceptor proximity	Cluster identification at nm scale; variety of detection methods
ICS, image correlation spectroscopy	Spatial dependence of fluorophore density	Image analysis method; characterizes lateral inhomogeneities
SICM, Scanning ion conductance microscopy	Topography of soft non-conducting surfaces	Can be used on living cells to understand the membrane topography
SPT, single particle tracking	Translational path of probe particle	Single particle sensitivity; detects domains as confinement zones
TIRFM, total internal reflection fluorescence microscopy	Evanescently excited fluorophores	Useful for substrate-proximal membranes; kinetic maps and membrane topology

Table 3. Different microscopic methods to study lipid rafts

Controversies in the field of lipid rafts

There are different numbers of studies that provide evidence for different types of nano-clusters and stabilized lipid rafts (Gomez-Mouton et al., 2001, Plowman et al., 2005, Alfalah et al., 2005, Hein et al., 2009, Castelletti et al., 2008, Madore et al., 1999, Roper et al., 2000, Wilson et al., 2004, Prior et al., 2003, Fujita et al., 2007, Karacsonyi et al., 2005). But there are many controversies in the field of lipid rafts; for example controversies about the lipid raft existence, their size and stability.

Lipid raft existence:

Most of the studies rely on the detergent extraction method of purifying DRMs and investigating the resistance to the different detergents, such as Triton X-100, Lubrol WX, Brij 58 and Tween 20. The method of detergent dependence to address he plasma membrane domains is a question of debate (Munro, 2003, Lichtenberg et al., 2005, Hancock, 2006) because, depending on the detergent type, its concentration, detergent to the cell ratio and the cell type used the amount and the type of proteins associated with DRMs vary. At the same time, during the detergent extraction and purification, the purified DRMs do not arise from the plasma membrane only, but also from all other membranes of the cell. Moreover, the use of detergent treatments for the purification of DRMs is questionable because the treatment of cells with detergents leads to the formation of domains (Heerklotz et al., 2003, Mayor and Maxfield, 1995, Lichtenberg et al., 2005, Heerklotz, 2002). The DRMs that are usually produced by detergent extraction method will eventually be dissolved completely if given enough time and excess detergent concentration (Heerklotz, 2002, Yu et al., 1973). So the detergent extraction method is not a direct approach of addressing lipid rafts in live cells. In the present study, 1% TX-100 is used to generate DRMs, as it is widely accepted that the use of 1% TX-100 leads to the purification of DRMs specifically containing proteins and lipids which are known to be associated with lipid rafts and exclude the non-raft proteins (Brown and Rose, 1992) (Paper I and paper III).

Lipid raft size and stability:

The significant uncertainty about the properties of lipid rafts such as their size, composition and stability in biological systems are mainly due to the difficulties in visualizing these dynamic entities *in vivo*. Various studies using different methods such as immunofluorescence, AFM, TIRF, cryo-EM and STED reported the size of the lipid rafts from few nanometers to 700 nm (Varma and Mayor, 1998, Eggeling et al., 2009, Pralle et al., 2000, Friedrichson and Kurzchalia, 1998, Sharma et al., 2004). The underlying mechanism for the formation of lipid rafts, their maintenance, stability and dynamics of lipid rafts is also a question of debate (Pike, 2009); for example, what is their size and how stable are these lipid domains in the plasma membrane at the physiological temperatures. At the same time, it is a question of debate that, how these nanodomains are important for the cellular functions such as plasma membrane organization and cell signaling (Munro, 2003) as these nanodomains are very dynamic and their formation and stability during various cellular functions is also a question of debate (Pike, 2009, Munro, 2003).
To answer these questions much more sophisticated techniques with higher resolution are needed. As biological imaging has undergone tremendous advancements in recent years and are now increasingly applied to identify and characterize lipid rafts in eukaryotic cells. The local organization of lipids seems to be determined by the summation of weak, non covalent interactions among lipids and proteins and individual components may be associated only transiently with nanodomains. Recently, dyes such as laurdan and DiI-4 have been widely applied to study lipid rafts and the plasma membrane order (Parasassi et al., 1994, Parasassi et al., 1991).
Therefore the spatial and temporal sensitivity of the applied methods are often critical factors also because raft domains can be very small and very dynamic (Lagerholm et al., 2005). Often applications require sample preparation and labeling or produce only indirect evidence.

T cell signaling

Over many years significant advances have been made in the field of lipid rafts and their role in cell signaling. In general, T lymphocyte activation is triggered by interaction of the TCR (T cell receptor) by antigens associated with the cognate major histocompatibility complex (MHC) molecules. The proximal events leading to the TCR signaling include activation of the Src-family kinases Lck and Fyn leading to phosphorylation of immunoreceptor tyrosine based

activation motifs (ITAMs) in the CD3ζ chain (Irving et al., 1993, Shores et al., 1997) which is associated with the TCR as well as the activation of zeta chain associated protein (ZAP-70) and Syk kinase. The substrates of ZAP-70 are LAT (linker for activation of T cells) and SLP-76. Phosphorylation of LAT and SLP-76 leads to recruitment of molecules for the activation Ras and calcium signaling pathways leading to T cell activation, proliferation and differentiation into effector cells.

Receptor mediated signaling in B and T cells provides information for understanding the interplay between lipid rafts, the actin cytoskeleton and cell signaling. One of the earliest signs of T cell activation is the compartmentalization of the TCR signaling to lipid rafts and the outcome of this is actin polymerization and actin driven clustering of lipid rafts in the plasma membrane (Chichili and Rodgers, 2009). Acute cholesterol depletion by methyl beta cyclodextrin or the depolymerization actin cytoskeleton (by Latranculin/cytochalasin B), inhibits the T cell activation, suggesting the importance of lipid rafts and signaling driven actin polymerization (Miceli et al., 2001, Wulfing et al., 2003). The lipid raft clustering following the TCR engagement and in combination with the actin polymerization leads to the formation of the immunological synapse; a region in which the T cell is in contact with the antigen presenting cell (Janes et al., 1999). Disruption of the immunological synapse inhibits the T cell activation, activation dependent cell proliferation and cytokine secretion (Montixi et al., 1998, Cemerski et al., 2008, Lee et al., 2002, Grakoui et al., 1999).

T cell activation requires a TCR to recognize cognate peptide antigen in the context of MHC molecule. In addition to this, for the effector signaling, T cell requires a second signal from co-stimulatory molecules such as CD28, LFA-1 and CD2 (Kiefer et al., 2002). In some cases, crosslinking of co-stimulatory molecules itself is needed for activation of T cells. CD28 is the principal co stimulatory receptor and its engagement is necessary to stimulate IL-2 production (Schneider et al., 1995). Biochemically CD28 has no intrinsic activity but rather functions as a linker protein that recruits specific effector molecules including PI3K, Lck, Itk and filamin A (Schneider et al., 1995, Tavano et al., 2006, Tavano et al., 2004, Holdorf et al., 1999). These effectors also activate actin polymerization or activate the binding of the plasma membrane proteins or lipids to the actin cytoskeleton. The co-stimulatory receptor CD28 provides signals that activate actin polymerization and lipid raft clustering (Kaga et al., 1998, Salazar-Fontana et al., 2003). Various proteins that are known to reside in lipid rafts in T cells are summarized in

the table below (Arcaro et al., 2001, Rodgers et al., 1994, Saint-Ruf et al., 2000, Foti et al., 2002, Zhang et al., 1998).

