INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

University of Alberta

TETRASPANINS: ROLE IN MHC CLASS II DISTRIBUTION AND TRAFFICKING

By

HONG ZHANG 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science

in

Medical Science-Oncology

Edmonton, Alberta

Fall 2000

University of Alberta

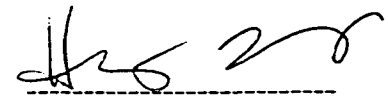
Library Release Form

Name of Author: Hong Zhang

Title of thesis: Tetraspanins: role in MHC class II distribution and trafficking

Degree: Master of Science

Year this Degree Granted: 2000



Hong Zhang
Department of Oncology
11560 University Avenue
Edmonton, Alberta, Canada

Date: August 30, 2000

ABSTRACT

How the differential requirements of stabilizing MHC class II at the cell surface and directing it to endosomes to acquire fresh peptide are regulated is not well understood. When CD9, a B cell activation antigen, was ectopically expressed in the Burkitts lymphoma cell line Raji, internalization of MHC class II was compromised. CD9 co-capped and co-immunoprecipitated MHC class II indicating the two proteins are physically and biochemically associated. The tetraspanins CD9 and CD81 co-localized with MHC class II on the cell surface whereas tetraspanins CD63 and CD82 colocalized with MHC class II intracellularly. Different tetraspanins may therefore enhance or impede MHC class II trafficking. CD9 and MHC class II are constitutively present within cholesterol-sensitive CHAPS insoluble lipid rafts and their presence increased by clustering. Our data suggest a role for CD9 in the stabilization of MHC class II at the cell surface possibly through redirection of MHC class II/CD9 complexes to lipid rafts.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Tetraspanins: role in MHC class II distribution and trafficking* submitted by *Hong Zhang* in partial fulfillment of the requirements for the degree of M.Sc. *(Medical Science-Oncology)*.

Dr. Andrew Shaw

Dr. Walter Dixon

Dr. Kevin Kane

Dr. Deborah Burshtyn

28th August 2000

DEDICATION

I would like to dedicate this thesis to

my dear wife Dr. Shurong Zhang

and

my lovely son Andrew Zhang.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Andrew Shaw for introducing me to Oncology during my studies at University of Alberta. I am deeply indebted to him for his valuable guidance, incessant encouragement, and generous support through out my entire M. Sc. program.

I would like to express my heart-felt thanks to Drs. Kevin Kane and Walter Dixon for serving on my supervisory and examining committees, and for their generous support and helpful discussion.

Special thanks go to Allan Mak, Dr. Xuejun Sun and Ywah Yau for their generous technical assistance with my research.

Finally, I would like to thank the University of Alberta and Department of Oncology for giving me the opportunity to study as a graduate student.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP1	Adaptor protein 1
APC	Antigen presenting cell
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-Sulfonate
CIIV	Class II containing vesicle
CLIP	Class II invariant chain peptide
DIG	Detergent insoluble glycolipid domain
ECL	Enhanced chemiluminescence
EE	Early endosome
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidylinsitol
HRP	Horse radish peroxidase
Ii	Invariant chain
LE	Late endosome
LIP	Leupeptin-induced peptide
MAb	Monoclonal antibody
MBCD	Methyl-β-cyclodextrin
MHC	Major Histocompatibility Complex
MIIC	MHC class II compartment
SLIP	Small leupeptin-induced peptide
TCR	T cell receptor
TfR	Transferrin receptor
TGN	Trans Golgi Network
TRITC	Tetramethylrhodamine-5(and6)-isothiocyanate

CHAPTER 1: INTRODUCTION

1.1 MHC class II and antigen presentation

B lymphocytes recruit T cells through presentation of antigenic peptide fragments to the T cell receptor (TCR) in association with major histocompatibility complex (MHC) class I and class II molecules [1]. MHC molecules are α/β heterodimers that trap peptides within a peptide-binding groove. In the case of MHC class I molecules the binding groove is formed through pairing of the α1 and α2 domains of the heavy chain [2] whereas in MHC class II molecules the α and β sub-units combine to generate the groove [3, 4]. MHC class I and class II molecules bind peptides in distinct ways and play different biological roles in antigen presentation. The peptide-binding groove is closed in MHC class I molecules and open in MHC class II. As a result, optimally-sized peptides for class I binding are limited to 8-10 amino acids, whereas 15-20 residues are typically found associated with mature MHC class II molecules [4-8]. Class I molecules are expressed on all nucleated cells and typically bind peptides derived from endogenous proteins. They therefore acquire peptides generated by intracellular pathogens such as viruses and parasites. Class II molecules, on the other hand, are expressed mainly on antigen processing cells such as B cells, macrophages, and dendritic cells, and acquire fragments of exogenous antigens within late compartments of the endocytic-lysosomal pathway. MHC class II antigens present exogenous peptides to CD4+ helper T cells. Recognition of immunogenic peptide by the T cell receptor complex results in T cell activation and cytokine release which in turn stimulates B cells to proliferate and differentiate into antibody secreting plasma cells.

Newly synthesized MHC class II molecules are assembled in the endoplasmic reticulum (ER) together with a third molecule, the so-called invariant chain (Ii). Ii trimerizes through its C-terminal region in the ER and a single Ii trimer associates with three αβ dimers of MHC class II to form a nonameric complex [9] in a process that is facilitated through association with the ER-resident chaperone calnexin [10]. The resulting trimer of class II α-β-Ii heterodimer is released from the ER. Association with Ii has been proposed to contribute in a number of different ways to the function of class II molecules. These include promoting the proper folding of MHC class II molecules in the ER [11, 12], facilitating their efficient transport from the ER through the trans-Golgi network (TGN) to the endocytic pathway [13, 14], and preventing class II molecules from loading peptides in the ER intended for MHC class I molecules [15].

From the TGN, the αβIi complexes are transported to the endocytic compartment for peptide loading. Ii targets MHC class II molecules to those compartments either by direct sorting in the TGN, or indirectly, through internalization from the plasma membrane. Electron microscopy has identified MHC class II molecules in uncoated vesicles in the TGN and in multi-membrane containing endocytic structures, in close proximity to, or even directly associated with, the TGN [16]. This suggests that direct sorting occurs in the TGN. However, some MHC class II molecules along with Ii reach the plasma membrane from which they rapidly internalize [17, 18].

Before MHC class II molecules bind antigen-derived peptide, the αβ heterodimers must first be released from interaction with Ii. The invariant chain is released through a step-wise process involving the combined action of proteases and acidic pH within the later

compartments of the endocytic pathway [19, 20]. The earliest products formed during Ii cleavage in vivo include the 21kDa leupeptin-induced polypeptide (LIP), and small leupeptin-induced peptides (SLIP) of 11kDa [21]. Finally, only a 7-25 amino acid fragment called CLIP (for class II-associated invariant chain peptide) remains which is protected from further degradation by the peptide binding groove of MHC class II molecules [22]. CLIP is not spontaneously exchanged for antigenic peptide. Two additional MHC-related complexes, HLA-DM and HLA-DO, play a role in the exchange process. HLA-DM interacts directly with class II molecules at acidic pH [23] and facilitates exchange of CLIP for antigen peptides [24-26]. By assisting the release of CLIP and other peptides of low affinity HLA-DM promotes the capture of high affinity peptides [27]. At the mildly acidic pH known to favor MHC class II peptide binding [28], DM associates with class II/invariant chain complexes stabilizing the empty forms of MHC class II generated in the peptide-loading compartment [29]. Another MHC-related complex, HLA-DO, binds directly to HLA-DM through a high-affinity reaction initiated in the ER, after which the DM/DO complexes are transported to the peptide-loading compartment [30]. HLA-DO is thought to inhibit the ability of HLA-DM to remove CLIP and to facilitate peptide loading [29, 31].

A key question is precisely where the class II-Ii complexes enter the endocytic pathway and where antigen processing and peptide-loading actually occur. Considerable evidence indicates that late endosomes (LEs)/lysosomes play a crucial role in class II mediated antigen presentation [32-35]. Immuno-electron microscopy, demonstrates the accumulation of MHC class II molecules in late endocytic structures with numerous internal membrane vesicles and membrane sheets typical of LE/lysosomes [36]. These structure are collectively designated as the MIIC (MHC class II compartment) [36, 37]. In addition to high levels of

MHC class II molecules, MIICs contain lysosomal membrane, and lysosomal enzymes and little or no transferrin receptor (TfR), a marker of early endosomes. Endocytic tracers accumulate in multi-vesicular MIICs with about ten minutes of uptake and progress through intermediate MIICs containing both vesicles and sheets, to multi-lamellar MIICs with membrane sheet only [38]. Cell-fraction studies, however, indicate that at least some APCs contain MHC class II-positive vesicles that are physically and functionally distinct from conventional endosomes or lysosomes [39-41]. Using free-flow electrophoresis, a morphologically similar compartment (designated CIIV for class II-containing vesicles) has been identified in murine B cell line, A20 [39]. Like MIICs, CIIVs are MHC class II-enriched and accessed by internalized antigen. However, unlike MIIC, these compartments contain the transferrin receptor and may therefore represent earlier stages in the endocytic pathway. Indeed recent immuno-electron microscopy studies identified an early MIIC type situated downstream of early endosomes (EEs) that contains abundant invariant chain Ii [37, 42]. Many characteristics of early MIIC match CIIV; both have internal vesicles, contain low levels of TfR and HLA-DM, and are morphologically distinct from LEs and lysosomes [37]. However, the main difference between CIIV and early MIICs is that the latter are enriched in Ii. CIIV may therefore represent an earlier peptide-loading compartment associated with earlier endosomes into which recycled MHC class II is directed. Taken together, recent biochemical [43] and morphological [37] evidence indicates that the major biosynthetic route of MHC class II-Ii complexes merges with the endocytic pathway downstream of EEs (the main TfR recycling compartment).

Following endocytosis and transport through early endosomes, antigens are proteolytically processed into peptides that can be loaded onto MHC class II molecules. To

date, it has not been possible to pin-point the precise sites at which the MHC class II molecules are loaded with peptide. It seems likely that more than one compartment is involved, depending on the source of peptide, the class II haplotype and individual APC type. One characteristic often used to determine the peptide-loaded state of MHC class II in subcellular fractions is the SDS-stability of high affinity class II-peptide complexes [44]. Antibodies selectively recognizing peptide-loaded class II have been used to define loading sites by immuno-electronmicroscopy [37]. Both approaches indicate that it is the MIICs in B cells and macrophages that represent the primary sites of peptide loading [32-34, 43-45]. However, this does not exclude the possibility that some peptides, perhaps those that are formed under mild proteolytic conditions, bind to MHC class II in EEs [46]. This may involved recycling surface-derived mature class II molecules that have lost their peptide or that exchange low-affinity peptides for higher affinity ones [43].

Once antigen peptide has been loaded onto the groove of MHC class II molecules, the complexes are ready to be transported to the plasma membrane. This final trafficking event is not well understood. A possible pathway for delivery of MHC class II-peptide complexes to the cell surface in B cells could involve the fusion of multi-vesicular MIIC with the plasma membrane (exocytosis), thereby inserting MHC class II in the membrane of the MIIC into the plasma membrane [47]. Interestingly, MIIC exocytosis is accompanied by the release of the internal MIIC vesicles as so-called exosomes, which bear SDS stable class II-peptide complexes and contain high level of tetraspanin proteins on their surface [48]. Exosomes of human B cell and mouse B cell can stimulate specific T cells [48]. In a murine B cell, CIIVs may function as vesicles for the transport of peptide-loaded MHC class II to the cell surface [39].

1.2 Detergent-insoluble glycolipid-enriched microdomains or lipid rafts

The central event in the generation of both humoral and cell-mediated immune responses is the activation and clonal expansion of T helper (Th) cells. Th cell activation is initiated by interaction of a processed antigenic peptide bound to an MHC class II molecule on the surface of an antigen-presenting cell with the TCR-CD3 complexes on a CD4+ Th cell. Thus, it has been very important for the study of T cell recognition to have data on direct binding between specific TCRs and their class II-peptide ligands. TCR affinity for a series of related ligands has been investigated using competitive inhibition of TCR-anti-TCR binding by soluble peptide-MHC molecule complexes [49], Scatchard analysis using labeled soluble TCR analogs and surface displayed peptide-MHC molecule complexes [50], measurement of covalently cross-linked proteins during association or dissociation reactions using cell-bound TCR and soluble peptide-MHC molecule ligands [51, 52], and direct analysis by surface plasmon resonance of the binding and dissociation of soluble αβ TCR and soluble peptide-associated class II molecules [53, 54]. Although the absolute affinity or association/dissociation rates differ, the range of values are nearly all indicative of relatively weak binding characterized by moderately fast on- and off-rates[55, 56]. Affinity rates range between 0.1 and 50 μM, with dissociation *t* 1/2 in 10- to 50-s. In comparison to this strength of binding is three to six orders of magnitude less.

The slow rate of association may indicate that the binding of the TCR to its MHC ligand is in some way intrinsically limited, perhaps by a requirement for a conformational change in the TCR or MHC complex, or even both. It was concluded that, in addition to the

dissociation rate of the peptide from the MHC molecule, the dissociation rate of a peptide–MHC complex from TCR is also a critical parameter in determining the magnitude of a T cell response. Small changes in these rates may lead to dramatic changes in the level of T cell stimulation.

The engagement of TCRs with class II-peptide ligands deliver stimulatory signals to the T cell. This is necessary, but not sufficient, to induce T cell proliferation and clonal expansion. T cell proliferation will not occur until an additional, co-stimulatory signal is received. This secondary signal is provided by surface ligands on the antigen presenting cells. The identification of B7 (CD80) molecules on the surface of APCs and their interaction with CD28 on the surface of T cells led to the discovery that B7-CD28 pathway plays a role in co-stimulation of T cell response. A secondary receptor for the B7 ligand, CTLA4 (CD154), was also found on T cells and is up-regulated on the surface of T cells after CD28-B7 co-stimulated T cell activation. An additional member of B7 family was discovered recently, B7-2 (CD86), which also bound to CD28 and CTLA4, was present on the surface of APCs, and could co-stimulate T cells.

The importance of co-stimulation to T cell activation is illustrated by the following experiments. Mice lacking CD28 can still mount T cell [57, 58]. In vitro studies demonstrated that in the absence of CD28 engagement, T cells require a very high TCR occupancy and a prolonged stimulation, whereas when co-stimulated through CD28 they respond more rapidly to lower levels of TCR occupancy [59, 60]. The functional importance of the B7-1 molecule also has been demonstrated in a number of studies of T cell activation. Both anti-CD3 and PMA-induced T cell proliferation was augmented by the addition of B7-1 transfectants. The proliferation was enhanced in a CD28-dependent fashion since T cell

activation was blocked by anti-CD28 mAbs [42, 43, 61]. Furthermore, the potent co-stimulatory role of B7-1 has been demonstrated in vivo in transgenic mice in which B7-1 was ectopically expressed on the cells of the islets of Langerhans [62].

Initial experiments demonstrating that anti-CTLA4 monoclonal antibodies enhance T cell proliferation in vitro were interpreted to support the notion that CTLA4 synergized with CD28 in enhancing the co-stimulation of human T lymphocytes. This result, however, may be a consequence of the removal of an inhibitory signal by interrupting CTLA4-B7 interactions rather than a consequence of enhancement of T cell proliferation through CTLA4 cross-linking. In support of this idea, it has indeed been shown in two independent reports [63, 64] that blockade of CTLA4-B7 interaction with anti-CTLA4 antibodies enhances T cell proliferation. Both Fab fragment and intact anti-CTLA4 antibodies augmented alloreactive and peptide-specific T cells responses in vitro. Furthermore, CTLA4 cross-linking, by immobilized monoclonal antibody or by soluble antibody cross-linked with a secondary antibody, inhibited T cell response induced by anti-CD3 or anti-CD28 antibodies [64]. This decreased response was due to lack of cell division, not to the induction of apoptosis. These results indicate that CTLA4 transduces an inhibitory signal that modulates the CD3 and/or CD28 signals.