Protein(s)	Function	Example
GPI-anchored proteins	Either co-stimulatory molecules or receptors for specific molecules	CD48, CD52, CD55, CD59,CD90 and CD230
Src-family kinases	Involved in signal transduction	Lck and FYN
Receptor/co-receptor proteins	Involved in signal transduction	CD4, pre-TCR, TCR ζ chain and CD8β
Adhesion receptors	Involved in cell adhesion and signal transduction	CD44 and proteolipid MAL
Adaptor proteins	Involved in signal transduction	LAT and PAG

Table. 4 proteins with various functions associated with lipid rafts in T cells

Several acylated proteins involved in the early phase of the TCR signaling, such as Lck, Fyn (Resh, 1994), the adaptor protein LAT (Zhang et al., 1998), phosphoprotein associated with glycosphingolipid-enriched domains (PAG) (Brdicka et al., 2000) or Csk activating protein (CBP) (Kawabuchi et al., 2000) and Lck interacting molecule (LIME) (Hur et al., 2003, Brdickova et al., 2003) reside constitutively in the lipid rafts. This suggested an intimate link between the lipid rafts and immunoreceptor signaling. Indeed biochemical studies in mast cells, T cells and B cells revealed that crosslinking of the respective immunoreceptors (TCR, and BCR) results in an increased association of the clustered receptors with lipid rafts (Harder and Kuhn, 2000, Harder et al., 1998, Zech et al., 2009) or *de novo* formation of lipid rafts. The change in the clustering or de novo formation of lipid rafts might be due to the underlying attached actin filament system making these proteins associate with lipid rafts. In T cells, upon TCR cross-linking, the ITAMs in the cytoplasmic tail of immunoreceptor complexes (CD3ζ chain and immunoglobulin α, β chain) become exposed to Src-kinases that are present in lipid rafts, leading to the downstream signaling pathways. During resting conditions lipid rafts are very small, accommodating specific proteins and excluding others; this helps regulation of cell

signaling. During cell activation, the aggregated lipid rafts contain segregated signaling molecules (Lck, LAT, TCR and associated proteins) which lead to the cell activation. These aggregated lipid rafts specifically exclude the inactivating phosphatases so that the signal transduction proceeds. In this present study, during the T cell activation mediated by cholesterol depletion, the aggregated lipid rafts in the plasma membrane bring together the signaling molecules such as LAT and Lck compared to resting T cells. This indicates that the lipid rafts which are at nano-scale level in resting T cells, aggregate and form large scale domain concentrated with signaling molecules upon cell activation (Paper III).

During TCR signaling the phosphorylated adaptor protein LAT binds to several cytoplasmic SH2 domain containing molecules such as Grb2 and Gads, and indirectly Sos1, SLP-76, Vav and Itk. LAT interacts with a key enzyme, phospholipase c γ1, which cleaves another molecule; PIP2 (Parmryd et al., 2003). One of the products, DAG interacts with protein kinase C isoenzymes leading to downstream cell signaling. Another important aspect of TCR signaling is that the co-receptors CD4 and CD8 molecules are also palmitoylated and therefore associate with lipid rafts. Due to this, their co-engagement with the TCR after contact with the antigen presenting cell may lead to the association of the receptor complex with lipid rafts. Studies have revealed aggregation of the lipid domains in the plasma membrane and the recruitment of co-stimulatory signaling molecules into the aggregated domains, by patching the receptor molecules on the plasma membrane, using specific antibodies, or treating the T cells with the beads coated with antibodies against TCR or co-stimulatory molecules (Gaus et al., 2005, Harder et al., 1998, Zech et al., 2009, Harder and Kuhn, 2000). These studies have provided direct evidence, that during T cell activation, the TCR resides in distinct domains composed of lipid composition similar to the lo phase of model membranes. These TCR activation domains are also enriched in specific set of lipids coincided with the plasma membrane condensation at the sites of TCR activation (Zech et al., 2009).

Many studies have shown that T cell signaling is mediated by lipid raft aggregation or *vice versa*. Crosslinking of lipid raft associated proteins or sphingolipids leads to activation of various signaling pathways in T cells (Janes et al., 1999, Parmryd et al., 2003, Zech et al., 2009, Gaus et al., 2005). These findings show synergistic interactions between the TCR and the lipid rafts in T cell signaling. Lipid rafts are enriched in signaling proteins, modulators of cortical actin and with the factors that anchor actin filaments to the plasma membrane. In paper III, using PP2; a specific inhibitor of the activation of the Src-kinases, we have revealed that the formation of

aggregated domains in the plasma membrane occurs independently of the activation of Src-kinases. This result gives clues about the initial formation of lipid domains in the plasma membrane during T cell activation. The formation of the lipid domains in the plasma membrane occur, independent of the Src family kinases and may also be independent of actin polymerization (Paper III). But the interplay between the molecules which reside in lipid rafts and the actin filament system leads to the formation and stabilization of large scale domains in the plasma membrane. However, further studies are necessary to fully resolve the mechanism for the lipid raft dependent actin cytoskeletal changes and *vice versa.*

The role of cholesterol in lipid rafts

A widely used technique to study the role of lipid rafts in a cellular process is depleting the components that are enriched in lipid rafts by metabolic or chemical methods and checking the consequences of that, indirectly showing the importance of lipid rafts. A number of studies have examined the influence of cholesterol on the physical and functional properties of various proteins residing in lipid rafts using different methods to lower cellular cholesterol such as culturing cells in the absence of exogenous cholesterol (Esfahani et al., 1993), inhibition of cholesterol biosynthesis by statins (Taraboulos et al., 1995), oxidation of cholesterol (Smart et al., 1994) or by using cholesterol binding agents such as filipin, digitonin, saponin and cyclodextrin (Kabouridis et al., 2000, Nguyen and Taub, 2002, Niggli et al., 2004, Pike and Casey, 2002, Pizzo et al., 2004, Yang et al., 1996). These studies have shown that lowering cellular cholesterol markedly affected the properties of lipid raft associated proteins, and leads their dispersion on the cell surface (Rothberg et al., 1990), to decrease in their surface expression (Cerneus et al., 1993), to their release in membrane vesicles, to an increase in solubility in non ionic detergents (Draberova et al., 1996, Hannan and Edidin, 1996, Melkonian et al., 1995, Cerneus et al., 1993) and to an impairment of their signaling properties.

In paper III, we have shown that cholesterol depletion leads to T cell activation mediated by clustering of signaling proteins into lipid rafts in the plasma membrane and polymerization of actin filaments underneath the plasma membrane. The different ways in which cellular cholesterol levels were lowered reported to have different consequences on GPI anchored proteins and cell signaling, depending on the amount of cholesterol depleted and the secondary

effects of that on cell viability. For example, treatment of cells with high concentrations such as 10 mM-20 mM of MBCD leads to extraction of 70-80% cholesterol from the T cells, at these levels of cholesterol depletions, cell membranes leak and cells die. At these acute levels of cholesterol depletions, the cells do not signal and this has been interpreted as cholesterol depletion leading to inhibition of cell signaling (Rouquette-Jazdanian et al., 2006). In the present study, using lower concentrations of MBCD such as 2.5 mM-5 mM, revealed that this procedure extracts a maximum of 50% of cholesterol from T cells, at these levels of cholesterol depletions the cells remain viable and they restore cholesterol to the plasma membrane from internal pools (Paper I). Upon these moderate cholesterol depletions, activation of MAP kinase pathways, tyrosine phosphorylations of early signaling proteins, clustering of signaling proteins and aggregation of lipid rafts in the plasma membrane and polymerization of the actin filament system were observed (Paper III). Furthermore, in paper III, we have reproduced the cholesterol depletion conditions used by other studies (Xavier et al., 1998, Kabouridis et al., 2000, Pizzo et al., 2002) and shown that treating cells with high concentrations of MBCD to low cell densities leads to the extraction of more than 75% of cholesterol from T cells. Using these MBCD treatment conditions we have demonstrated that the cells die (paper III).

Cholesterol and its biology

Cholesterol is an important structural component of cellular membranes, myelin and the precursor of steroid hormones and bile acids. Cell gets cholesterol either by *de novo* synthesis or by the uptake from low density lipoproteins, which contains esterified cholesterol. In cells cholesterol content varies depending on the organelle and compartment. The ER, where the cholesterol is synthesized, contains 0.1-2% of cholesterol in mole% depending on cell type (Lange et al., 2004). The plasma membrane contains 30-40% cholesterol out of total lipids in mole%. The amount of cholesterol in different compartments varies depending on the cell type.

Compartment	% of cholesterol	Ref
Plasma membrane	30-95	(Liscum and Munn, 1999, Blanchette-Mackie, 2000, van Meer, 1989, Lange et al., 1989)
ER	0.1-2%	(Lange, 1991)
Endocytotic recycling compartment	Small fraction	(Lange, 1991, Lange et al., 1989, van Meer, 1989)
Trans Golgi network	Small fraction	(Lange et al., 1989, Lange, 1991, van Meer, 1989)

Table 5. Cholesterol content of various compartments in eukaryotic cells

Cholesterol biosynthesis

The ER is the primary site of cholesterol biosynthesis. The 27 carbon tetra cyclic compound is synthesized from acetate in a series of enzymatic reactions. The rate limiting enzyme of the pathway is hydroxymethyl glutaryl CoA reductase (HMG-CoA reductase), whose enzymatic activity is controlled by multiple mechanisms. These include regulation at the gene transcription level, the efficiency of mRNA translation, the rate of proteins synthesis, degradation and enzyme activity (Goldstein and Brown, 1990). This enzyme contains a conserved sterol sensing domain (SSD), which is important for its association with the ER. It interacts with the ER resident protein Insig; which binds to the cholesterol. The binding of HMG-CoA reductase to Insig leads to the degradation of HMG-CoA reductase and in turn the regulation of cholesterol biosynthesis.

Transcriptional regulation of cholesterol biosynthesis

The ER cholesterol levels are sensed by the cholesterol homeostatic machinery of ER embedded proteins. The cholesterol levels of the ER are very low and very minute changes in ER cholesterol levels leads to high fluctuations (Tabas, 2002). The transcriptional regulation of cholesterol biosynthesis occurs by feedback regulation in response to sterol levels and it is

mediated by two transcription factors SREBP1 and SREBP2; sterol regulatory element binding proteins. Usually, these transcription factors are associated with SCAP (SREBP/cleavage activating protein) forming a complex with Insig; a resident protein in the ER which binds to cholesterol (Goldstein et al., 2006). When the cholesterol levels are low, the Insig cannot bind to cholesterol, this leads to the release of SREBP/SCAP from the ER to the Golgi complex due to weaker interaction between SREBP/SCAP complex and Insig. The transcriptional activating domain of SREBP will be cleaved by S1p and S2p enzymes.

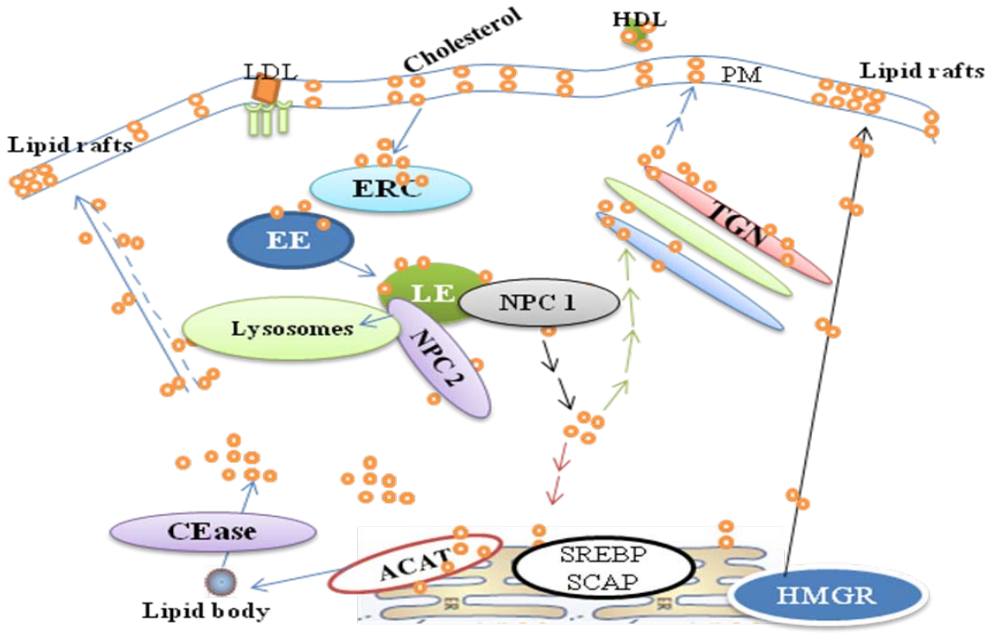
This leads to the translocation of the transcriptional activating domains of SREBP to the nucleus to activate the genes involved in cholesterol synthesis and uptake (Goldstein et al., 2006, Rawson, 2003). In parallel, HMG-CoA reductase and SCAP contain sterol sensing domain (SSD), and at high concentrations of cholesterol, both SCAP and HMG-CoA reductase interacts with cholesterol and will be retained in the ER, binding to Insig (Rawson, 2003). During the low levels of cholesterol due to the weaker interactions between SREBP/SCAP-Insig and HMG-CoA reductase-Insig, the SREBP/SCAP complex and HMG-CoA reductase will be released from the ER. Retention of SREBP/SCAP complex and HMG-CoA in the ER binding to Insig leads to the declined transcription of cholesterol synthesizing proteins and cholesterol biosynthesis (Sever et al., 2003). Other transcription factors that are involved in cholesterol biosynthesis or in the regulation of cholesterol biosynthesis are Liver X receptor (LXR) and peroxisome proliferating activated receptor (PPAR) (Chinetti et al., 2006).

Cholesterol transport

All nucleated cells synthesize cholesterol. Cholesterol, which is synthesized in the ER reaches the plasma membrane by both vesicular and non vesicular transport mechanisms. The vesicular transport is mediated by cholesterol transporting vesicles budding off from intracellular organelles or the plasma membrane. The non-vesicular transport is a very rapid mechanism and it is a direct transport mediated by the carrier (SCPs) from one region of cell to the other (Heino et al., 2000, Baumann et al., 2005, Maxfield and Mondal, 2006). Most of the cholesterol taken up by the cells is via receptor mediated internalization by lipoproteins. The core of lipoproteins is composed of triglycerides and cholesteryl esters. The LDLs (low density lipo-proteins) are internalized by clathrin mediated endocytosis into early endosomes and the receptors are

recycled to the membrane surface, while the LDL particle containing cholesteryl esters and triglycerides are subjected to lipolytic degradation in endosomes and lysosomes by the cholesterol ester hydrolases and acid lipases. The hydrolyzed cholesterol is used for various cellular processes such as maintaining membrane organization and cell signaling.

Cholesterol is re-cycled by both vesicular and non-vesicular transports from various compartments of cells to others. Endosomal cholesterol reaches the plasma membrane directly or via the Golgi apparatus or reaches the ER to regulate cholesterol homeostasis. The mechanisms and routes of cholesterol exit from endocytic circuits are not well understood. Two proteins, NPC1 (Niemann-Pick Type C1) and NPC2 (Niemann-Pick Type C2), are involved in endosomal recycling and trafficking of cholesterol (Chang et al., 2005). The NPC1 and NPC2 proteins contain a sterol sensing domain, which is required for the functioning of NPC1 and NPC2 (Karten et al., 2009, Storch and Xu, 2009) . NPC1 is majorly located in the late endosomal compartment but also transiently associated with lysosomes and the trans-Golgi network (Zhang et al., 2001). Whereas NPC2 is a soluble protein that can be secreted from the cells, it is also targeted to late endosomes (Naureckiene et al., 2000). The cholesterol carrier LDL binds to its cognate receptor on the plasma membrane internalizes and enters the endocytic compartments. The hydrolysis of cholesteryl esters to cholesterol by lipases occurs in the endocytic compartments and the cholesterol ends up in the late endosomes/lysosomes. The transport of cholesterol from late endosomes to various destinations in the cells occurs by NPC1 and NPC2 proteins. The defects in this process lead to the Neimann-pick disease (Karten et al., 2009, Storch and Xu, 2009) characterized by the defects in cholesterol storage and transport.