Interaction between CD40 on B cells and CD40 ligand (CD40L, CD154) on activated T cells up-regulates B7 expression on B cells [65]. The role of CD40-CD154 in regulation of B cell APC functions was further exemplified in an in vivo system, showing that combined administration in mice of anti-CD154 antibodies and allogeneic B cells elicited poor mixed lymphocytes and cytotoxic T cell responses [66].

Biological membranes are "highly selective permeability barriers" that enclose the entire cell or internal cellular spaces. Three major types of lipids are found in membrane bilayer structure: phospholipids, glycolipids, and cholesterol. Phospholipids are derived from either glycerol (phosphoglycerides) or sphingosine. Major phosphoglycerides are derived from phosphatidate and include phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl glycerol (cardiolipin) and phosphatidyl inositol. Glycolipids are sugar-containing lipids derived from sphingosine. In glycolipids, phosphoryl choline is replaced by a glucose or by galactose in cerebrosides. Cholesterol, which is present in mammalian cell membranes, contains a tetracyclic hydrocarbon ring system which has little flexibility, a polar 3L-hydroxyl group and a hydrophobic side chain.

One of the most intriguing questions about cell membranes is why they contain so many different types and species of lipids. After all, if the chief purpose of membranes is to compartmentalize cells while serving as selective permeability barriers in the export and import of various cellular materials, then only a handful of lipid types should be necessary. Recent advances from the biologic observations in the fields of membrane transport and signaling have not only provided enlightenment regarding the need for diversity among membrane lipids but have also served to renew interest in the nonrandom organization of lipids within biological membranes to form lipid microdomains.

1.2.1 Lipid phase-separation, and lipid raft formation

Phospholipid bilayers are thought to exist predominantly in a fluid liquid-disordered (l*d*) phase because most biologic phospholipids have low acyl chain melting temperatures

(T*m*). In eukaryotic cell membranes, although glycerolipids generally have low Tm, sphingolipids (especially glycosphingolipids) have much higher Tm. This disparity suggests that a fluid phase separation between glycerolipid- and sphingolipid-rich domains might occur. Indeed, phase separation has been described in model membranes containing binary mixtures of saturated-chain lipids (which have high Tm) and cholesterol [67-69]. When the temperature exceeds the Tm of the lipid, a liquid-ordered (l*o*) phase can separate from the l*d* phase as the amount of cholesterol is increased. Acyl chains of lipids in the l*o* phase have properties that differ from those in the l*d* phases. being extended and ordered [70]. Cholesterol may enhance the motional order of acyl chains in the liquid-disordered phase by diminishing the amount of trans-gauche isomerization and increasing the fraction of trans-dihedrals about the carbon-carbon bonds [67, 71]. Thus cholesterol may contribute not only to lipid ordering but also to diffusional movement of acylated molecules within the l*o* phase.

Early studies suggested that glycosphingolipids might cluster in membranes [72]. This raises the questions whether membranes contain lipid rafts in different phases. Detergent-insolubility studies strongly suggest that plasma membranes are not entirely in the l*d* state, and that detergent-insoluble glycolipid-cholesterol-enriched fractions with properties similar to those of l*o* phase membranes can be isolated from mammalian cell lysates. It is envisioned that microdomains exist in intact cell membranes as rafts floating in a detergent-soluble l*d* phase sea. [73]. Liposomes with sphingolipid and cholesterol levels similar to those in the plasma membrane are partially detergent insoluble and may undergo phase separation [74, 75]. In addition model membranes may be present in a single uniform phase with properties that are intermediate between the l*o* and l*d* phases. It has been suggested that such transition phases may also occur in-vivo [76].

Proteins (such as GPI-anchored proteins) and lipids (such as glycosphingolipids) that have an affinity for ordered environments should partition into rafts and act as morphological markers. The conclusion of several attempts to observe rafts directly is that if rafts exist, they are difficult to directly visualize. GPI-anchored proteins generally appear uniformly distributed in the plasma membrane [77], and it has been difficult to obtain evidence of glycosphingolipid clusters larger than a few molecules [78].

However, there is much better evidence that rafts exist after certain proteins and lipids are clustered in the membrane. When proteins associate with rafts, they are expected to prefer an ordered environment and to co-cluster when both are clustered independently. This has been shown for two different GPI-anchored proteins [79] and recently also for a GPI-anchored protein and a transmembrane raft protein [80, 81]. The affinity of these independently clustered proteins for each other suggests that both are present in rafts.

Recently, more direct evidence of lipid raft in living cell membranes was reported by two different groups. Using fluorescence resonance energy transfer technique, GPI-anchored folate receptors were observed to be non-randomly and density-independently distributed in sub-pixel-sized microdomains. These domains are likely to be less than 70 nm in diameter and are disrupted by removal of cell surface cholesterol [82]. By chemical crosslinking, it was also observed that GPI-anchored growth hormones reside in the microdomains of living cells that are cholesterol dependent and much smaller than those seen after detergent extraction [83]. These results provide the first evidence for the existence of rafts in living cell and also illustrate some the difficulties of using morphological studies to validate lipid rafts.

The original method for raft isolation was based on the resistance of certain membrane components (e.g. GPI-anchored proteins, sphingolipids, cholesterol) to solubilization by the detergent, Triton X-100, at 4°C. Indeed, most of the sphingolipids and some of the cholesterol in mammalian cell membranes are detergent-resistant in the cold, and can be isolated as rafts [84]. In contrast, most of the cellular phospholipid is detergent soluble. Rafts can be isolated by density gradient centrifugation because of their high lipid content and low buoyant density. The raft fraction floats at low density, and can therefore be easily separated from other TX-100-insoluble material such as the actin cytoskeleton that have a higher density.

In addition to sphingolipid and cholesterol, a specific group of membrane linked to saturated acyl chains are proteins is present in rafts. Saturated acyl chains can be linked to proteins in two ways: either in the form of a glycosylphosphatidylinositol (GPI) anchor [85], or through acylation with myristate or palmitate. Most GPI-anchored proteins in mammalian cells generally associate with rafts in a GPI anchor-dependent manner [86, 87]. The association of non-receptor Src protein tyrosine kinases with rafts is another example of acylation assisting in raft targeting [88-90]. The majority of these enzymes requires dual modification by myristate and palmitate to associate with rafts [90, 91]. Several other palmitoylated raft proteins have been identified [92] suggesting that acylation may be a widely used raft targeting signal. Indeed mutation of either palmitoylation site in the dually palmitoylated neuronal protein GAP-43 [93] blocks its association with rafts [94].

However, a key issue regarding detergent insolubility is whether the biochemical method used to isolate these structures actually harvests pre-existing microdomains or creates them artifactually. This might occur by selective extraction of low-T*m*

phospholipids, leaving the remaining sphingolipids to pack tightly with cholesterol to form rafts in an l*o* -like phase. Two studies indicate that this does not occur. In these studies, lipids that favored l*d* and l*o* phases were mixed in various ratios and subjected to detergent extraction. In both studies, detergent insolubility was found only when the l*o* phase was present prior to detergent addition, showing that detergent did not reorganize lipids in the l*d* phase to create insoluble domains [74, 75]. Further support comes from the observation that depletion of sphingolipid or cholesterol abolished the association of several proteins with the DIG fractions as predicted from their involvement in the lipid raft assemblies [95, 96]. Moreover, reconstitution experiment of GPI-anchored placental alkaline phosphatase into liposomes of different lipid composition showed that the TX-100 insolubility critically depends on the raft lipid environment [97]. Nevertheless the characterization of raft domains using detergent has its limitations. Rafts may not accurately represent the entire organization of glycolipid microdomains in cellular membranes as raft markers may coalesce upon TX-100 extraction. Also it is not possible to determine the size and subcellular localization of rafts or to distinguish between rafts of different composition. The development of detergent-free methods of isolating rafts may help to resolve these issues.

1.2.2 Lipid rafts and signaling

Lipid rafts are thought to be important for transmembrane signaling at the cell surface. Indeed antibody mediated cross-linking of GPI-anchored proteins induces the phosphorylation of various substrates on tyrosine residues, leading to calcium flux and phosphotyrosine signalling [98, 99]. p56 lck (Lck) is a src-like tyrosine kinase critical for T-

cell development and activation that is partly associated with rafts [86]. The raft-associated Lck, however, was hyper-phosphorylated which correlates with a lower kinase activity relative to TX-100 soluble Lck. In contrast, using a detergent-free procedure, a plasma membrane fraction was obtained from T lymphoma cells that was enriched in GPI-anchored proteins and Lck and had high specific tyrosine kinase activity [94]. Thus Lck activity may be high in rafts but diminished by the method of extraction. In the Jurkat T cell line, tyrosine phosphorylation dependent accumulation of actin filaments was observed upon clustering of GPI-anchored proteins and glycolipids indicating that lipid rafts are sites of phosphotyrosine-dependent actin polymerisation [100].

The raft-mediated signaling pathway is not restricted to GPI-linked proteins. In granulocytes as well as mast cells and basophils, aggregation of the high affinity IgE receptor FcεRI by receptor-bound IgE antibodies results in translocation of the receptor into the lipid raft with resulting interaction of the non-receptor tyrosine kinase Lyn with the β chain of FcεRI. The subsequent tyrosine phosphorylation of the L and Q subunits of the receptor triggers a signalling cascade leading to the release of mediators of the allergic response [101, 102].

Signal transduction following lateral assembly of raft components may play a role in T cell activation. GM3 was found enriched in a TX-100 insoluble fraction, together with the T-cell specific co-receptor CD4 and Lck [103]. CD4 and Lck interact via unique domains in both proteins and are involved in signal transduction through the T-cell antigen receptor [104]. Recently, direct recruitment of the TCR/CD3 to rafts, dependent on both TCR engagement and the activity of Src family kinases, was observed in T lymphocytes. The

raft-associated receptor complexes were highly enriched in hyperphosphorylated p23 chains and contained most of the TCR/CD3 associated and phosphorylation-activated ZAP-70 kinase [105]. The recruitment of rafts are also important for delivery of a co-stimulating signal. Engagement of CD28, a co-receptor for CD3-dependent signaling, led to the re-distribution and clustering of membrane and intracellular kinase-rich raft microdomains at sites of TCR engagement [106]. Multimolecular signalling complexes therefore appear to be constituted via raft association with its opportunities for additional protein-protein interaction. Possibly, receptor-ligand interactions trigger redistribution of receptors into rafts or stabilize larger raft assemblies, leading to association of receptors with non-receptor protein tyrosine kinases and regulation of actin assembly.

1.2.2.2 Lipid rafts, protein sorting and transport

It has been suggested that rafts may also function in the sorting of lipids and proteins into secretory and endocytic pathways. Most of the studies analyzing how the lipids and proteins are sorted and delivered to the cell surface have been carried out in polarized cells. Epithelial cells have to deliver newly synthesized proteins to the apical and basolateral plasma membrane domains of the polarized cell surface. Sorting takes place in the TNG into different pathways for apical and basolateral delivery. For membrane proteins, basolateral sorting signals appear to be dominant over apical ones [107-109]. The basolateral signals include tyrosine- or dileucine-based motifs similar to those implicated in clathrin-dependent endocytosis. In the apical pathway, glycosphingolipid-enriched rafts act as sorting platforms for the assembly of vesicles transporting GPI-anchored proteins and apical

transmembrane proteins [109]. GPI-anchored proteins partition into lipid rafts during biosynthetic transport, and are also targeted apically in epithelial cells [110].

Cholesterol and ceramide are synthesized in the ER and ceramide is further modified in the Golgi complex by the addition of phosphorylcholine to form sphingomyelin or of monosaccharides to form glycosphingolipids. Glycolipid enriched rafts can be formed in the Golgi apparatus where they serve as platforms for the selective transport of raft associated protein [111]. This is supported by the observation that in epithelial cells apically destined proteins like influenza HA and the GPI-linked placental alkaline phosphatase acquire detergent resistance in the Golgi complex [112]. Furthermore these proteins can be solubilized by TX-100 after extraction of cholesterol with saponin or methyl-β-cyclodextrin [95].

The transport of apical proteins through the secretory pathway also depends on sphingolipid-cholesterol-enriched raft. When sphingolipid biosynthesis is blocked in polarized Madin-Darby canine kidney (MDCK) epithelial cells, apical sorting of the GPI-linked protein GP2 is abolished [113]. Similarly depletion of cellular cholesterol by 60 to 70 % in MDCK cells caused mis-sorting of the apical influenza hemagglutinin (HA) protein, which associates with rafts, while the transport of the basolateral marker vesicular stomatitis virus glycoprotein was unaffected [114]. Similarly the preferential axonal delivery of HA and endogenous GPI-linked Thy-1 in neurons is also disturbed by cholesterol extraction or by inhibition of sphingolipid biosynthesis [115].

1.3 The tetraspanin family

The CD9 antigen is a 24 kDa surface glycoprotein and member of the tetraspanin family of polytopic proteins expressed in such evolutionarily diverse organisms as Schistosoma, *C. elegans*, *Drosophila*, and mammals [116]. Protein topology studies of CD9 identify four hydrophobic domains of membrane spanning length and two hydrophilic extracellular domains located between transmembrane 1 (TM1) and TM2 and between TM3 and TM4 [117]. The short hydrophilic regions at the amino and carboxyl termini and between TM2 and TM3 are most likely cytoplasmic. The region between TM2 and TM3 is the most conserved region in all TM4 proteins. These basic structural characters exist in all TM4 proteins and distinguish them from other proteins with four membrane-spanning domains such as CD20, ion channels and connexins. There is considerable sequence divergence in the extracellular loops of tetraspanins. However, three highly conserved cysteines in EC2 are located at defined distances from the TM regions in the majority of family members. In addition certain TM domains contain highly conserved polar amino acids (an asparagine in TM1 and a glutamate or glutamine in TM3 and TM4) that may play a role in the interaction of TM4 proteins with each other or with other membrane proteins.

In addition to being N-glycosylated within the first extracellular loop, CD9 is also modified by the addition of palmitic acid [118]. Two-dimensional limited proteolysis analysis with Staphylococcus aureus V8 proteinase indicated that CD9 contains more than one palmitoylation site [119]. Tetraspanins associate with a wide range of other proteins including integrins [120], the T cell–specific molecules CD4 and CD8 [121], MHC class II molecules [122] and themselves. The first published evidence for the tetraspanin-integrin complex showed anti-CD9 antibody induction of CD9/αIIbβ3 association in platelets [123]. Subsequently the integrins α4β1, α3β1, and α6β1 were found to associate constitutively

with CD9 molecule, as well as with other tetraspanin CD53, CD63, CD81 and CD82 [124]. CD81 and CD82, both expressed on T cells, have been found to associate with the co-receptors CD8 and CD4 [121, 125]. In addition CD37, CD53, CD81 and CD82 associate with MHC class II molecules in B cells [126]. Tetraspanins may also be involved in class II processing and antigen presentation. Of particular importance is the recent finding that the tetraspanins CD82, CD63 and CD151 are enriched in the MIIC of human B lymphoblastoid cell lines where they associate with MHC class II, HLA-DM and HLA-DO [127]. These tetraspanins, together with CD37, CD53 and CD81, are also enriched on the surface of MHC class II expressing immunogenic vesicles called exosomes released into the extracellular space on B cell stimulation [48]. Taken together these observations implicate tetraspanins in the sorting and intracellular trafficking of MHC class II.

Tetraspanins are involved in a broad range of cellular functions, as revealed by the binding of mAbs. Anti-CD9 antibody 50H.19 triggers platelet aggregation and induces association of CD9 with the integrin αIIb/β3 [123]. Anti-CD81 mAb, 5A6, is strong inducer of homotypic adhesion in hematolymphoid cells [128]. Recent studies demonstrate that antibodies to CD63 induce neutrophil adhesion to HUVEC and that adhesion is probably mediated by LFA-1 since antibodies to LFA-1 and Ca++ depletion block CD63-mediated adhesion [129]. CD9 can influence cell motility. Ectopic expression of human CD9 in the CD9-negative, poorly motile, human B cell line Raji dramatically enhances migration across fibronectin- and laminin-coated polycarbonate filters. The CD9-enhanced migration was inhibited by the protein tyrosine kinase inhibitor herbimycin A suggesting that tyrosine phosphorylation played a role in the generation of a motorgenic signal [130].