A model for intracellular cholesterol trafficking in a typical mammalian cell. Along with the *de novo* synthesis of cholesterol which occurs in the ER, cells take up cholesterol in the form of esters from LDL. These esters will be hydrolyzed by cholesterol esterases in the endosomes. From endosomes the free cholesterol is transported to various compartments by NPC1 and NPC2 proteins. The cholesterol after reaching to the ER will be converted to cholesteryl esters by the enzyme ACAT. The cholesteryl esters will be stored in lipid bodies. When cellular cholesterol levels are low, the sterol sensing domain containing SREBP will be released from the ER and the transcriptional activating domain of SREBP will be translocated to the nucleus inducing the transcription of the genes involved in cholesterol biosynthesis. During low levels of cholesterol in the cells, along with the transcription, the cholesteryl esters in the lipid bodies will be converted into cholesterol by cholesterol esterase. This cholesterol will be used for various cellular processes. At high levels of cellular cholesterol, the ACAT enzyme converts the cholesterol to cholesteryl esters and along with this, cell effluxes cholesterol via HDL receptors.

* In cells free cholesterol is toxic; it is always encapsulated by proteins (SCP) or transport vesicles.

Fig. 6 Schematic diagram showing cholesterol trafficking in a mammalian cell.

Cholesterol esterification and hydrolysis

Cholesterol can be esterified and stored in lipid droplets. The 3-OH group of cholesterol becomes fatty acylated to form cholesteryl esters; the enzyme responsible for the reaction is acyl CoA cholesterol acyl transferase (ACAT). This is an integral membrane protein of the ER (Chang et al., 1997). ACAT exists in two isoforms: ACAT-1 and ACAT-2, where the first one is expressed in many tissues with high expression levels in macrophages, adrenal glands and sebaceous glands. ACAT-2 has restricted tissue distribution; it is expressed in hepatocytes and enterocytes (Pramfalk et al., 2005). ACAT-1 produces cholesterol esters that are stored in lipid droplets, and ACAT-2 produces cholesteryl esters to be included in apolipoprotein B containing lipoproteins such as chlymicrons in enterocytes and VLDLs (very low density lipoproteins) in hepatocytes. Cellular cholesterol undergoes continuous esterification and hydrolysis, net breakdown of cholesterol esters takes place when the levels of cholesterol fall. Cholesteryl esters are breakdown by the enzyme cholesterol esterase. There are different esterases that differ between cell types (Momsen and Brockman, 1976, Tuhackova et al., 1980, Meyer, 1989).

Lipid droplets

The esterified cholesterol is stored in specialized organelles in the cell known as lipid droplets. Lipid droplets are also known as adiposomes or lipid bodies which are lipid rich organelles present in all organisms, although the number and size of lipid bodies differs in different cell types of the same organism (Bozza et al., 2007, Bozza et al., 2009, Martin and Parton, 2006). For example, white adipocytes contain numerous and larger sized lipid bodies compared to immune cells. Even though the lipid droplets are considered distinct membrane compartmentalized organelles, the biogenesis and formation of lipid droplets in not fully understood.

Lipid droplets will be formed very rapidly in response to elevated free cholesterol/fatty acid levels in the cell (Pol et al., 2004). When there is high availability of fatty acids in the medium provided by LDL particles, the cell take up these LDL associated fatty acids by LDL receptor. Upon internalization of LDL particles, the LDL associated fatty acids will be hydrolyzed in endosomes and then transported to the ER. At the ER the free cholesterol along with the *de novo*

synthesized cholesterol enters into the cellular pool which can be utilized in different processes such as membranes and lipid signaling.

The excess cholesterol will be converted to cholesteryl esters and stored in lipid droplets or effluxed from the cells via surface receptors (HDL) (Hill and McQueen, 1997). The cholesteryl esters stored in lipid droplets will be hydrolyzed to cholesterol and utilized for cellular processes whenever the cellular cholesterol levels fall. The levels of cholesterol, cholesteryl esters, and *de novo* synthesis of cholesterol and uptake of fatty acids by LDL, efflux of cholesterol by cell surface receptors are tightly regulated. One of the most acceptable models in eukaryotic lipid droplet formation is that these organelles originate from the ER, which can explain their organization as a core of neutral lipids surrounded by a phospholipid monolayer (Brown, 2001, Murphy, 2001, Tauchi-Sato et al., 2002) . Although, the lipid droplets are known to be associated with neutral lipid storage and transport, more and more evidence that are making clear that lipid droplets are highly regulated, dynamic and functionally active organelles (Bozza et al., 2009, Bozza and Bandeira-Melo, 2005, Bozza et al., 2007).

Unlike adipocytes, immune cells are not the primary sites for lipid storage. Nevertheless there has been increasing interest in understanding the formation and functional analysis of lipid droplets in immune cells especially lymphocytes. Findings in the field suggest the involvement of lipid droplets in a wide range of cellular functions in immune cells such as inflammation, key markers for leukocyte/lymphocyte activation and attractive targets for novel anti inflammatory therapies (Bozza et al., 2009, Bozza et al., 2007, Bozza and Bandeira-Melo, 2005). At the same time, the formation of lipid droplets is also a question of debate and recent reports suggesting the formation, size and number of lipid droplets is directly correlated to the immune cell signaling, activation and host defense (Bozza et al., 2007).

Cholesterol efflux and bile acid formation

Excess cholesterol from peripheral tissues is transported to the liver for excretion. This process is known as reverse cholesterol transport and is mediated by HDLs (Schmitz and Grandl, 2009, Rader, 2006, Lewis and Rader, 2005). These HDL proteins take the cholesterol from the plasma membranes and using the HDL associated enzyme LCAT (Kuivenhoven et al., 1997, Ng, 2004), esterifies the cholesterol and transport the esterified cholesterol back to the liver. The cholesterol

from peripheral tissues can be transferred to VLDLs and LDLs by the action of cholesteryl ester transfer protein (Apo-D) (Rassart et al., 2000); which is associated with HDLs (Oram and Yokoyama, 1996). By this mechanism the effluxed cholesterol from cells can be used by the LDLs for another round of transport. Cells in general cannot degrade cholesterol; however hepatocytes can excrete cholesterol in the form of bile. For this cholesterol has to be transported from extra hepatic tissues to the liver, which is done by HDLs (Fielding and Fielding, 1995). ABC transport proteins are involved in the mechanism by which cholesterol is secreted into bile, the heteromeric ABC transported ABCG5/G8 mediates the biliary cholesterol secretion at the apical membranes of hepatocytes (Graf et al., 2003, Yu et al., 2002).