Tetraspanins may also play a regulatory role in immunoresponses. When B lymphocytes present specific antigen to CD4+ T helper cells in vitro, cross-linking surface CD81 significantly enhanced IL-4 and IL-10 synthesis of activated T cells [131]. CD82 can provide a co-stimulatory signal to T cell activation initiated by CD3 or TCR cross-linking. Using anti-CD82 mAb or anti-CD3 antibody alone, no cell proliferation and IL-2 synthesis were observed. However, when Jurkat cells were treated with anti-CD3 and immobilized anti-CD82 together, a great increase in IL-2 producing and T cell proliferation was seen. In addition the cells became strongly adherent to plastic dishes, and developed dendritic processes [132]. Cross-linking CD81, which is associated with CD4 or CD8 on T cells, promotes the rapid induction of integrin-mediated cell-cell adhesion via lymphocyte function-associated molecule-1 (LFA-1) and is co-stimulatory for signaling through the TCR/CD3 complex inducing interleukin 2-dependent thymocyte proliferation. [133]. Similarly, anti-mouse CD9 mAb, with sub-optimal concentrations of anti-CD3 mAb, was an effective co-stimulator of T cells. Although the magnitude of activation was similar to co-stimulation by anti-CD28, it was CD28-independent since T cells derived from CD28-deficient mice showed the same degree of co-stimulation by CD3 and CD9 as normal mice [134].

1.4 Hypothesis

MHC class II molecules associate co-synthetically with a series of chaperone proteins that guide their assembly and targeting to peptide-loading compartments. At the start of my studies it was known that MHC class II could associate with CD9, CD81 and CD82 at the

cell surface, but the significance of the associations was not established. By analogy with the proteins that guide the early stages of MHC class II peptide acquisition we hypothesized that tetraspanins might play a role in MHC class II trafficking and antigen presentation at the cell surface. The overall objective of my investigation was therefore to determine whether CD9 could alter the trafficking of MHC class II proteins in the CD9 negative cell line Raji through the transfection of CD9. Recently it was reported that CD9 and CD81 were present within a low buoyant density fraction of CHAPS-detergent insoluble membranes in kidney cells [135] suggesting that tetraspanins might be constitutive within lipid rafts. We wondered whether CD9 might regulate the activity of MHC class II within the context of lipid rafts. A secondary aim of my proposal was therefore to determine whether CD9 expression could alter the association of MHC class II molecules with lipid rafts.

CHAPTER 2: MATERIALS AND METHODS

2.1 Antibodies

Anti-CD9 monoclonal antibody 50H.19 was raised against a melanoma cell line [136]. Monoclonal antibody 7H.3 and 9H.1 recognize conformational determinants on MHC class II heterodimer and MHC class I heterodimer respectively [136]. Anti-sera obtained from commercial sources were Alexa 488nM (green) and Alexa 594nM (red) conjugated goat anti-mouse IgG (Molecular Probes, Eugene, Or), FITC (green) and TRITC (red) conjugated Extra-avidin (Sigma), and anti-talin mAb (Sigma). mAb against CD81 was the kind gift of Dr. Shoshana Levy (Stanford University) and mAb γC11 against CD82 the kind gift of Dr. Helene Conjeaud (INSERM Paris), anti-CD45 mAb was the kind gift of Dr. Sibrand Poppema (University of Alberta) and anti-human MHC class II heavy and light chain rabbit antisera the kind gift of Dr. Hidde Ploegh (Harvard Medical School).

2.2 Cell lines

The lymphoblastoid human B cell line Raji was maintained in RPMI 1640, 10% fetal calf serum (Gibco, BRL) plus 100 U/ml Penicillin, 100 U/ml streptomycin and 2mM glutamine. A full-length cDNA encoding human CD9 was cloned into pCDNA3.1 and transfected into the Raji cells strain by electroporation. 48 hours after transfection, cells were immunoselected by anti-CD9 antibody followed by anti-mouse coated dynabeads.

For FACScan analysis 10^6 cells were stained with 10 μg/ml 7H.3 or 50H.19 mAb for 1 hour at 4°C in PBS supplemented with 0.1% BSA. The cells were washed twice with same

solution then incubated with FITC-conjugated goat anti-mouse IgG (Jackson Laboratories) for 30 minutes at 4°C. Analysis of 4% formaldehyde fixed cells was performed using a FACScan (Becton Dickinson). Staining with second antibody alone was included as control. The stably transfected clones bearing different levels of CD9 expression were selected using a FACScan sorter (Becton Dickinson).

2.3 Biotinylation of the cell surface

Cells were washed 3 times with ice cold PBS and then incubated with freshly prepared 1.5mg/ml Sulfo-NHS-LC-biotin or Sulfo-NHS-SS-biotin (Pierce) at 2 x 10^7 cells/ml for 30 min on ice. The reaction was stopped by the addition of 3 ml of ice-cold RPMI-1640, 0.1% BSA followed by washing two times with the same buffer.

2.4 Immunoprecipitation and immunoblotting

$2x10^6$ biotinylated cells were lysed in 0.7ml CHAPS detergent lysis buffer (50 mM Tris-HCl, pH 7.4, 1% CHAPS, 137 mM NaCl, 1mM EDTA and protease inhibitors) for 2 hours. For immunoprecipitation of CD9 and MHC class II in transfected cells, 2 µl of 1 mg/ml purified 50H.19 or 7H.3 was added to the lysate for at least 2 hours at 4°C, followed by 35 µl of protein A-Sepharose 1:1 slurry for another 2 hours. The unbound fractions were removed and the precipitates were washed three times with lysis buffer at 4°C. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with streptavidin-HRP or anti-Ig-HRP.

2.5 Co-capping and confocal microscopy

$1x10^6$ RAJI/CD9 transfected cells were washed twice with ice-cold RPMI-1640, 1% FCS and incubated with anti-MHC class II antibody 7H.3(10 μg/ml in 100 μl PBS) at 4°C for 30 min. After 2x washing, cells were incubated with Alexa-488 goat anti-mouse (10 μg/ml in 100 μl PBS) at 4°C for 30 min. MHC class II capping was performed at 37°C after 1 hour incubation and subsequently fixed with 2% paraformaldehyde for 1 hour. Cells were incubated with anti-CD9 antibody 50H.19-biotin at 4°C for 1 hour and then with Extra-avidin TRITC for 30 min. After washing, cells were mounted in PBS, 80% glycerol and 4% DEBCO (Molecular Probes) as anti-fade.

2.6 Assay of MHC class II internalization

Cell surface proteins on Raji and Raji/CD9 cells were biotinylated with NHS-SS-biotin. Internalization of MHC Class II molecules was performed by incubating 2 $x10^6$ cells/1 ml in pre-warmed RPMI-1640, 10% FCS at 37°C for various periods of time. The surface NHS-SS-biotin was displaced by incubating the cells with 50 mM reduced glutathione wash solution (50 mM glutathione, reduced type, 75 mM NaCl, and 75 mM NaOH, 10 mM EDTA, pH value adjusted to 8.6). 1 ml this solution was used for 2 $x10^6$ cells. After 15 minutes another 1 ml fresh solution was added for a total time of 30 minutes. After washing with ice-cold PBS, the cells were lysed with RIPA lysis buffer (1% NP-40, 0.25% DOC, pH 7.5, and protease inhibitors). MHC class II molecules were immunoprecipitated with mAb 7H.3 and protein-A Sepharose. Proteins were separated on 5-20 % non-reducing SDS-

PAGE. Internalized MHC class II molecules were visualized by transferring to a nitrocellulose membrane and blotting with streptavidin-HRP.

2.7 Pulse-chase labeling and protein trafficking

Intracellular methionine/cysteines were depleted by incubating cells in Met/Cys free RPMI-1640, at 37°C for 2 hrs. The cells were metabolically labeled for 30 min with [^{35}S] methionine/cysteine labeling mix (0.1 mCi/ml; Amersham Corp., Arlington Heights, IL) at 37°C and chased for various periods of time in complete medium supplemented with cold 0.45mg/ml methionine and 0.75mg/ml cysteine at 1 x10^6 cells/ per ml.

For the surface delivery assay of new synthesized class II or class I, cells were surface biotinylated with NHS-SS-biotin and lysed in RIPA buffer for 1 hour at 4°C and MHC class II or class I molecules were precipitated with mAb 7H.3 or 9H.1 respectively. The MHC class II or class I molecules were eluted from protein A-Sepharose CL-4B (Amersham Phamacia Biotech.) beads by a 30 minute incubation at room temperature in 100 µl PBS containing 2% SDS. 80 µl were used to recover the biotinylated proteins by 1:10 dilution with PBS and adsorption onto streptavidin-agarose (Sigma). After washing the beads were resuspended in 30 µl of reduced SDS sample buffer and heated to 95°C for 3 minutes (this treatment releases class II or class I from beads and thus permits the visualization of the class II or class I molecules accessible to biotinylation on the surface). The total amount of MHC class II or class I was evaluated using the rest of 20 µl sample. The surface and total class II or class I were visualized by SDS-PAGE and autoradiography.

2.8 Buoyant density flotation gradient analysis

Raji cells ($1x10^7$ per gradient) were lysed in 200μl 1% CHAPS lysis buffer (100mM Tris-Hcl, pH7.4, 125mM NaCl, 1%CHAPS, protease inhibitors), 10% sucrose at 4°C for 1 hour. The cell pellet was resuspended thoroughly by pipetting through a 200-μl pipettor tip and incubated for 20 minutes on ice and then mixed again with 500 μl of cold 60% Optiprep (Sigma). The mix was transferred to an SW60 centrifuge tube (Beckman) and the sample was overlaid with a 600-μl step of each of 35%, 25%, 20%, 0% Optiprep in 1% CHAPS lysis buffer, 10% sucrose. The gradients were spun for 4 hours at 40,000 rpm at 4°C. Six fractions from the top to the bottom of the gradient were collected. TCA was added to the final concentration of 10% and the precipitated proteins were pelleted on an Eppendoff table centrifuge at top speed. 50 μl of 1 M Tris base was added to nutralize the TCA and the dissolved protein were analyzed by Western blot with anti CD9-mAb 50H.19, anti-β1 mAb #138, anti-talin mAb or anti-class II rabbit polyclonal antibodies followed HRP-conjugated secondary antibodies and ECL (Amersham, Pharmacia Biotech.). In some cases, the CD9 or class II was first clustered by cross-linking with relative antibodies.

2.9 Cholesterol depletion

Cells were depleted of membrane cholesterol with 10 mM methyl-β-cyclodextrin in PBS containing 0.9 mM Ca^{2+} and 0.5 mM Mg^{2+} for 1 hour at 37°C. Cells tested with trypan

blue for membrane integrity showed no more than ~4% staining indicating that membranes were still functionally intact.

CHAPTER 3. ASSOCIATION BETWEEN CD9 AND MHC CLASS II

3.1. Introduction

The most striking property of tetraspanins is the extent to which they associate with other integral membrane proteins. Since it was first demonstrated that anti-CD9 antibody induces association of CD9 with the integrin αIIb/β3 on platelets and triggers platelet aggregation [123], various integrins have been reported to associate with tetraspanins. For the most part these interactions are demonstrable using so-called mild detergents that retain non-covalent association. For example α6β1 and α3β1 co-immunoprecipitate CD9, CD81 and CD63 from mild detergents [120, 124, 137] whereas α4β1 and α4β7 associate with CD53, CD81 and CD82 [138]. Only CD9 associates with the β1 integrin precursor suggesting that some tetraspanin interactions with transmembrane proteins are direct whereas other proceed via tetraspanin-tetraspanin interactions [139].

Tetraspanins also associate with potential signaling molecules. Pro HB-EGF, a membrane anchored heparin-binding epidermal like growth factor transduces biological signals in a non-diffusible manner to neighboring cells, and associates with both CD9 and the integrin α3β1[140]. CD19, a membrane protein of the Ig superfamily, associates with CD81 in a signaling complex together with CD21 and Leu-13 [141]. Recently, phosphatidylinositol 4 kinase (PI 4-K), an enzyme that controls the first step in the biosynthesis of phosphatidylinositol bisphosphate (PIP_2), was found to associate with the tetraspanins CD63, and CD81 and with the integrin α3β1 [142]. CD151 also associates with PI 4-Kinase and with α3β1 integrin possibly acting as a bridge between the two molecules

[143]. The recent finding that the mild detergent digitonin fails to preserve tetraspanin-tetraspanin interactions and therefore identifies only direct associations between tetraspanins and non-tetraspanin proteins promises to clarify much of this complexity [144]. In digitonin CD81 associates with $\alpha 3\beta 1$ and CD151 with $\alpha 6\beta 1$ in the absence of any tetraspanin interaction. Dr. Rubinstein's discovery not only offers an approach to the identification of direct interactions but also raises the interesting question why detergents differ in their ability to maintain protein-protein associations. It seems likely that different detergents differentially solubilise lipids and that the lipid environment plays an important role in determining particularly tetraspanin-tetraspanin associations.

Another potentially important interaction occurs between tetraspanins and MHC class II molecules. By immuno-precipitation and co-capping, CD81 appears to be noncovalently associated with MHC class II on human B cells. Because CD81 also exists in a B cell-specific complex including CD19, CD21 and Leu-13 CD81 may link this complex to MHC class II [126, 145]. Using flow cytometric energy transfer (FCET) to probe the proximity of molecules on the cell surface the B cell membrane was shown to contain multi-component complexes containing MHC class I, MHC class II, CD20, CD53, CD81, and CD82 [146]. It has been proposed that MHC class II is a part of a cell surface tetraspanin web or network that provides opportunities for crosstalk between signaling complexes [122].

Another way to assess protein-protein association is to look for co-capping of a surface protein after its potential partner is crosslinked by antibody. Like co-localization experiments, co-capping does not provide unequivocal evidence of physical association between two proteins. However, co-capping does provide evidence of spatial association between two proteins, and in conjunction with biochemical evidence obtained by

immunoprecipitation, corroborates the concept that the proteins are interactive. More importantly, functional information may be derived from co-capping experiments. Capping of surface proteins is an energy-dependent process [147] that is mediated by the contractile activity of the actomyosin peripheral cytoskeleton [148, 149]. Consequently, treatment of lymphocytes with metabolic inhibitors or with drugs that impair cytoskeletal function results in the inhibition of surface protein capping [147, 150]. Previous studies also suggest that capping is a lipid-related process. When low concentrations of cis unsaturated, but not saturated free fatty acids are intercalated into the lymphocyte plasma membrane, capping is inhibited [151]. Similarly if cellular cholesterol is depleted by incubation with phospholipid vesicles, capping of surface immunoglobulin decreases and can be reversed by replacement of cholesterol [152]. Capping may therefore occur through coalescence of lipid rafts. Indeed, capping of leucocyte sialoglycoprotein and Thy-1 significant increase their detergent Triton X-100 insolubility [153]. Indeed capping of the raft-localized ganglioside GM1 was inhibited by cholera toxin and anti-toxin antibody, sodium azide, and cytochalasin B and accompanied by the co-capping of alpha-actinin [154].

Here we report our use of co-capping and confocal microscope to study the interaction between CD9 and MHC class II in CD9 transfected Raji cells and to examine the effect of clustering MHC class II on the distribution of other tetraspanins.