Cholesterol homeostasis

Organisms must maintain the proper function of their membranes in response to various changes. Defects in cholesterol synthesis or metabolism lead to the change in the cholesterol levels in the cells (Liu et al., 2010, Martins et al., 2009). As cholesterol is the major player in the regulation of lipid organization, its cellular levels should be maintained and the cells have variety of mechanisms to accomplish this (Soccio and Breslow, 2004, Lange and Steck, 2008, Lange et al., 2004, Maxfield and Wustner, 2002, Maxfield and Mondal, 2006). Rapid transport of sterol by vesicular or non vesicular transport mechanisms leads to changes in cholesterol content in the membranes and organelles. A quick response to the increase in cholesterol levels is the esterification of cholesterol by ACAT (Chang et al., 1997) and storage of cholesteryl esters in lipid bodies. When required, cholesterol esters will be hydrolyzed and cholesterol released from the hydrolysis of esters will be used for the cellular processes. The activity of ACAT is regulated as a homeostatic sensor. ACAT is allosterically regulated by cholesterol levels and cholesterol loading of cells leads to rapid increase in the plasma membrane cholesterol (Tabas and Boykow, 1987, Spector et al., 1979, Doolittle and Chang, 1982, Suckling and Stange, 1985, Tabas, 2002). Most of these studies tested the role of cholesterol in regulating the ACAT activity *in vitro by* isolating the ACAT enzyme from the microsomes. In paper II, we have shown that cholesterol activates ACAT enzyme in a concentration dependent manner *in vivo* by looking at the levels of cholesteryl esters at various levels of cellular cholesterol manipulations.

Along with ACAT and cholesterol esterases, the family of ABC transporter proteins such as ABCA1, ABCG subfamily proteins and SR-B1protein play key roles in delivering cholesterol and lipids to apolipoproteins (Hassan et al., 2006, Oram and Lawn, 2001, Klein et al., 1999). The endosomal proteins NPC1 and NPC2 are crucial for the transport of cholesterol to various compartments in the cells (Naureckiene et al., 2000, Storch and Xu, 2009). Defects in the above mechanisms for cholesterol homeostasis leads to cholesterol mediated disorders (Karten et al., 2009, Liu et al., 2010, Zhang et al., 2001, Chang et al., 2005) characterized by the defects in cholesterol storage and transport.

Cholesterol depletion

Cholesterol depletion is widely used technique to study lipid rafts (Zidovetzki and Levitan, 2007, Christian et al., 1997), and their involvement in cellular processes (Matthews et al., 2003, Kabouridis et al., 2000, Levitan et al., 2000, Fujita et al., 2007). Cholesterol depletion by chemical or metabolic methods is used as a tool to manipulate the cholesterol content of the cells. Out of these methods; cholesterol depletion by cyclodextrin has advantages such as it is very quick and cell surface acting method. Metabolic depletion of cholesterol needs longer time of treatment with the inhibitors, as it takes long time to inhibit the *de novo* synthesis of cholesterol. Chemicals like filipin (Behnke et al., 1984) do not extract cholesterol from cells but form complexes with it, so that the cholesterol is immobilized. At the same time, to reduce cellular cholesterol content, cells can be grown in a medium devoid of lipoproteins, but this method do not inhibit the *de novo* synthesis of cholesterol. Depending on the method used and extent of cholesterol depletion results vary a lot.

Cyclodextrins

Cyclodextrins are the oligosaccharides, primarily degradation products of starch and contain α (1-4) linked D-glycopyranose units (Davis and Brewster, 2004, van de Manakker et al., 2009). Cyclodextrins are water soluble and they have been used as carriers for hydrophobic drugs, as they contain a hydrophobic cavity. The cyclodextrins exists as hexamers (α-CD), heptamers (β-CD) and octamers (γ- CD). The degree of polymerization and the number of glycopyranose units

in the ring structure of cyclodextrins, gives the size of the hydrophobic cavity and affinity for specific classes of compounds (Davis and Brewster, 2004, van de Manakker et al., 2009). The water solubility of the cyclodextrins also varies and it can be improved by modifications, such as methylation, hydroxyl propylation and sulfobutylether modifications (Davis and Brewster, 2004, van de Manakker et al., 2009). Of these various modifications, MBCD which has high water solubility, is widely used to deplete cholesterol from cells (Puglisi et al., 1995). α-cyclodextrins have the highest affinity to phospholipids and β-cyclodextrins have affinity towards cholesterol (Ohtani et al., 1989, Ohvo and Slotte, 1996). Depending on the cyclodextrin concentration, type of cells and number of cells there is wide a range of cholesterol depleted from cells. Some examples are given in table 3.

MBCD concentration	Cell type	Percentage of depletion	Temperature	Time of treatment	Ref
20 mM	Cos-7	50	37ºC	90 min	(Matthews et al., 2003)
10 mM	Mouse fibroblasts	100	37ºC	120 min	(Kilsdonk et al., 1995)
10 mM	Mast cells	60	37ºC	60 min	(Sheets et al., 1999)
5 mM	CHO	60	37ºC	120 min	(Romanenko et al., 2004)
2.5 mM	T-cells	40	37ºC	15 min	Paper I and III
0.5mM	T-cells	10	37ºC	15 min	Paper I

Table 6. Cholesterol extraction by different concentrations of MBCD.

The depletion of cholesterol by cyclodextrins depends on the temperature of treatment, cell density, cell type and the duration of the treatment. For example at 37ºC using 10-15 mM cyclodextrin for 15 min leads ≥70% depletion of cholesterol from T lymphocytes (Paper I). When T cells ($10x10^6$ cells/ml) treated with 2.5 mM cyclodextrin at 37ºC for 15 min leads to extraction of 40% cholesterol from cells. In T cells lower concentrations like 0.5 mM-3.0 mM of cyclodextrin treatment for 15 min can deplete 10-50% of cholesterol from T lymphocytes. After sensing low cholesterol levels the cells start cholesterol homeostatic mechanisms. At 0ºC higher concentrations like 30 mM -120 mM of cyclodextrin could deplete only 35% of cholesterol from T lymphocytes (Paper I).

The high affinity of MBCD towards cholesterol can be used not only to extract cholesterol but also to add cholesterol to the biological membrane by generating MBCD-cholesterol complexes. MBCD pre-loaded with cholesterol can deliver cholesterol to biological membranes and increase the membrane cholesterol level. Only a fraction of the cholesterol delivered by MBCD will be retained in the plasma membrane and most of the remaining loaded cholesterol ends up in the internal pools due to the cellular cholesterol homeostatic machinery (Paper II).

The MBCD-cholesterol complexes can be generated by mixing MBCD and cholesterol at different ratios. The ratio of the MBCD and cholesterol determines the functioning of MBCD either as cholesterol acceptor or cholesterol donor. At high MBCD/cholesterol ratio, MBCD works as the cholesterol acceptor and at low MBCD/Cholesterol ratio mixture works as cholesterol donor (Christian et al., 1997, Levitan et al., 2000). It is important to know that the MBCD treatment will have different effects depending on the treatment methods. At the same time MBCD can interact with non cholesterol membrane components in the cell; for example MBCD treatment, along with cholesterol depletion can also lead to the extraction of GM1 from the plasma membrane (Paper III).

Studies have demonstrated that when cells are treated with various MBCD concentrations, MBCD may interact with membrane phospholipids. Leventis and Silvius showed that higher concentrations of MBCD interacts with DPPC which is available on the outer leaflet of the plasma membrane (Leventis and Silvius, 2001). These studies open a new dimension of the artifacts of MBCD treatment. During treatments with high concentrations of MBCD, the cell membranes get leaky and the cell viability will be critical; this is a known non-specific effect of MBCD treatment other than cholesterol depletion (Paper III). So in order to confirm or reassure the effects seen upon MBCD treatment are actually due to the cholesterol depletion but not due to the secondary effects of MBCD (such as phospholipid or sphingomyelin extractions), there should be some control strategies. One such strategy for showing the involvement of cholesterol in a particular cellular function, is keeping the cellular cholesterol content at an equilibrium state using cyclodextrin. Cellular cholesterol can be kept in equilibrium by treating with MBCD-cholesterol complexes so that there will not be any net change in cholesterol content of cell.

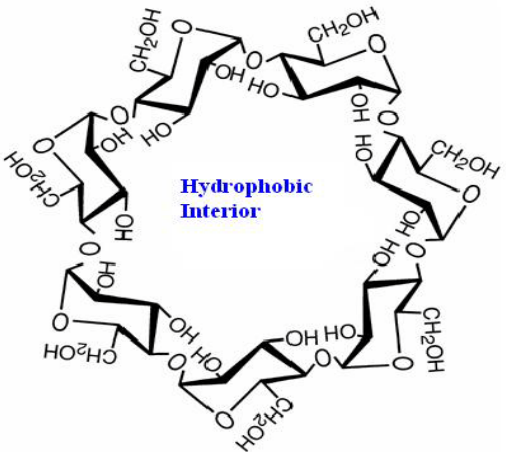


Fig 8. Cyclodextrin molecule

Many studies claim that using cyclodextrin for cholesterol depletion leads to lipid raft disruption and cyclodextrins are even referred as lipid raft inhibitors (Keller and Simons, 1998, Haglund et al., 2004, Allen et al., 2005, Raghu et al., 2007, Gaus et al., 2004) but recent studies with dehydroergosterol (DHE) have shown that cholesterol is distributed evenly in the plasma membrane (Wustner et al., 2005), and in the present study this topic has been studied systematically by using MBCD to deplete cholesterol in a controlled fashion; for example 10%, 20%, 30% 40% and 50%. In paper I, we have demonstrated that, MBCD treatment does not preferentially extract cholesterol from lipid rafts. The analysis of various fractions of sucrose density centrifugations made after cholesterol depletions from T cells revealed that the MBCD treatment leads to the reduction of cholesterol equally in different fractions of sucrose density centrifugation such as the TX-DRMs, the intermediate and the TX-soluble fraction (Paper I).

Cholesterol depletion and T cell signaling

Various studies have shown that cholesterol depletion leads both to the activation and inhibition of T cell signaling. Different studies show confusing results, where in some cases cholesterol depletion leads to activation of T cell signaling (Kabouridis et al., 2000). Using filipin to sequester cholesterol leads to MAP kinase pathway activation (Bolard, 1986) whereas in other cases cholesterol depletion inhibits T cell signaling (Rouquette-Jazdanian et al., 2006, Xavier et al., 1998, Kabouridis et al., 2000, Pizzo et al., 2002, Nazarov-Stoica et al., 2009). Cholesterol sequestration by filipin inhibits tyrosine phosphorylation of early signaling proteins by anti-CD3

antibodies (Xavier et al., 1998). These results vary, depending on the amount of cholesterol depleted and on the viability of the cells upon cholesterol depletion. In the paper III, we have shown that the limited cholesterol depletion by MBCD leads to the activation of T cell signaling mediated by aggregation of lipid rafts in the plasma membrane leading to the downstream signaling. In this study, MBCD is used to deplete cholesterol from T cells up to an extent where cells are still viable and signal. Unlike other studies (Xavier et al., 1998, Kabouridis et al., 2000, Pizzo et al., 2002) in which higher amounts of cholesterol was depleted from cells, due to which the cells get leaky and die. During this state of the cell, the cells may not respond to induce activation signals (Paper III). Studies have looked at the activation strategies of T cells by cross linking membrane receptors after cholesterol depletion, but not many have looked at the cell signaling by just removing cholesterol from cells. Some studies have looked at the effects on T cell signaling by cross linking membrane receptors after cholesterol depletion but not many studies have looked at the actual role of cholesterol depletion itself on T cell signaling. Understanding the role of cholesterol depletion in cell signaling, membrane organization and cholesterol homeostasis is much needed to elucidate the role of lipid rafts in those above mentioned processes. In this present study we have addressed the role of cholesterol in membrane organization, cell signaling and cholesterol homeostasis (Paper I, III and II).

Present study

In this present study we have established a method where cholesterol is depleted from T cells in a controlled fashion using MBCD. Using this method, we studied the role of cholesterol in different aspects of T cell biology.

The main categories of the present study were,

I. Cholesterol depletion and homeostasis in T lymphocytes. (Paper I)
II. Characterization of cholesterol substrate accessibility as limiting factor for ACAT activity in cells. (Paper II)
III. Effect of cholesterol depletion on T cell signaling. (Paper III)

Summary of the results

Paper I

Cholesterol homeostasis in T cells. Methyl-beta-cyclodextrin treatment results in equal loss of cholesterol from Triton X-100 soluble and insoluble fractions

MBCD is a widely used chemical to deplete cholesterol from cells. In order to better understand MBCD treatment and effects on membrane cholesterol and cholesterol homeostasis in T-cell, a progressive cholesterol depletion procedure was established using ^{3}[H]-cholesterol and MBCD treatment.

The major findings of this study were

1. *Cyclodextrin treatment at 37ºC results in equal loss of cholesterol from TX-DRMs and TX-soluble fractions.*

Many studies claim that MBCD extracts cholesterol only from lipid rafts; to analyze this, after cyclodextrin treatment, T cells were subjected to progressive cholesterol extraction followed by sucrose density centrifugation and analyzed for the reduction in cholesterol levels in each fraction such as the TX soluble, the Intermediate and the DRM fraction. This reveals that the cholesterol is lost equally from all fractions of sucrose density centrifugation fractions, i.e. cyclodextrin treatment at 37°C is not specific for DRM cholesterol.

2. *At 37ºC the plasma membrane cholesterol recovers very rapidly after MBCD treatment*

MBCD treatment extracts cholesterol from T cells, which can be assessed by filipin staining. As expected, the plasma membrane staining for filipin was reduced upon cholesterol depletion. Soon after the cyclodextrin treatment the plasma membrane recovers its cholesterol even though the medium did not contain any serum supplements (growth factors, glucose, fatty acids, amino acids, lipids and cholesterol). After 60 min of MBCD treatment 75% of recovery in membrane cholesterol was observed. This recovery was not inhibited by simvastatin, an inhibitor of cholesterol synthesis, but the recovery of cholesterol after 60 min was related to the decrease in the number of lipid bodies.

3. *At 0ºC MBCD treatment results in the loss of more cholesterol from TX-DRM fraction compared to other fractions of sucrose density centrifugation*

At 0°C the lipid rafts aggregate to form large clusters enriched in signaling molecules. To analyze the ability of cyclodextrin to extract cholesterol from these aggregated regions of plasma membrane, cells were treated with cyclodextrin at 0°C, lysed and subjected to sucrose density centrifugation. At 0°C cyclodextrin treatment results in reduction of more cholesterol from TX-DRMs compared to other fractions. The vesicular transport at lower temperatures is very low or limited at 0°C, due to this, the cholesterol from internal pools cannot be equilibrated to the plasma membrane leading to the extraction of cholesterol mostly from the lipid rafts in the plasma membrane.

4. *Incubating T cells at 0°C leads to the increase in the levels of cholesteryl esters and the number of lipid bodies.*

T cells incubated at 0°C have less plasma membrane cholesterol than the cells incubated at 37°C. The reduced filipin staining in the plasma membrane is related to the concomitant increase in the number of lipid bodies. This result indicates that upon cold induction, in order to maintain membrane fluidity, cells loose cholesterol from the plasma membrane. This plasma membrane cholesterol is converted to cholesteryl esters and stored in lipid bodies. This was confirmed by the results showing that the cells incubated at 0°C contain more lipid bodies compared to the cells at 37°C.