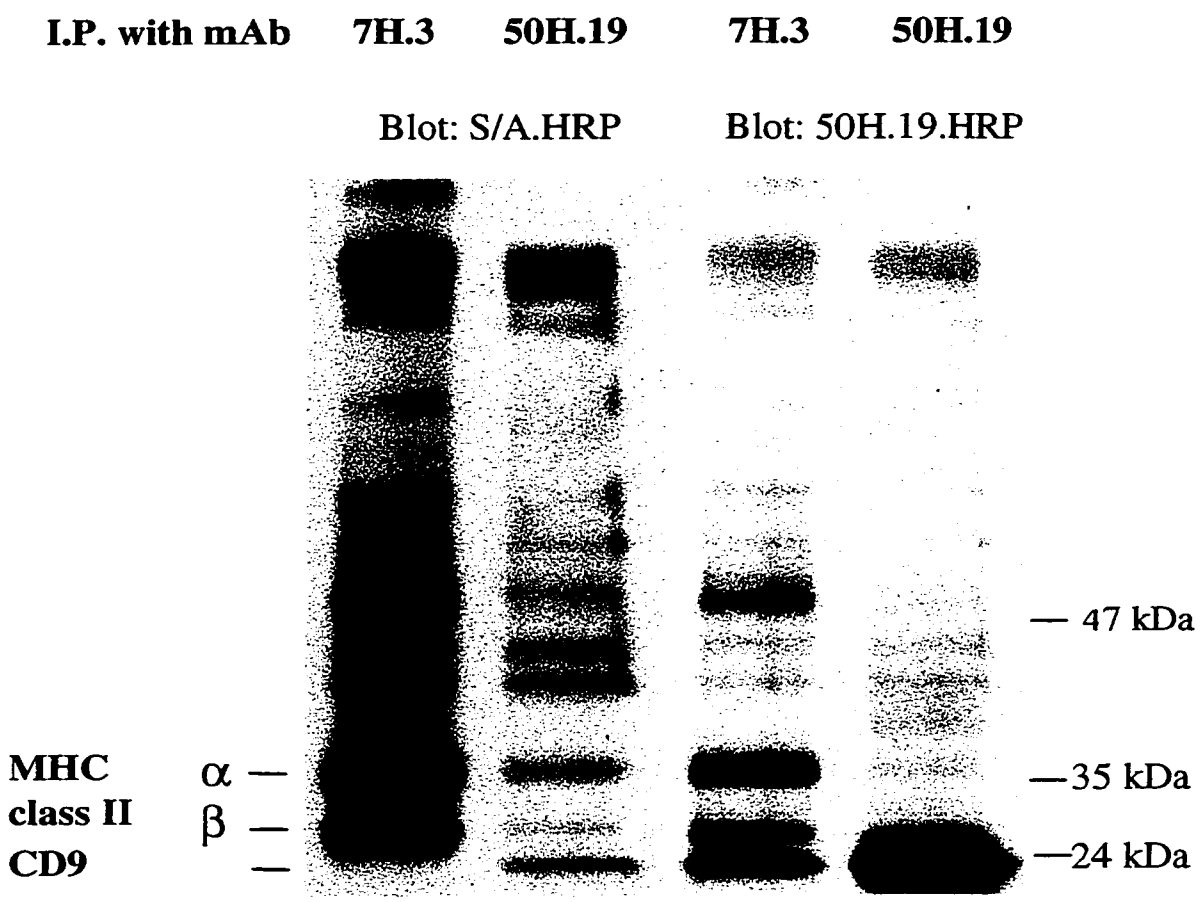


Fig. 1 *MHC class II associates with CD9 in Raji/CD9*

Biotinylated Raji/CD9 cells were lysed in 1% CHAPS, and immunoprecipitated with mAb 7H.3 against MHC class II, or mAb 50H.19 against CD9. Following SDS-PAGE, the proteins were transferred onto a nitro-cellulose and resolved with S/A-HRP or 50H.19-HRP and enhanced chemiluminescence. CD9 (24 kDa) is co-immunoprecipitated by mAb 7H.3 as detected by immunoblotting with anti-CD9 mAb, but not by SA.HRP since CD9 is only weakly biotinylated. MHC class II α and β are co-immunoprecipitated by the anti-CD9 mAb 50H.19.

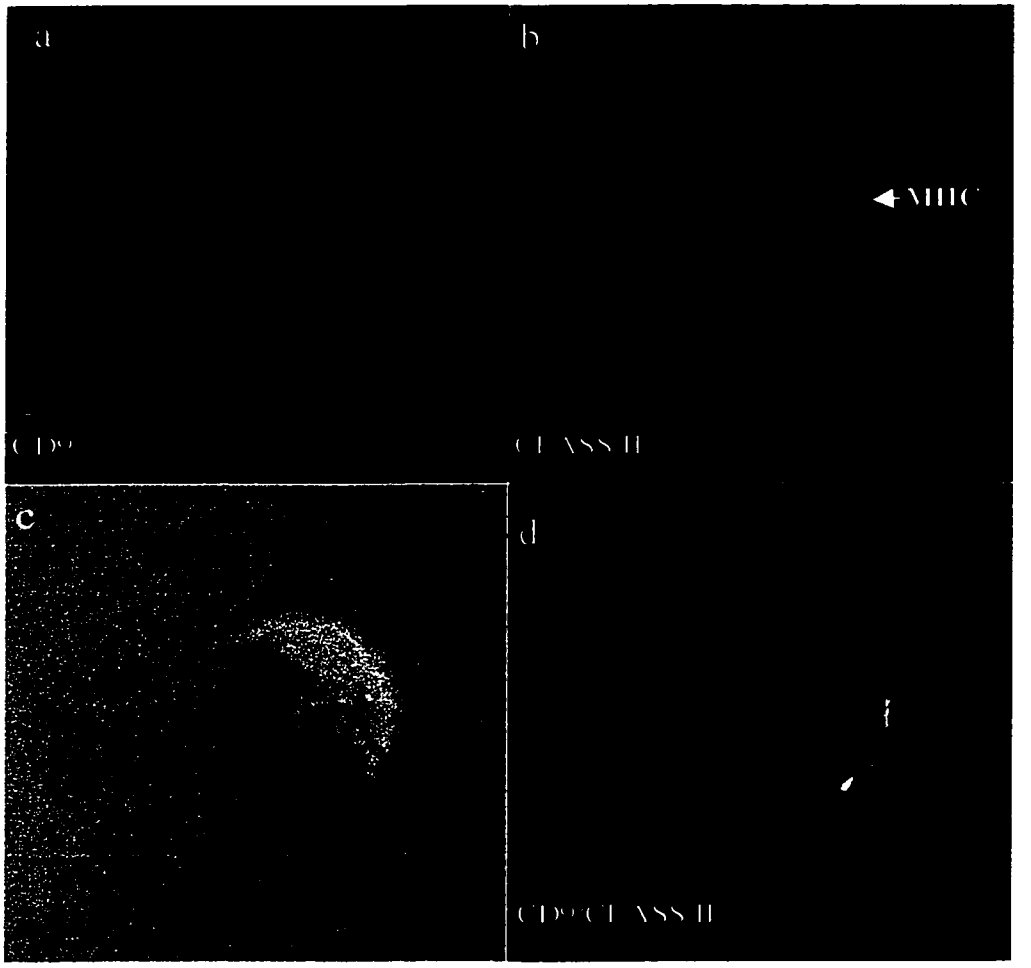


Fig. 2. *Capping CD9 co-caps MHC class II in Raji/CD9.*

CD9 capping was induced by binding mAb 50H.19 and cross-linking with goat anti-mouse-Alexa 594 (red) at 37C for 1 hr. After fixation and permeabilization non-specific binding sites were blocked by normal mouse IgG. MHC class II was detected with mAb 7H.3-FITC (green). (a) CD9 stain. (b) MHC class II stain. (c) visible (d) composite. The arrow denotes the MIIC peptide-loading compartment that contains intracellular MHC class II. Note that CD9 does not internalize.

3. 2. Co-immunoprecipitation of CD9 and MHC class II in Raji/CD9

First we analyzed CD9 and MHC class II association in CD9 transfected Raji cells by co-immunoprecipitation. Biotinylated cells were lysed in 1% CHAPS to maintain protein-protein association. CD9 and MHC class II were immunoprecipitated using mAb 50H.19 and 7H.3 respectively. After separating the proteins by SDS-PAGE and transferring them to nitrocellulose, the membrane was probed using S/A-HRP (Fig 1). mAb 7H.3 against MHC class II did not co-immunoprecipitate biotinylated CD9, however mAb 50H.19 against CD9 co-immunoprecipitated biotinylated MHC class II (Fig 1. Panel A). The failure to detect CD9 as a component of the MHC class II immunoprecipitate is due to the low efficiency with which CD9 was biotinylated since CD9 was detected by re-blotting with an HRP conjugated anti-CD9 mAb and detecting the signal by enhanced chemiluminescence (panel B). On the basis of immunoblotting it can be estimated that approximately 20% of CD9 is co-precipitable with MHC class II.

3. 3. MHC class II co-caps with CD9 on CD9 transfected Raji cells

It is possible that associations in weak detergents do not exactly reproduce the associations occurring between molecules in biological membranes. We therefore performed co-capping experiments to investigate the interaction of CD9 and MHC class II on the plasma-membrane. Fig. 2 is a confocal microscope picture of MHC class II co-capping with antibody induced CD9 capping. The cells were incubated at 4°C with mAb 50H.19, followed by crosslinking with goat anti-mouse IgG ALEXA-594 at 37°C for 1

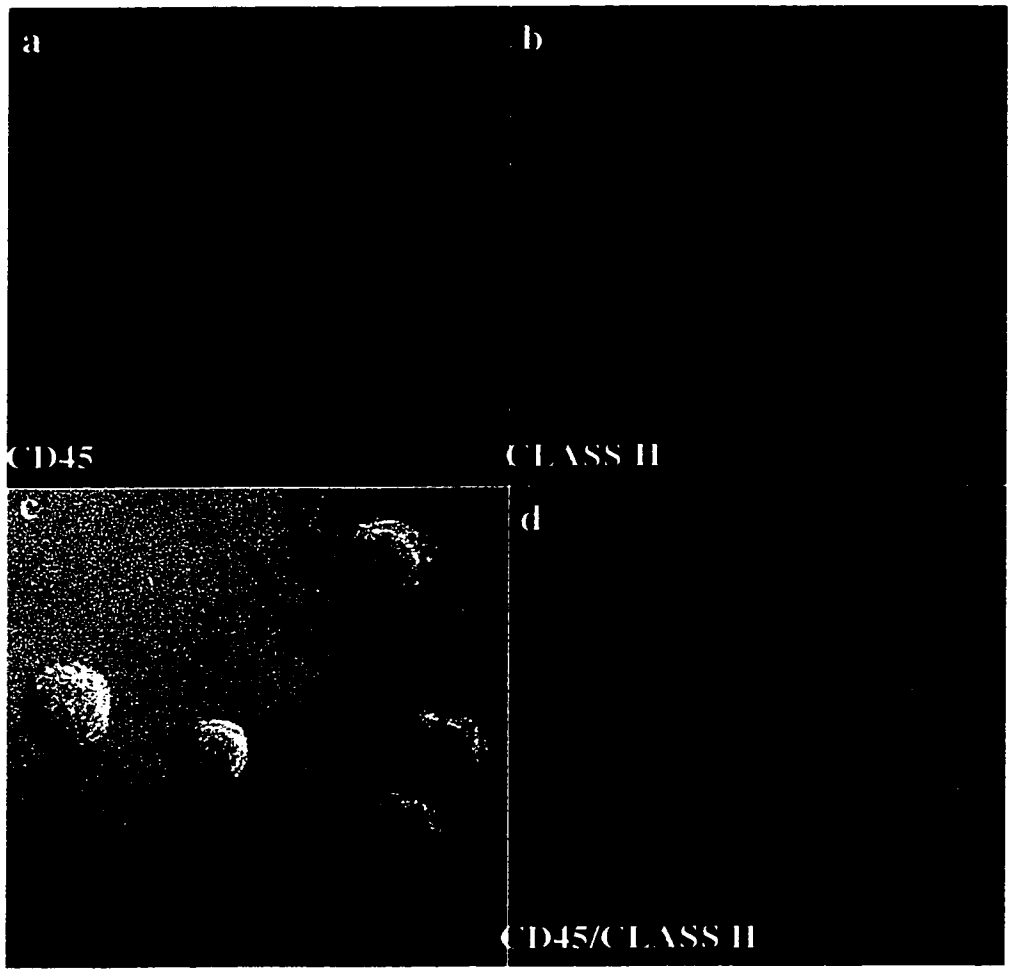


Fig. 3 *Capping CD45 does not co-cap MHC class II.*

Raji/CD9 cells were labeled with anti-CD45 mAb and incubated at 37C for 1 hour with goat anti-mouse IgG Alexa-594 (red). After fixation, the cells were blocked with normal mouse serum. MHC class II was detected using mAb 7H.3-FITC (green). a)CD45. b) MHC class II. c) visible d) composite image. Bars: 10 μm.

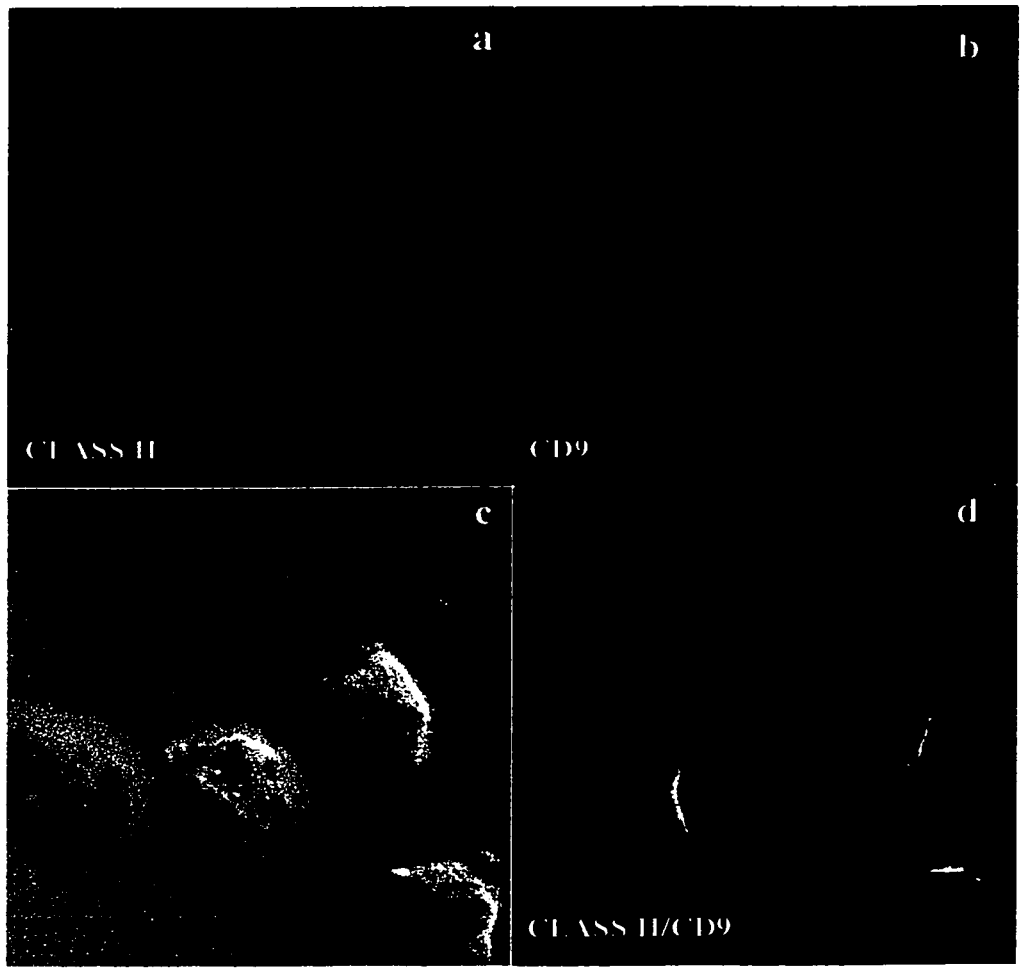


Fig. 4 *MHC class II capping co-caps CD9 in Raji/CD9 cells.*

MHC class II capping was induced by incubating with mAb 7H.3 and goat anti-mouse IgG.Alexa 594 for 1 hr at 37C. After fixation and blocking with normal mouse serum the cells were stained with FITC-conjugated anti-CD9 mAb 50H.19. a) MHC class II. b) CD9 c): visible. d) composite. CD9 and MHC class II co-cap extensively on the cell surface.

hour. After fixing and permeabilizing, the cells were blocked with saturating levels of murine IgG to block any unoccupied sites on the anti-mouse IgG and stained with the anti-MHC class II mAb 7H.3-FITC to show the MHC class II distribution. Cells formed strong CD9 caps with MHC class II largely co-localized to the capping area. Since capping CD9 causes MHC class II to co-cap CD9 and MHC class II must be associated in some way on the cell surface. It is of interest that despite the 60 minute incubation no CD9 is observed to internalize. MHC class II is detected intracellularly in a compartment that may be the MIIC. To further control for possible non-specific co-capping events and to show that co-capping is not a general membrane phenomenon, we tested the ability of MHC class II to co-cap with CD45. CD45 was cross-linked using anti-CD45 mAb followed by Alexa-594 conjugated goat anti-mouse IgG. When the capped cells were probed with the anti-MHC class II mAb 7H.3-FITC conjugate, no significant capping of MHC class II was observed (Fig. 3). Therefore, the co-capping of MHC class II with CD9 capping is not a general membrane response to capping conditions, but rather a specific response to the ligation of CD9.