Paper II

Cholesterol delivery from the plasma membrane to the ER is the rate limiting factor of Acyl-Coenzyme A acyltransferase activity *in vivo*

The molecular activators of the ACAT enzyme and the actual mechanisms behind the activation of ACAT are not well understood, to address this question many studies have used the methods of culturing cells in the medium containing high concentrations of fatty acids or lipids thus inducing the formation of cholesteryl esters but in the present study the cells were incubated at 0°C to address the question. Incubating cells without any serum supplements at 0°C, leads to the formation of more lipid bodies and cholesteryl esters in Jurkat T cells, in mouse embryonic fibroblasts and in HeLa cells. During the cold incubation condition, the cells try to maintain their

membrane fluidity by reducing the levels of their plasma membrane cholesterol. This is related to concomitant increase in the levels of cholesteryl esters and to the number of lipid bodies. At the same time, to further study the involvement of ACAT in converting cholesterol to cholesteryl esters, cholesterol levels of T cells were lowered or increased by using MBCD and then their cholesteryl ester pools were analyzed. Thus addressing a very important question, sterol substrate limited activation of ACAT.

Specific findings of this study are

1. Cold induced effect on the order of the plasma membrane

The effect of temperature on the order of the plasma membrane was studied by using laurdan; a fluorescent dye that changes its emission depending on the order of the membrane. Upon cold induction the total plasma membrane order of Jurkat T cells was reduced, showing that a larger fraction of the plasma membrane was in a ld-like state upon cold induction, compared to the membrane order of the same cells at 37°C. The reduction in the membrane order at low temperatures may be due to a weakened interaction between the plasma membrane and underlying actin filament system.

2. Involvement of ACAT in cold induced cholesteryl ester formation

To understand the role ACAT on the plasma membrane cholesterol levels, control and Sandoz 58035 (an inhibitor for ACAT activity) treated cells were incubated at 37°C and 0°C. The filipin staining of cholesterol is estimated in these cells. The plasma membrane cholesterol staining was higher in Sandoz 58035 treated cells compared to control cells at 37°C indicating that the cholesterol could not be converted to cholesteryl esters. This leads to the increased amount of cholesterol in the plasma membrane. Upon cold induction the filipin intensity of cholesterol did not reduce in Sandoz 58035 treated cells, compared to control cells, showing that the inhibition of ACAT effects the conversion of plasma membrane cholesterol to cholesteryl esters. In control cells which were not pre treated with Sandoz 58035, the cholesteryl ester pool was increased upon cold induction, but the cells treated with Sandoz 58035 did not show increase in cholesteryl ester pool. This result shows that upon cold induction the increase in cholesteryl esters in related to the activation of ACAT.

At the same time, if the cholesterol is not converted to cholesteryl esters, the number of lipid bodies should not increase in Sandoz treated cells upon cold induction. To assess this, the control cells and Sandoz 58035 treated cells were incubated at 37°C and 0°C, fixed and stained with Nile red. As expected, the number of lipid bodies did not increase in Sandoz 58035 treated cells and surprisingly the Sandoz 58035 treated cells contained lower number of lipid bodies upon cold induction. These above mentioned results provide insights into the substrate threshold/concentration specific activation of ACAT in converting cholesterol to esters.

3. Activity of ACAT is limited by substrate availability

To further understand the cholesterol concentration specific activation of ACAT enzyme, Jurkat T cells were pre treated with MBCD to either load cholesterol or deplete cholesterol and then incubated at lower temperatures either in the presence or absence of Sandoz 58035. Upon cholesterol loading, in control cells, there is a clear increase in cholesterol esters at 37°C and at 0°C even higher % of cholesteryl esters have been observed compared to the control cells at 37°C and at 0°C. In Sandoz 58035 treated cells, even though same amount of cholesterol is loaded onto T cells by MBCD, no increase in cholesteryl esters is observed neither at 37°C or 0°C. These above mentioned results stress that the sterol concentration specific activation of ACAT in converting cholesterol to cholesteryl esters upon cold induction and cholesterol loading conditions. The levels of cholesteryl esters did not increase in the samples treated with MBCD to extract cholesterol. This result supports the earlier result of sterol substrate specific activation of ACAT in converting cholesterol to cholesteryl esters.

4. Membrane order of the plasma membrane in the presence of ACAT inhibitor

To understand further the role ACAT inhibition on the order of the plasma membrane at 37°C and 0°C, live cell imaging studies were performed using laurdan. In cells treated with Sandoz 58035, the order of the plasma membrane was observed to be very low compared to control cells both at 37°C and 0°C. This result shows that a larger fraction of the plasma membrane was in a ld-like state in cells treated with Sandoz 58035 although the cholesterol content in the plasma membranes is higher than the control cells. The possible mechanism for the observed low membrane order may be due to the weak or reduced interactions between the plasma membrane and underlying actin filament system.

Paper III

Limited cholesterol depletion causes aggregation of plasma membrane lipid rafts inducing T cell activation.

Cholesterol and lipid rafts are implicated in numerous signaling pathways, including T and B cell activation, neuronal signaling and apoptotic pathways. A number of studies have examined the influence of cholesterol on the physical and functional properties of signaling proteins in T cells. Many studies have used receptor cross-linking after cholesterol depletion to check the role of lipid rafts and their involvement in T cell signaling, but the effects of cholesterol depletion by itself on T cell membrane organization, signaling and on the actin filament system has not been done systematically. Addressing this would provide the direct answers in understanding role of cholesterol in the plasma membrane organization, lipid rafts and in cell signaling.

For this study, Jurkat T cells were treated with different concentrations of cyclodextrin for progressive cholesterol depletion. At each level of cholesterol depletion the cells were subjected to analysis to address the effect of cholesterol depletion on phosphorylation of early signaling proteins, the distribution of signaling proteins and lipids in the membrane and actin filament changes. Furthermore, to establish all the effects seen by MBCD treatment were actually due to cholesterol depletion and not by due to the nonspecific effects of MBCD treatment, cells were treated with MBCD-cholesterol complexes. During the treatments with MBCD-cholesterol complexes the cellular cholesterol content remained unchanged. This control strategy confirmed that the effects seen by MBCD treatment were actually due to cholesterol depletion.

Major findings of this study are

1. *Moderate cholesterol depletion initiates T-cell signaling events, tyrosine phosphorylation of early signaling proteins, phosphorylation of ERK1/2 and Ca^{+2} fluxes.*

Jurkat T cells were treated with MBCD to extract cholesterol in a progressive and controlled fashion. At each level of extraction cells were lysed and analyzed by Western blot for phosphorylation of signaling proteins by specific antibodies. Analysis of this shows moderate cholesterol depletion by cyclodextrin leads to activation of T cells mediated by the MAP

kinase/ERK pathway. The phosphorylation of early signaling proteins like Lck, LAT, ZAP-70 increases by mere cholesterol depletion and reaches maximum at 20% cholesterol depletion. Cholesterol loading did not have any effect on the ERK pathway activation and tyrosine phosphorylation of the early signaling proteins.

To see the effect of MBCD on Ca^{+2} signaling Jurkat T cells labeled with Flou-4, calcium sensing fluorescent dye and analyzed by FACS during MBCD treatment and this revealed that cholesterol depletion leads to a small flux of Ca^{+2} from internal pools.

2. *TX-DRM associated signaling proteins shift to the TX-soluble fraction after cholesterol depletion and glycosphingolipid GM1 is lost from T-cells.*

To understand the effect of cyclodextrin treatment on TX-DRM associated signaling proteins, cells were treated with MBCD and subjected to sucrose density centrifugation. This revealed that the signaling proteins such as Lck and LAT that are associated with lipid rafts shift from the TX-DRMs to the TX-soluble fraction upon cholesterol extraction, confirming previously reported results. The glycosphingolipid GM1 is a marker for lipid rafts. When cholesterol is depleted the GM1 molecule is lost from T-cells. When the MBCD treated cells stained with alexa 594-cholera toxin-B and analyzed by FACS for total intensity, a 27% reduction of total intensity of GM1 is observed. This might be due to non specific extraction by MBCD or GM1 shedding off as cholesterol is extracted by MBCD.