Crosslinking MHC class II with mAb 7H.3 followed by goat anti-mouse IgG ALEXA-594 caused mAb CD9 to re-distribute into the cap (Fig. 4). Since CD9 co-localized so completely with MHC class II it suggests that the proteins are closely associated. However, densitormetry assay indicates only about 20% of CD9 co-immunoprecipitated with MHC class II. It is therefore possible that mild-detergents fail to maintain the full extent of non-covalent interactions that occur on the cell surface.

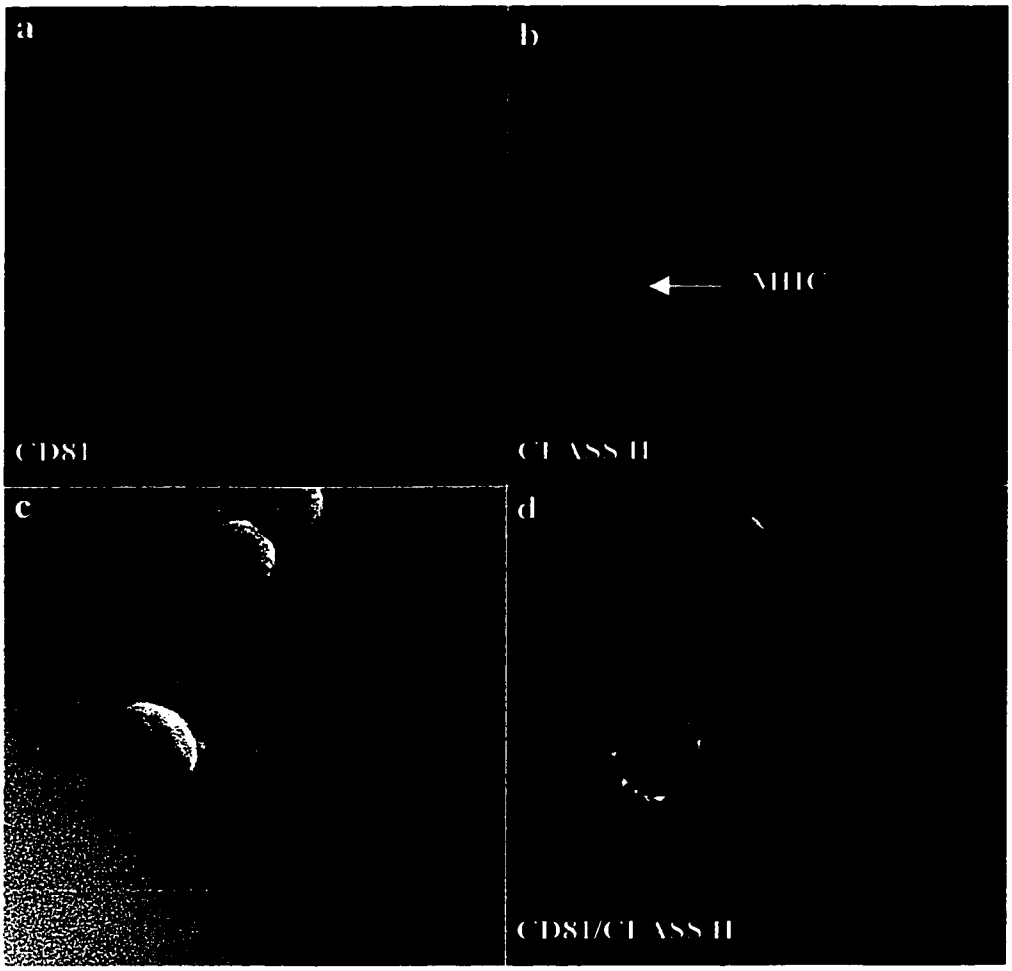


Fig. 5 *CD81 co-localizes with MHC class II on the surface of Raji/CD9, but not with internal MHC II.*

Raji/CD9 cells were fixed and stained with anti-CD81 and goat anti-mouse IgG Alexa-594 blocked with mouse IgG and stained with FITC conjugated anti-MHC class II mAb 7H.3. a) CD81 b) MHC class II c) visible light d) composite. Note that CD81 weakly co-localizes with the pool of intracellular MHC class II (MIIC).

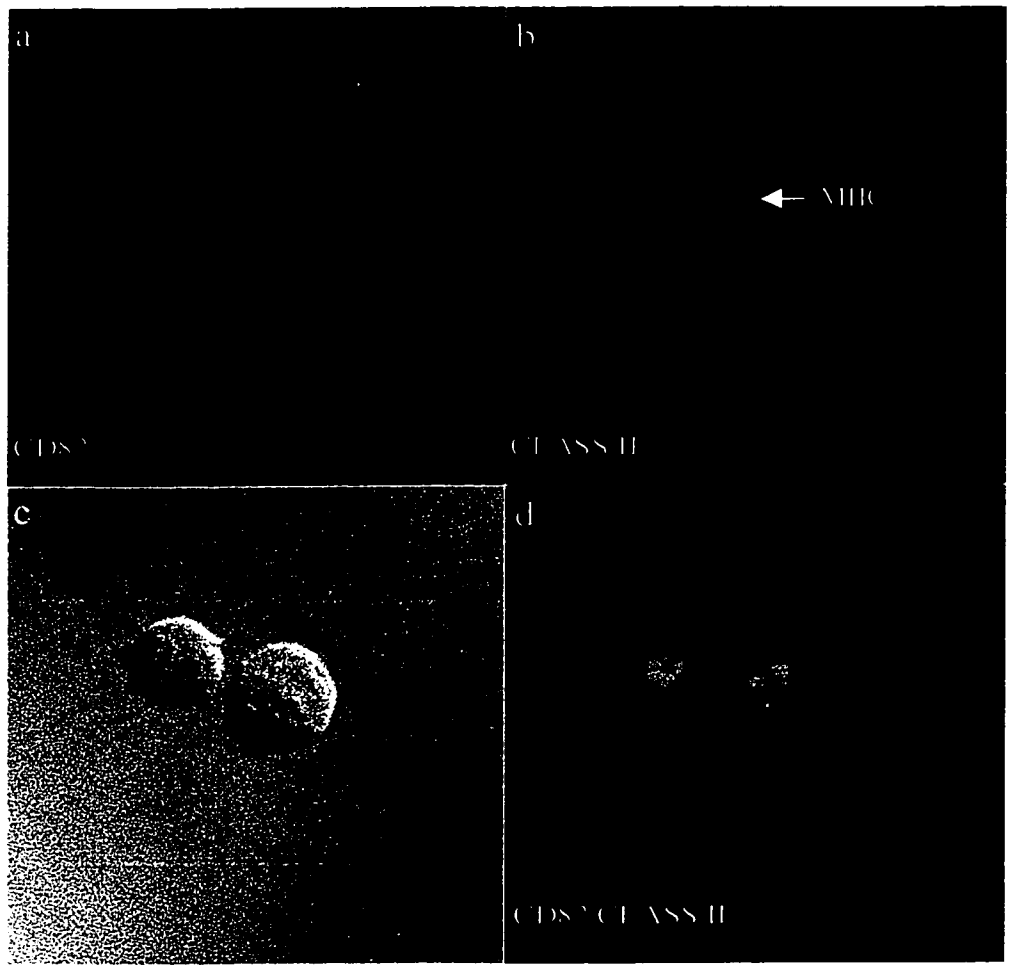


Fig. 6 *CD82 and MHC class II co-localize intracellularly in Raji/CD9.*

Raji/CD9 cells were fixed and stained with anti-CD82 mAb γC11 and with goat anti-mouse Alexa-594 (red), blocked with mouse serum, and stained with FITC conjugated anti-MHC class II mAb 7H.3 (green). a) CD82 b) MHC class II c) visible light d) composite. Note that CD82 weakly stains the cell surface but strongly associates with MHC class II in the MIIC

3. 4. CD81 co-localizes with MHC class II on the cell surface

CD81 is almost ubiquitously expressed among different tissues and is abundant on germinal center B cells. Using double-label immunofluorescence to investigate fixed cells we found that CD81 co-localized extensively with MHC class II on the cell surface (Fig. 5). Raji cells were attached to the poly-L-lysine coated slides for 15 min at room temperature. After fixing with paraformaldehyde and permeabilizing with 0.2% CHAPS cells were stained for CD81 using an anti-CD81 mAb followed by goat anti-mouse Alexa-594. MHC class II was detected by staining cells with mAb 7H.3-FITC. CD81 co-localized extensively with MHC class II on the cell surface. A small amount of anti-CD81 mAb was detected internally where it co-localized extensively with MHC class II. CD81 therefore associates to a minor extent with intracellular MHC class II in contrast to CD9 that is not present intracellularly.

3.5. Association of CD82 in MHC class II enriched vesicles

Like CD81, CD82 is broadly expressed on various leukocytes and other cell types. Double immunofluorescence staining of MHC class II and CD82 on fixed cells following attachment to the poly-L-lysine coated slides demonstrated that CD82 was present mainly in MHC class II enriched intracellular vesicles and only weakly on the cell surface (Fig. 6). The major presence of CD82 in MIIC compartments, suggests CD82 may play a major role in peptide acquisition.

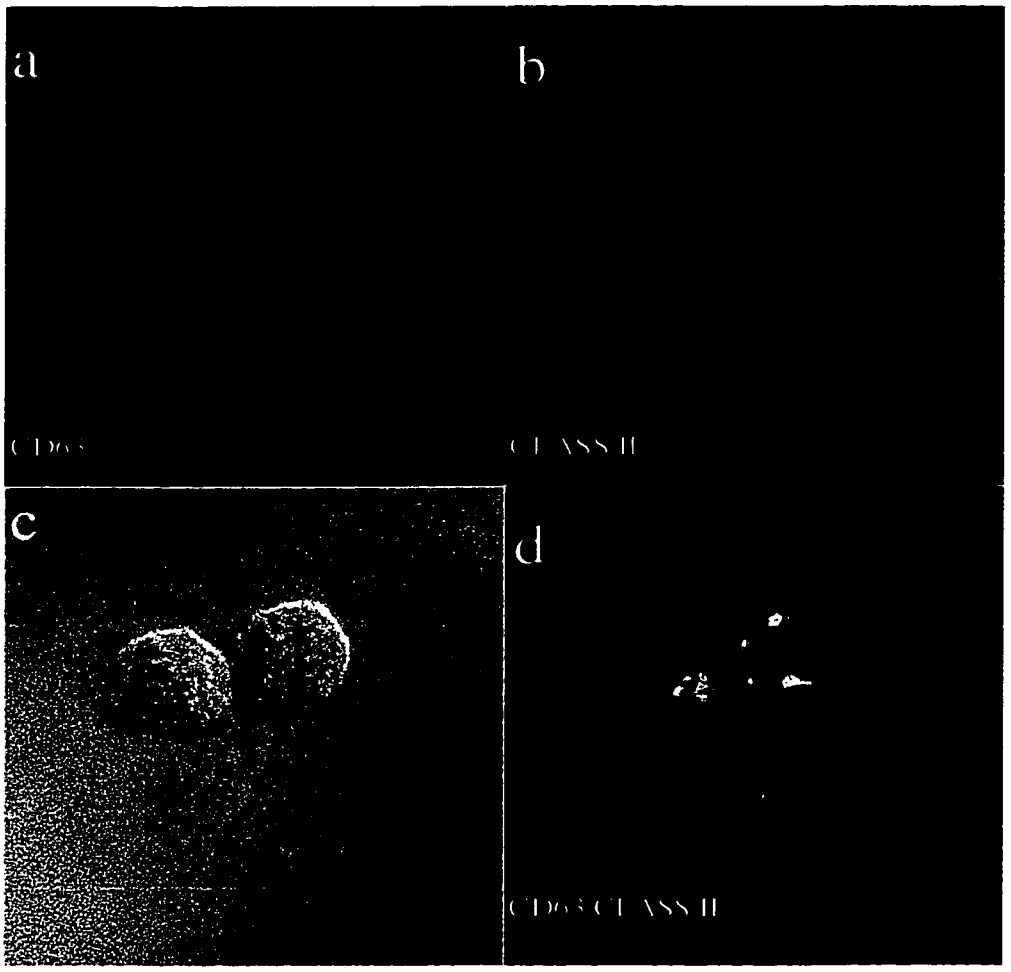


Fig. 7 *CD63 co-localizes with intracellular MHC class II in Raji/CD9 cells but is not expressed on the cell surface.*

Fixed cells were stained by anti-CD63 monoclonal antibody AP1.100 followed by goat anti-mouse Alexa 594 (red) and anti-MHC class II mAb 7H.3-FITC (green) a) CD63 b) MHC class II c) visible light d) composite

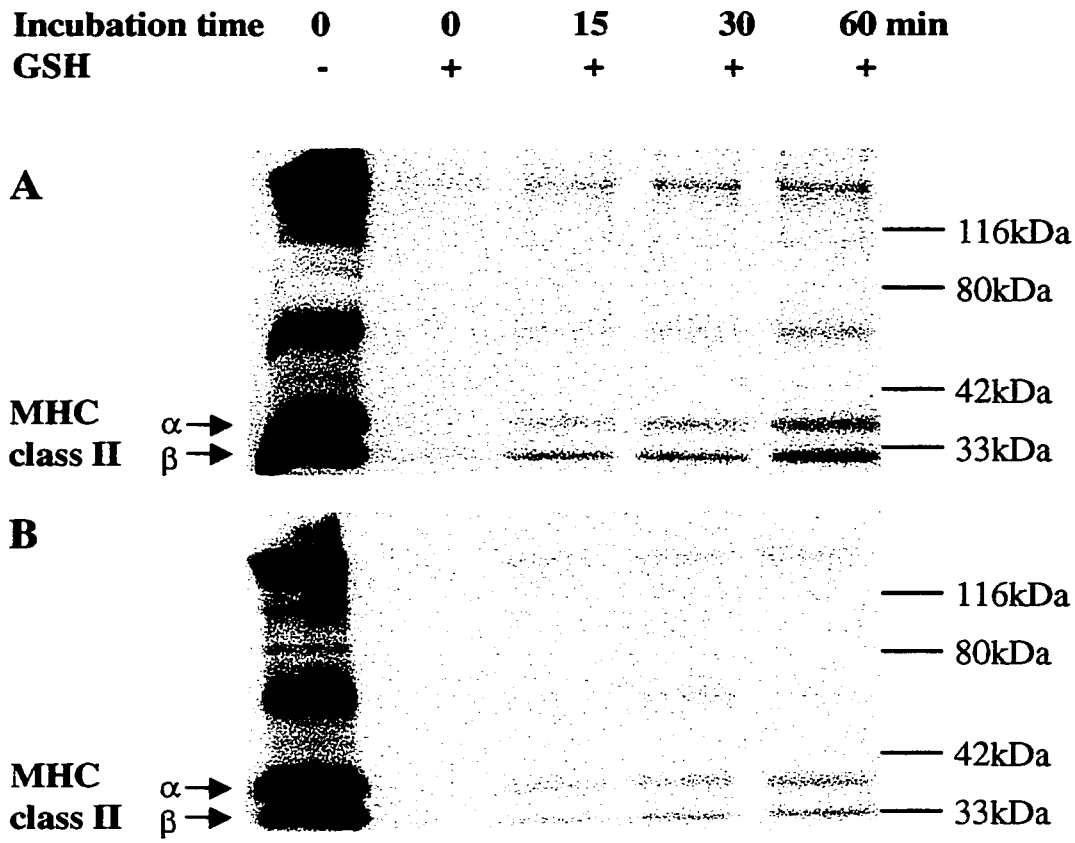


Fig. 8 *CD9 expression reduces the rate of spontaneous MHC class II internalization.*

Raji (A) or Raji/CD9 (B) cells were biotinylated with NHS-SS-biotin and incubated at 37C for the times indicated. Surface biotin was purged by reduction with glutathione. Since glutathione is membrane impermeable internalized MHC class II is protected from reduction by GSH and could be assayed by lysing the cells in 1% NP-40, immunoprecipitating with the anti-MHC class II mAb 7H.3, resolving the immunoprecipitated proteins by SDS-PAGE and blotting with S/A-HRP.