3. *Cholesterol depletion induces actin polymerization.*

The actin filament system is important for the maintenance of the cell structure. During cell activation actin filament rearrangements are necessary for signaling and morphological changes. To understand the effects of cholesterol depletion on the polymerization of actin, T cells, control or MBCD treated, were stained for filamentous actin by FITC-phalloidin. The analysis for actin intensity underneath the plasma membrane in control and MBCD treated cells for 10, 30% cholesterol depletions revealed that there is 41% and 36% increase in actin intensity upon cholesterol depletion. These results were confirmed by the spread cell morphology of the cells upon cholesterol depletion compared to the round control and cholesterol equilibrated cell morphology. These results further confirm that cholesterol depletion causes to T cell activation and actin polymerization leading to spread cell morphology. Along with this, upon cholesterol depletion, the formation of the membrane

protrusions containing actin filaments was observed. The control and cholesterol equilibrated cells show a round morphology with very few or no membrane protrusions.

4. Moderate cholesterol depletion leads to the aggregation of lipid rafts in the plasma membrane

Cholesterol depletion has obvious effects on membrane signaling proteins and their association with TX-DRMs and moderate cholesterol depletion leads to the activation of signaling pathways in T -cells. This might imply the clustering of signaling molecules in the plasma membrane leading to activation. To understand this GM1 distribution in the plasma membrane was analyzed and revealed that cholesterol depletion leads clustering of GM1 in the plasma membrane compared to the control cells as well as cholesterol equilibrated cells. This result shows the cellular activation by MBCD treatment is mediated by clustering of signaling molecules in the plasma membrane. By aggregation the signaling molecules cluster into large domains and cell signaling will be initiated. The aggregation of the GM1 molecules in the plasma membrane and cell spreading was not inhibited by PP2; an inhibitor for Src family kinases, before and during the MBCD treatment. This result indicates that the formation of aggregated domains in the plasma membrane during the cell activation occurs prior to the activation of Src-kinases.

5. Cholesterol depletion induces ordered domains in the membrane order in T cells

To further elucidate the role of cholesterol in T cell signaling and membrane order, Jurkat T cells were labeled with laurdan and observed live by fluorescent microscopy during MBCD treatment. The cell images were taken and analyzed for the plasma membrane order during the early and late phases of cholesterol extraction. This revealed the cholesterol depletion itself makes the plasma membrane more ordered, indicating that a larger fraction of the plasma membrane was in lo-like state. Similar to the results observed when membrane receptors are cross linked by specific antibodies leading to changes in the order of the plasma membrane and T cell activation. The membrane order of the cells treated with MBCD-cholesterol complexes remained unchanged. The change in the plasma membrane order is related to the polymerization of the actin filament system underneath the plasma membrane.

Final conclusions

In the present study, the role of membrane cholesterol in various aspects of the T cells such as membrane organization, lipid rafts, cell signaling and cholesterol homeostasis was addressed. For this T cells were treated with MBCD to deplete or load cholesterol on to T cells. Prior to our studies there was a widespread assumption that MBCD preferentially targets cholesterol in lipid rafts. To analyze this in T cells using MBCD a progressive cholesterol extraction protocols was established. We have demonstrated that MBCD does not preferentially extract cholesterol from lipid rafts.

At the same time, we have studied the role of cholesterol in substrate limited activation of the ACAT enzyme in T cells. For this we have established a method of incubating T cells at 0ºC in serum free medium. This method leads to the reduction of cholesterol in the plasma membrane and concomitant increase in cholesteryl esters and lipid bodies. Using MBCD to deplete or load cholesterol on to T cells we have demonstrated that ACAT activity is influenced by cholesterol in a substrate limited manner. Inhibitor of the ACAT enzyme by Sandoz 58035 has confirmed our results.

Cholesterol depletion is generally associated with both increased and abolished T cell signaling. In this study this anomaly has been investigated by analyzing Jurkat T cells for activation upon extraction cholesterol using MBCD. We have demonstrated that moderate cholesterol depletion leads to activation of T cell signaling correlated with the aggregation of lipid rafts in the plasma membrane, peripheral actin polymerization, cell spreading and membrane protrusions. We have shown that moderate cholesterol depletion leads to an increase in overall plasma membrane order.

In summary, the levels of cholesterol are tightly regulated in eukaryotic cells. The plasma membrane cholesterol plays crucial roles in maintaining the membrane organization; in the formation and stabilization of lipid rafts, in cell signaling and in cholesterol homeostasis in T cells.

Future directions

The molecular activators of the ACAT enzyme and the mechanisms behind the activation of ACAT are not well understood. From our studies in paper II, we have demonstrated that substrate limited activation of the ACAT enzyme by cholesterol. It is very intriguing to further study the exact mechanisms by which cholesterol does this activation of ACAT.

In my future directions, I would like to study the molecular mechanisms behind the role of cholesterol in activating ACAT in detail. To address this, I will use various microscopic and molecular biology methods.

Specific experiments

a. I will perform time course analysis of the intracellular localization of cholesterol by filipin staining in respect to the ER and ERC at various time points of the cholesterol loading or cholesterol depletion or cold induction conditions. These experiments would reveal the localization of the excess cholesterol which is either loaded on to the cells or released from the plasma membrane into the cell upon cold induction. This will help in understanding the intracellular localization and distribution of cholesterol at different time points of the experimental treatments.

b. I will perform Western blot analysis to study the localizations of the proteins involved in regulating cholesterol biosynthesis in cells (Radhakrishnan et al., 2008). I will treat the cells with MBCD to load cholesterol on to cells or deplete cholesterol and analyze to nuclear and cytosolic localizations of SREBP. Along with these experiments I will perform similar studies in cold induced cells. I will confirm the results of these experiments by immunofluorescence using confocal microscopy. These studies provide details about the levels of cholesterol to induce the translocation of SREBP to the nucleus and levels of cholesterol to retain SREBP in cytoplasm.

c. To confirm that cholesterol activates the ACAT enzyme, I will purify the ER compartment by density centrifugation methods after cholesterol loading or cholesterol depletion or cold induction to see the changes in cholesterol content of the ER after these

treatments (Radhakrishnan et al., 2008). Along with this, I will purify the ACAT enzyme residing in the ER compartment to study the kinetics of converting cholesterol to cholesteryl esters at different temperatures such as 37°C, 20°C and 0°C.

d. To understand the correlation between cellular cholesterol and sphingomyelin levels (Okwu et al., 1994), I will perform quantitative TLC assays for assessing the levels of sphingomyelin upon various treatments to alter cellular cholesterol content. Alterations in sphingomyelin levels upon cold induction or cholesterol loading or depletion will be very interesting and further studies will be done to deeper understanding the mechanisms behind.

e. In order to understand the role of cholesterol in activating ACAT, along with T cells, I will carry out these experiments in white adipocytes as well. This will provide direct evidence for the cholesterol homeostatic mechanisms in fat storing cells.

f. To further confirm the cholesterol substrate limited activation of ACAT, I will perform Si-RNA experiments to knock down the ACAT enzyme (Huttunen et al., 2007) to study the levels of cholesterol and cholesteryl esters upon cholesterol loading or cholesterol depletion or upon cold induction.

g. I will study the effect of inhibiting ACAT by Sandoz 58035, on the actin filament system to relate it to the reduced membrane order observed in the plasma membrane of T cells (Paper II). I will perform experiments to study the membrane order in T cells upon cholesterol loading in control and Sandoz 58035 treated cells.

With these above mentioned experimental strategies, I will address the role and molecular mechanisms of cholesterol in activating the ACAT enzyme *in vivo*

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