3.6. CD63 co-localizes with MHC class II in lysosomes

CD63, is a known component of MIIC and lysosomes but also appears on the surface of activated and tumor cells. Double immunofluorescence staining of fixed Raji cells for CD63 and MHC class II revealed that CD63, like CD82, is predominantly localized within the MIIC compartment (Fig. 7).

In this chapter, we demonstrate association of CD9 and MHC class II by immunoprecipitation and co-capping. By confocal microscopy and immunofluorescent staining we show that MHC class II distributes between an intracellular compartment and the cell surface and that different members of the tetraspanin family vary in their relative distribution between intracellular sites of MHC class II sequestration and the cell membrane. CD9 lies at one end of the spectrum being present only on the cell surface, whereas CD81 is well represented on the cell surface with only a minor presence internally, and CD82 and CD63 are poorly represented on the cell surface, but are well represented internally. Our data therefore corroborates and complements new published evidence that the tetraspanins CD82 and CD63 are present within internal vesicular compartments in association with MHC class II, HLA-DM, and HLA-DO and that exosomes, the externalized vesicles released by fusing multi-vesicular MIIC with plasma membrane, are enriched in MHC class II and the tetraspanins CD37, CD53, CD81, and CD82.

MHC class II molecules follow a unique intracellular pathway to bind processed exogenous antigen peptide and present it to helper T cells. The extensive association of different tetraspanins with MHC class II at different stages of MHC class II transport and in

different compartments strongly suggest the proteins are aggressively involved in MHC class II trafficking.

MIIC is the location of antigen peptide binding and Ii chain degradation. The fact that CD82 and CD63 form a complex with MHC class II and HLA-DM indicates that the peptide exchange process may be regulated by this association. Another possibility for CD63 and CD82 function in the MIIC is suggested by the finding that CD63 and CD82 contain a recently identified clathrin activating protein-2 binding motif (YSKV) that may serve as an endosome targeting signal. By travelling between the MIIC and the plasma membrane, CD63 and CD82 could promote both MHC class II-peptide trafficking to the cell surface and internalization of mature MHC class II through clathrin coated pits. Possibly CD82 and CD63 have a low presence on the cell surface (Fig. 6. and Fig. 7) because they do internalize so readily. Structurally CD9 and CD81 differ from CD82 and CD63, in the absence of internalization sequences. They may therefore lack the ability to internalize through clathrin-coated pits. Since both CD9 and CD81 are present within detergent insoluble compartments of the plasma membrane that are not thought to support clathrin-mediated endocytosis CD9 and CD81 may help stabilize MHC class II on the cell surface. The status of CD9 as a B cell activation antigen is compatible with a role in stabilizing MHC class II peptide complexes on the cell surface to facilitate further stimulation of helper T cells. In next chapter, we compare the internalization of MHC class II in Raji and Raji/CD9 transfectants to see whether CD9 plays a role in the internalization of MHC class II.

CHAPTER4: CD9 EXPRESSION AND MHC CLASS II TRAFFICKING

4.1. Introduction

MHC class II molecules are rapidly endocytosed from the cell surface [155, 156]. However, at present the function of such rapid and massive internalization is unclear. Two possibilities have been proposed based on the studies of MHC class II trafficking and antigen loading. The first possibility is that internalization involves sorting of newly synthesized MHC class II-Ii chain complexes into the endocytic pathway. Although studies have led to general agreement on the endocytic pathway as the site of MHC class II-Ii chain targeting and peptide loading, there is no consensus as the exact route that the class II-Ii chain complex takes from the TNG to the endocytic compartment. The classic model accepted by many researchers proposes that the MHC class II-Ii complex is directed to a unique pre-lysosomal compartment named the MIIC [16, 35]. It was therefore suggested that newly synthesized class II molecules do not access all of the endocytic pathway, but are directly transported from the TGN to this specialized MIIC compartment, where they accumulate and are loaded with incoming processed antigen peptides [42, 157]. However, other studies have yielded data inconsistent with this model by reporting the presence of MHC class II-Ii chain complexes on the cell surface [158, 159]. In a human B-cell line, a large population of MHC class II-Ii chain complexes reached endosomes by rapid internalization from the cell surface [15]. Similarly the majority of newly synthesized MHC class II-Ii complexes in dendritic cells appear to be first routed to the plasma membrane from which they are rapidly internalized and Ii degraded [160].

Although MHC class II-Ii complexes enter the endosomal pathway from the cell surface in dendritic cells that capture antigen on their cell surface the majority of MHC molecules on the B cell surface are not associated with Ii. It seems more likely that MHC class II internalization in B cells occurs independently of the Ii chain and serve to direct MHC class II molecules that already contain peptide through the early stages of the endosomal pathway where they have the opportunity to exchange peptide with peptides generated in early endosomes. The first direct evidence for an alternative antigen presentation pathway was the demonstration that immunodominant epitopes in the haemagglutinin protein of influenza virus and myelin basic protein require recycling of surface MHC class II molecules. Truncation of either one of the α or β cytoplasmic tails virtually eliminated internalization of MHC class II molecules as well as presentation of haemagglutinin from inactive virus particles. In contrast, the invariant chain-dependent presentation of matrix antigen from the same virus particles was unaffected by these truncations [161]. The peptide-loading site for the alternative pathway involves an earlier compartment from the traditional pathway [162]. Interestingly, it had already been shown that the efficient internalization of MHC class II-Ii chain into early endosomal compartments is not sufficient for MHC class II antigen presentation using chimeric proteins in which the Ii cytoplasmic tail is replaced with the cytoplasmic tail of the transferrin receptor [163]. Maybe different proteolysis requirements exist for different antigen presentation pathways. This is supported by a recent observation that antigen presentation controlled by a dileucine-based internalization signal didn't require newly synthesized MHC class II and appeared to involve determinants requiring only limited proteolysis [164].

Although the mechanism of MHC class II sorting in the Golgi and of internalization from the cell surface is not well defined, evidence suggests that clathrin-coated vesicles (CCVs) may be involved. In eukaryotic cells, clathrin-coated vesicles mediate the internalization of plasma membrane protein receptors and the sorting of transmembrane proteins in the TGN for transport to endosomes [165-167]. The first step in CCV formation is translocation of the cytosolic adaptor protein complex adaptor protein 1 (AP1) or adaptor protein 2 (AP2) onto the target membrane where transmembrane proteins bearing the CCV sorting signals are localized. These targeting signals consist of a tyrosine-based motif (Asn-Phe-X-Tyr) and a dileucine-based motif (two leucine residues or one leucine and a small hydrophobic residue) [168] in the cytoplasmic domains. Adaptor proteins not only concentrate transmembrane proteins by interacting with the sorting signals but also interact with clathrin triskelions which, when organized into cages, constitute the outer layer of the coat. AP1 is found associated with TGN-derived vesicles, and AP2 with those derived from the plasma membrane [169], indicating their involvement in sorting and internalization respectively.

Class II-Ii chain complexes only account for a small amount of total MHC class II internalization. The internalization signal in the Ii chain-free, mature surface MHC class II molecules is still unknown. MHC class II molecules with truncated cytoplasmic tails, expressed in transfected fibroblasts, failed to internalize, suggesting that the cytoplasmic tails of MHC class II molecules mediate internalization [161]. A recent report indicated that dileucine-based signal in the cytoplasmic tail of MHC class II β chain may be responsible for the Ii-independent class II internalization [164]. The finding that surface MHC class II molecules associate with various other membrane proteins especially tetraspanins, suggests

that these associations may regulate the internalization of MHC class II. Indeed, CD63 and CD82, two tetraspanins having internalization sequences in their cytoplasmic domains, associate extensively with MHC class II in MIIC compartments. We have demonstrated that CD9-MHC class II form complexes on the cell surface. In this study, we examine the role for CD9 in the internalization and trafficking of MHC class II molecules in Raji cells by ectopic expression of CD9.

4.2. Expression of CD9 in a B cell line inhibits spontaneous internalization of MHC class II. The degree of inhibition correlates directly with the level of CD9 in cloned CD9 transfectants.

In most internalization experiments, MHC class II molecules are tagged with an agent that could affect endocytosis and/or modify its intracellular routing. We have chosen to employ a less invasive and antibody-independent protocol to follow the internalization of MHC class II molecules on the surface of Raji and CD9 transfected Raji cells. After surface biotinylation with reduction-cleavable NHS-SS-biotin at 4°C for 30 minutes in PBS, MHC class II internalization was assessed by incubating cells at 37° C in complete medium for various periods of time. Non-internalized cell surface label was removed by stripping surface biotin with the membrane impermeable reducing reagent glutathione. Internalized biotinylated MHC class II is protected against purging and can be recovered by immunoprecipitation. MHC class II was immunoprecipitated from NP40 detergent lysates with mAb 7H.3. The amount of internalized MHC class II was then assayed following SDS-PAGE by western blotting with S/A-FITC (Fig. 8).

MHC class II is strongly expressed on the surface of Raji cells. Stable transfection of CD9 into Raji did not affect the expression of MHC class II as judged by the amount of immunoprecipitable biotinylated MHC class II prior to incubation (Fig. 8. lane 1). The efficiency of surface biotin purging by glutathione is better than 95% as demonstrated in lane 2. Internalization of biotinylated MHC class II molecules could be observed as early as 15 min, indicating rapid internalization of MHC class II from the cell surface (Fig. 8A). After 60 min at 37°C, about 20% of total MHC class II molecules (compared to lane 1) were internalized, ~80% of molecules remaining at the cell surface. Because no antibodies were used we could rule out the possibility that internalization was affected by the engagement of Fc receptors or by antibody-induced class II clustering. Interestingly we found that CD9 transfection reduced the amount of MHC class II internalization in Raji cells (Fig. 8B). At all three time points about 50% less internalization of MHC class II was observed in the transfectant. Thus our experiment demonstrates that CD9 reduces MHC class II internalization as might be predicted if CD9 increased the residency of MHC class II in lipid rafts.

The effect of CD9 expression upon the internalization of MHC class II was also investigated by confocal immunofluorescence microscopy. Anti-class II mAb 7H.3 was bound to Raji or Raji/CD9 on ice for 30min, washed and labeled with Alexa-488 conjugated goat anti-mouse IgG. Since first and second antibodies were used to label MHC class II the conditions were very different from those of the biotinylation experiment. Internalization was initiated by 37°C incubation in RPMI-1640 in 10% FCS, in the presence of 5 μg/ml of TRITC conjugated transferrin (Tfn). In Raji cells (Fig. 9), after 15 min at 37°C, MHC class II began to cap and internalize indicating rapid

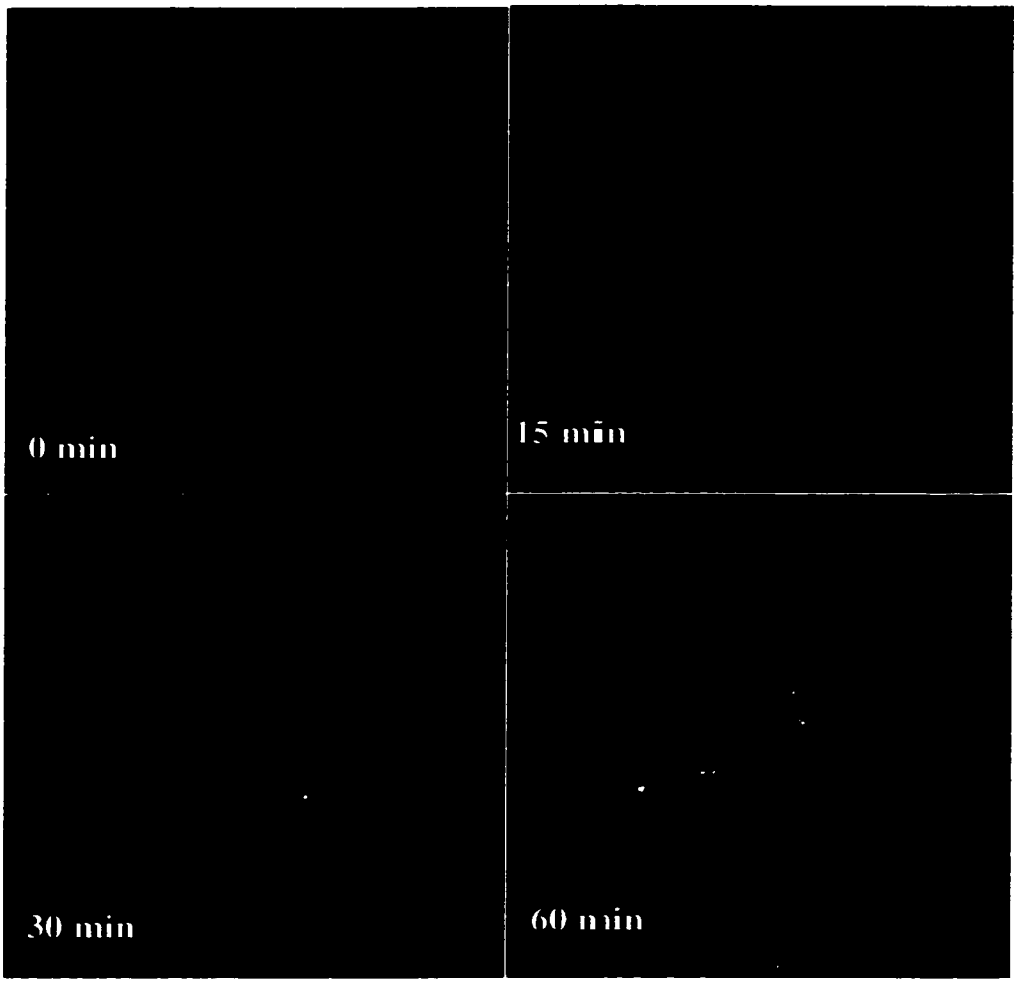


Fig. 9 *MHC class II does not colocalize with the early endosome marker transferrin.*

MHC class II internalization was followed by labeling Raji cells with anti-MHC class II mAb 7H.3 and goat anti-mouse IgG ALEXA-488 (green) and incubating for the intervals specified. TRITC-conjugated transferrin (red) was maintained in the medium to map early endosomes. Transferrin internalized rapidly in contrast to MHC class II that internalized more slowly. Although MHC class II accumulated in vesicles in similar subcellular locations as transferrin it did not co-localize with it.

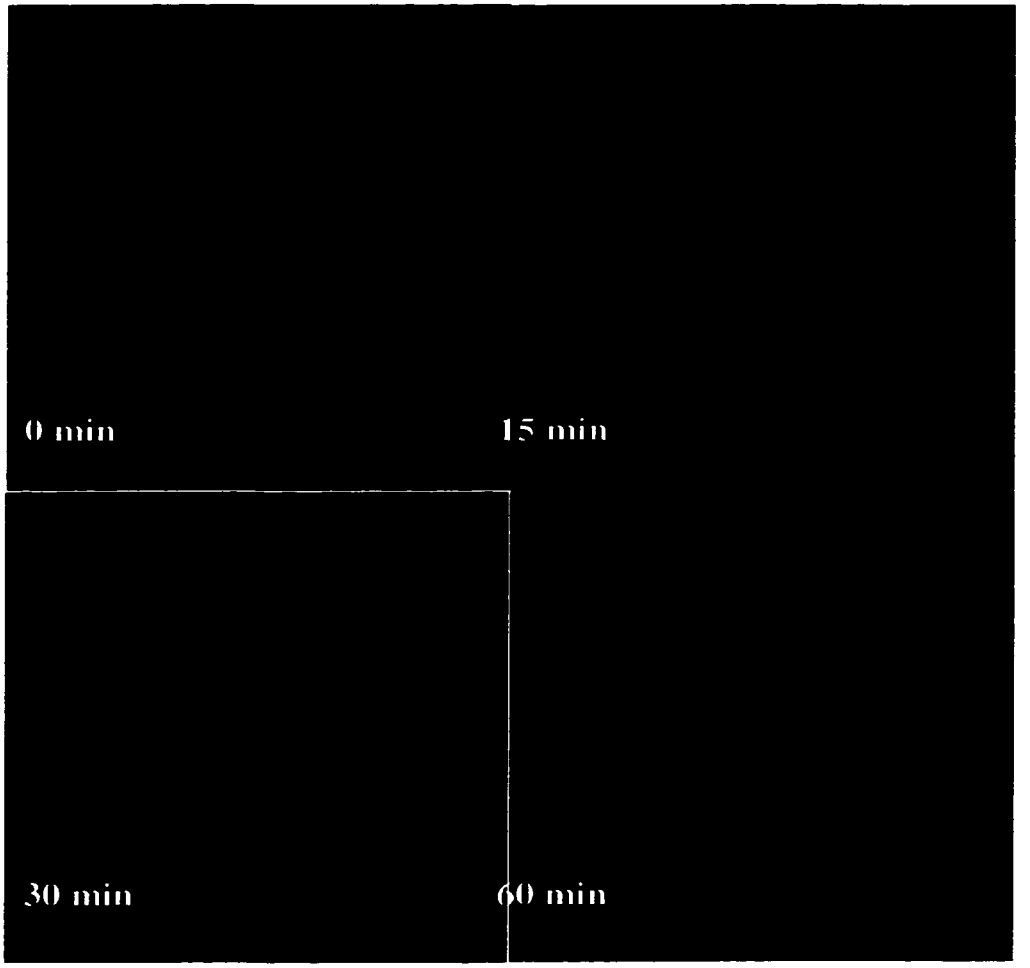


Fig. 10 *CD9 expression reduces the rate of internalization of MHC class II.*

Raji/CD9 cells were labeled with anti-MHC class II mAb 7H.3 and goat anti-mouse IgG ALEXA-488 (green) and incubated at 37C for the times indicated. TRITC-labeled transferrin (red) was added at the start of incubation. Very little MHC class II had internalized even after an hour (compare Fig.9).

internalization of MHC class II. The size and number of MHC class II intracellular vesicles increased at 30 min and 60 min intervals of incubation. However, the majority of MHC class II remained on the cell surface. In contrast less MHC class II internalization was observed in Raji/CD9 transfectants (Fig.10). After 15 min 37°C incubation, no internalized class II vesicles could be detected, in concordance with the result obtained by biotin labeling MHC class II. Compared to Raji cells, Raji/CD9 cells contained less internalized antibody at 30 min and 60 min of incubation. Thus using two separate approaches to follow MHC class II trafficking we observed that CD9 reduced recycling of MHC class II from the cell surface. When single clones of cells are compared there is a danger that the characteristics observed are those of the selected clone and not of the transfected gene. We therefore compared the internalization rates of MHC class II in clones of Raji/CD9 selected by FACS for different levels of CD9 expression (Fig.11). The low, medium and high level of CD9 expression in the selected clones remained stable as confirmed by FACS analysis (Fig 11. left panel). Internalization was performed as before and samples of internalized MHC class II examined at 45 min and 90 min intervals at 37°C. The results (Fig.11 right panel) demonstrate that MHC class II internalization is decreased in CD9 transfected Raji cells in a manner that correlates directly with the level of CD9 expression. Thus our data are consistent with CD9 expression negatively influencing the trafficking of the MHC class II.

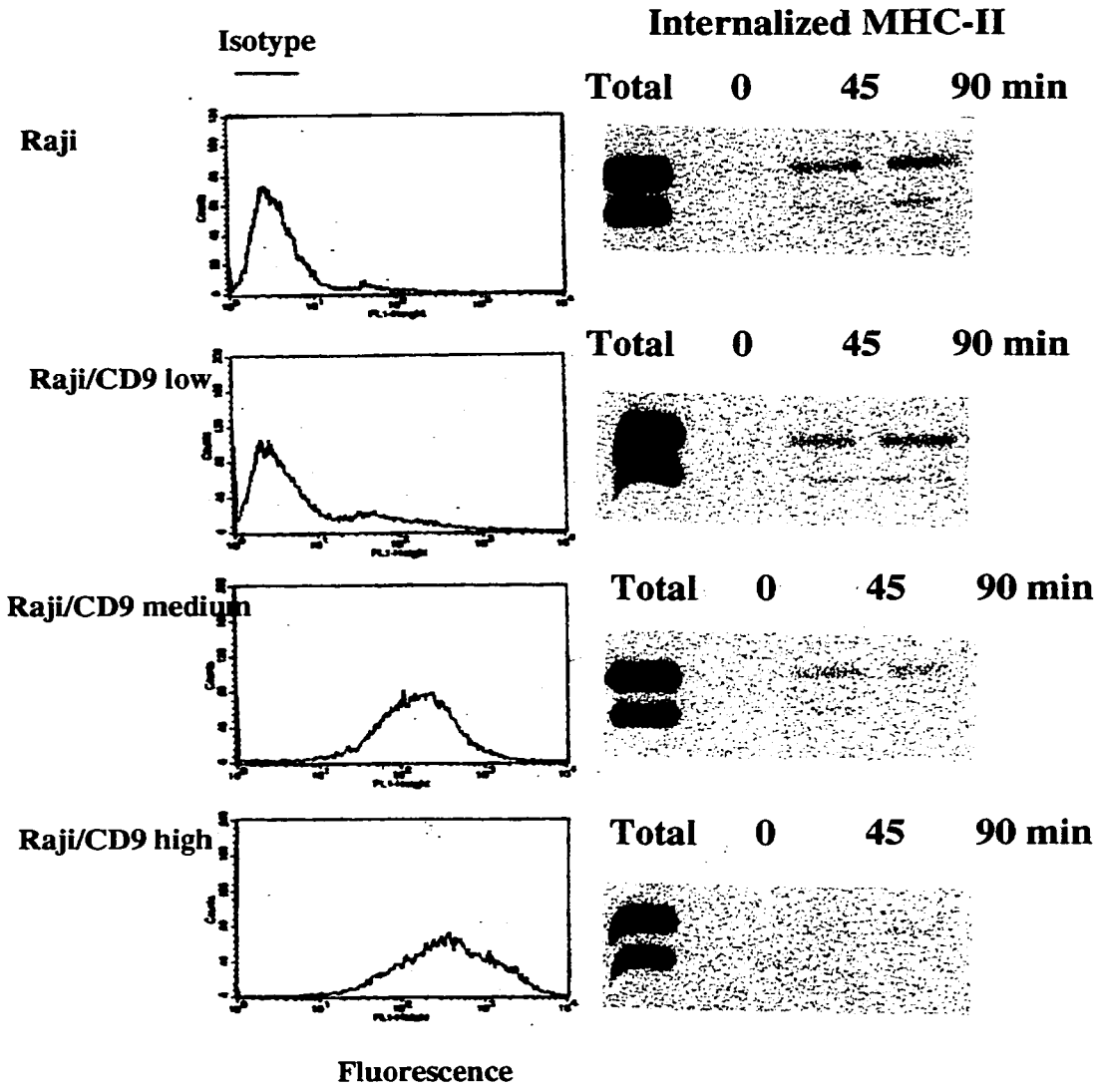


Fig. 11 *Spontaneous internalization of MHC class II is reduced in proportion to the level of CD9 expression in cloned CD9 transfectants.*

Raji/CD9 was sorted into low, medium, and high CD9 expressor clones and compared for MHC class II internalization. Cells were surface-labeled with NHS-SS-biotin on ice and incubated for the times indicated before purging surface biotin with glutathione. Internalized MHC class II was immunoprecipitated with mAb 7H.3 and probed with SA.HRP following resolution by SDS-PAGE.

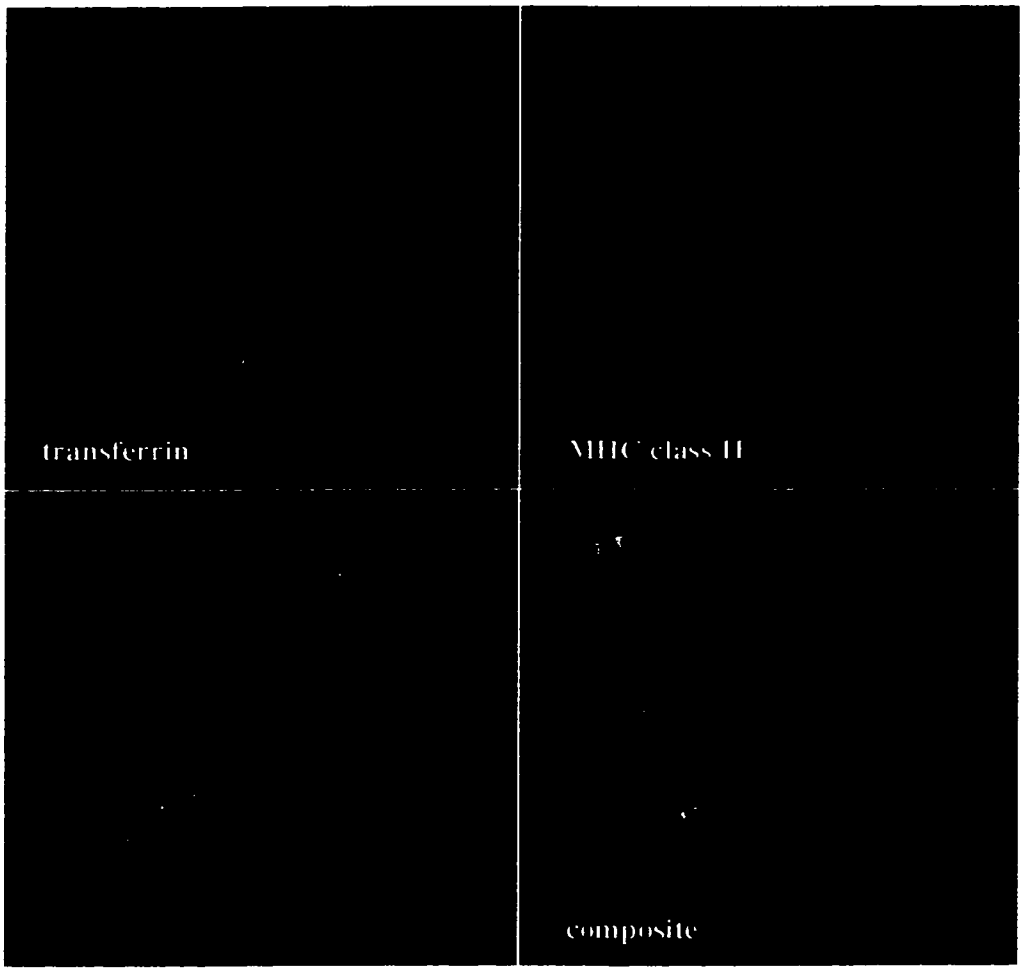


Fig. 12 *Internalized MHC class II does not co-localize with transferrin in Raji cells.*

Raji cells were labeled with mAb 7H.3 and goat anti-mouse IgG ALEXA-488 (green) for 60 minutes in the presence of transferrin-TRITC (red). MHC class II did not co-localize with subcellular vesicles within the early endosomal pathway that are labeled by transferrin.

4.3. Localization of internalized MHC class II by confocal microscopy

Successful presentation of peptides by MHC class II from different antigens involves distinct pools of MHC class II molecules and utilization of different pathways for antigen acquisition. The fast internalization of surface MHC class II molecules may play a role in the processing and loading of peptides in endocytic compartments of different proteolytic and physiologic properties. We therefore asked where in the endocytic pathway internalized MHC class II was localized. For this purpose, two markers were used for labeling the different endocytic compartments. The intracellular transferrin receptor (TfnR) is extensively localized in early endosomes. By 37°C incubation in TRITC-conjugated Tfn solution, we are able to label early endosomes. The late endosome and lysosome compartments were labeled by Lysotracker, a weak basic amine conjugated to a red fluor that selectively accumulates in cellular compartments of low internal pH.

Cells were incubated for 30 min at 4°C with the anti-MHC class II mAb 7H.3 and for 30 min at 4°C with GAM Alexa-488. After washing twice, the pre-warmed (37°C) probe-containing (5 μg/ml for Tfn and 1:10000 dilution for Lysotracker) medium was added and cells incubated for 1 hour at 37°C. The co-localization of internalized MHC class II with Tfn or Lysotracker was studied by confocal microscopy. In Raji (Fig. 12), after 1 hour of incubation, intracellular MHC class II (green) and Tfn (red) were observed in large amounts. However, most of the internalized MHC class II and TfnR did not have the same intracellular distribution, suggesting that the majority of MHC class II was not retained within early endosomes.

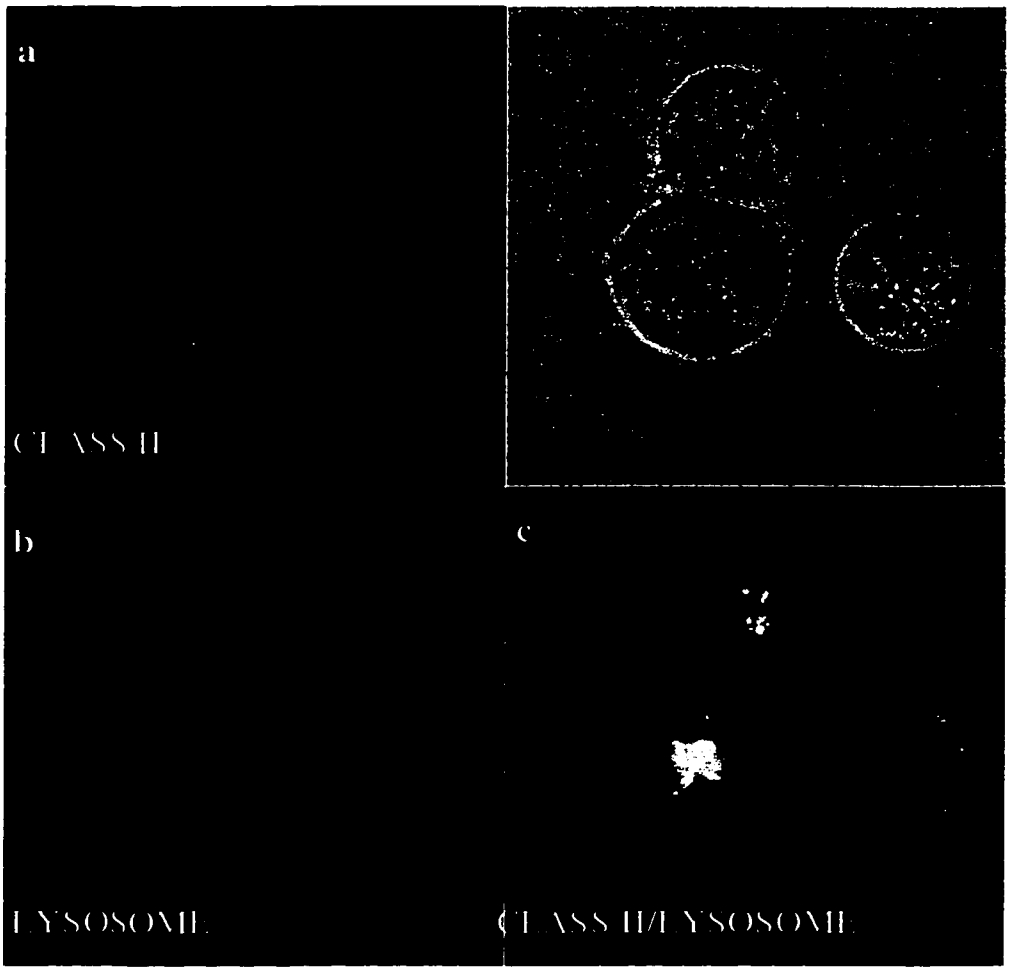


Fig. 13 *Internalized MHC class II in RAJI cells accumulates in a lysosomal compartment.*

RAJI cells were labeled with anti-class II mAb 7H.3 followed by goat anti-mouse Alexa 488 (green). After 1 hr incubation at 37C in medium containing 1:10,000 dilution of lyso-tracker, the cells were examined by confocal microscopy. Internalized MHC class II was localized largely to lysosomes.

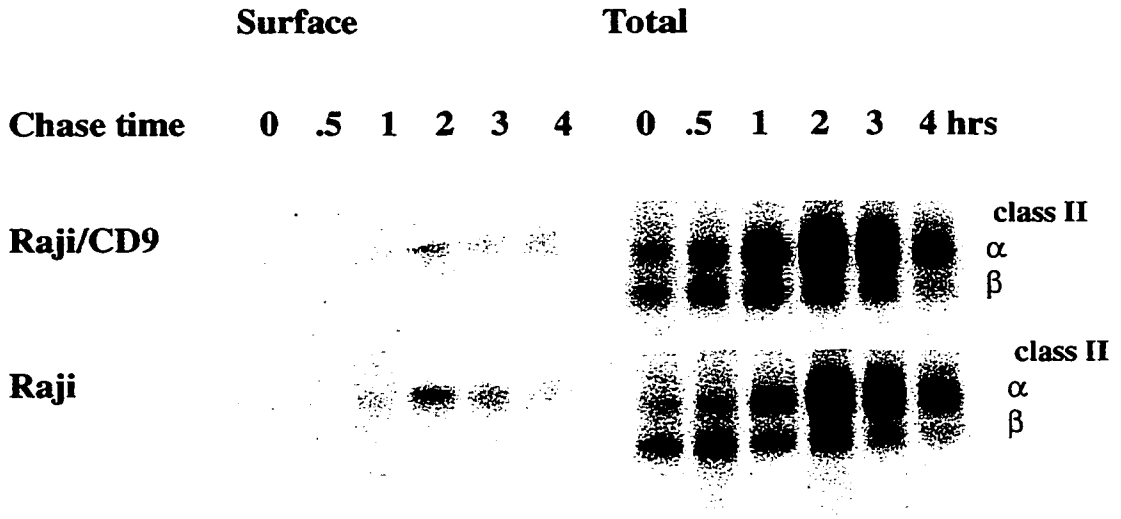


Fig. 14 *Expression of CD9 reduces MHC class II trafficking to the cell surface.*

Raji or Raji/CD9 cells were pulse-labeled with ^{35}S methionine for 30 min and chased for the intervals indicated. At each time point cell surface proteins were biotinylated and total and cell surface MHC class II sequentially immunoprecipitated with anti-MHC class II mAb 7H.3 (total MHC class II) and streptavidin-agarose (surface MHC class II). ^{35}S labeled MHC class II were visualised by autoradiography of SDS-PAGE resolved proteins.

The internalized MHC class II did not overlap with internalized TfnR and less intracellular MHC class II was observed in the transfectants. Our results therefore demonstrate that internalized MHC class II does not accumulate in early endosomes. When Lysotracker was presented during 37°C incubation, extensive co-localization of MHC class II and Lysotracker was observed in Raji cells (Fig. 13). Because lysosomes are classically defined as terminal degradation compartments [170], the vesicles seen by confocal microscope are likely to represent MIIC compartments (late endosomes and early lysosomes).

4.4 Trafficking of newly synthesized class II, but not MHC class I, was delayed by ectopic CD9 expression

Membrane and secreted proteins synthesized in the ER and transported to the Golgi follow a default pathway to the cell surface, deviation from which requires the presence of sorting signals. The major site of signal-dependent protein sorting within the secretory pathway is in the TGN. Protein either enters the endocytic pathway directly from the TGN, or via transient expression on the cell surface followed by rapid internalization and delivery to endocytic compartments.

MHC class II molecules are routed through the endocytic pathway via a signal-dependent mechanism and require enzymatic activity of proteases for Ii breakdown, peptide binding, and transport to the final destination on the cell surface. MHC class-II associated Ii chain not only promotes the proper folding of MHC class II molecules in the ER, but also

contain signals for sorting in the N-terminal cytoplasmic tail. This cytoplasmic tail lacks tyrosine based motifs but contains multiple dileucine based signals that finally sort MHC class II αβIi complexes to an endocytic compartment with endosomal/lysosomal characteristics. It is therefore possible that MHC class II transportation from the ER via the endocytic pathway to the cell surface may be regulated by associative proteins such as tetraspanins.

To address this question, Raji or Raji/CD9 were pulse-labeled with ^{35}S-Methionine for 30 min and chased for various periods of time. At intervals, the radiolabeled MHC class II or MHC class I molecules that reached the cell surface were detected by derivatization with NHS-SS-biotin and sequentially immunoprecipitating with antibody against MHC class II with streptavidin agarose.

In the absence of CD9, newly synthesized MHC class II molecules appeared on the cell surface of Raji cells at 1 hour (Fig 14. Bottom panel). The kinetics were similar to those previously reported [40]. In the cells expressing CD9, cell surface appearance of MHC class II was delayed and reduced in amount (Fig. 14 upper panel). Transport of MHC class I to the cell surface was also investigated. As with MHC class II it took 2 hours to reach maximal levels of surface MHC class I expression (Fig 15). The level of expression was similar in the Raji and Raji/CD9. The pulse-labeling experiments therefore suggest that CD9 affects MHC class II and but not MHC class I trafficking to the cell surface. CD9 may therefore influence both trafficking of MHC molecules to the cell surface and their rate of internalization. If CD9 regulates the association of MHC molecules with lipid rafts and lipid rafts serve as platforms for sorting and trafficking then CD9 expression could alter the kinetics of peptide surveillance.

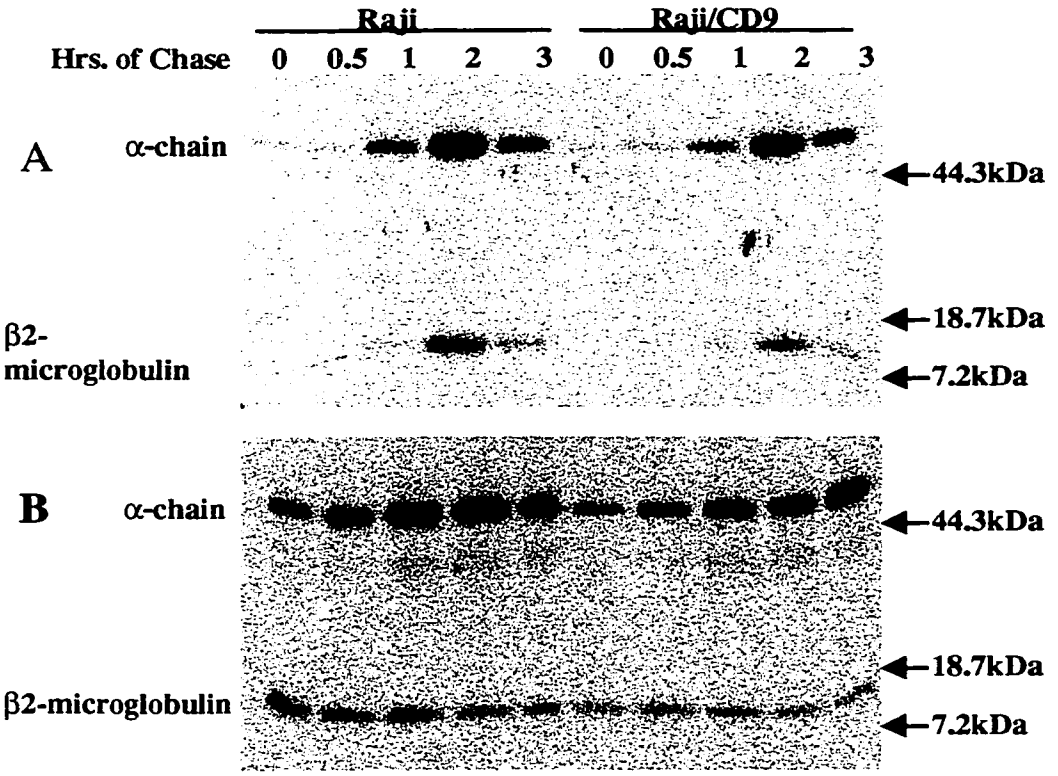


Fig. 15 *Expression of CD9 does not reduce MHC class I trafficking to the cell surface.*

Raji or Raji/CD9 cells were pulse-labeled with ^{35}S methionine and chased for the times indicated. Cell surface proteins were biotinylated and total and cell surface-labeled MHC class I sequentially immunoprecipitated with mAb 9H.1 (B) and streptavidin-agarose (A). ^{35}S labeled MHC class I was visualised by autoradiography of SDS-PAGE resolved proteins

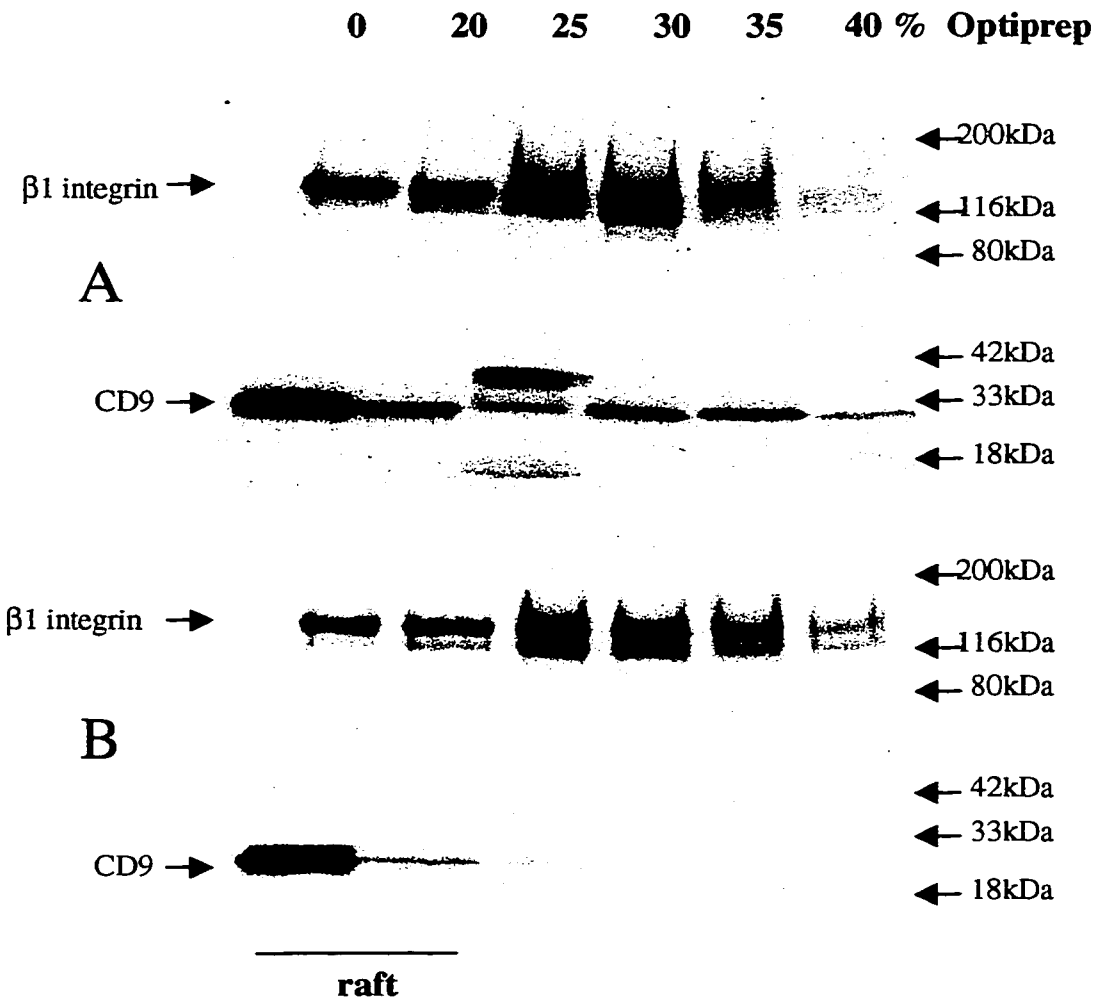


Fig. 16 *CD9, but not β1 integrin translocates to a low buoyant density fraction on antibody crosslinking.*

Cell surface CD9 was crosslinked with anti-CD9 mAb 50H.19 and goat anti-mouse IgG for 60 minutes at 37°C. Cells were lysed in 1% CHAPS and separated on an Optiprep buoyant density gradient before SDS-PAGE and immunoblotting with HRP-conjugated mAb AP1.138 against β1 integrin and HRP-conjugated mAb 50H.19. HRP-conjugated antibodies were visualised by enhanced chemiluminescence.

Membrane trafficking is a very complex process and involves the movement of lipid membranes and associated proteins among intracellular compartments and the plasma membrane. The generation of transport vesicles through budding processes and the fusion of vesicles with acceptor membranes clearly involves significant alterations in membrane structure. Recent progress in membrane structure is the lateral assembly of sphingolipids and cholesterol to form platforms that serve to support numerous cellular events in membrane traffic and signal transduction [109]. These "lipid raft" microdomains perform their function by preferentially associating with specific proteins while excluding others. In the next chapter, we investigate whether CD9, and MHC class II associate with low buoyant density detergent insoluble domains.

CHAPTER 5: CD9-MHC CLASS II COMPLEXES ARE ASSOCIATED WITH LIPID RAFTS

5.1. Introduction

T cell activation is initiated by engagement of MHC class II-peptide complexes on the surface of an antigen-presenting cell with the TCR-CD3 complex on a CD4+ T helper cell [171]. Recent evidence suggests that T cell activation may be closely related to lipid raft re-organization. Cross-linking T cell GPI-anchored proteins may induce signaling in a raft-dependent manner. For example, cross-linking GPI-anchored CD59 and CD48 in Jurkat T cells promotes calcium flux that is inhibited by reducing cholesterol [172]. Similarly activation of T cells through the T cell receptor (TCR) is impaired in cells defective in GPI anchor synthesis [173] and Jurkat cells cultured in polyunsaturated fatty acids supplemented medium have diminished calcium responses to CD3 complex or GPI- anchored CD59 clustering [172]. Cholesterol may influence T cell activation by regulating raft assembly in response to TCR engagement. In support of this notion the TCR translocates to a low-density, detergent-insoluble microdomain on antibody induced engagement accompanied by the accumulation of tyrosine-phosphorylated substrates and an increase in Lck activity within the raft [105]. T cell co-stimulatory signals also require lipid raft integrity. CD28 engagement by anti-CD3/anti-CD28 coated beads redistributed and clustered protein tyrosine kinase-enriched raft microdomains at the site of TCR engagement resulting in higher and more stable tyrosine phosphorylation of several substrates and higher consumption of Lck [106]. CD2 engagement of GPI-linked CD48 on T cells recruits rafts to

sites of T cell-B cell interaction. CD48/TCR interaction enhances raft-dependent associations of TCR with the actin cytoskeleton and tyrosine phosphorylation suggesting that rafts provide sites for the integration of receptor-induced signals and cytoskeletal reorganization [174].

Since T cell activation requires engagement of TCR/CD3 complexes by class II-peptide ligands on B cells, and both the TCR complex and peptide-bound MHC molecules interact with low affinity, it is likely that cognate signaling requires clustering and stabilization of both the TCR and MHC complexes in their respective membranes [175]. Recruitment of cognitive proteins into lipid rafts could facilitate protein-protein interaction and signal transduction. Whether B cells organize lipid rafts in response to MHC class II clustering is not known. However, it was recently established that MHC class II clustering resulted in translocation of MHC class II molecules into a lipid raft upstream of phosphotyrosine signaling in a cytokine stimulated monocytic cell line [176]. In this chapter, we investigate the possible association of MHC class II, CD9 and their complexes with low-density detergent-insoluble microdomains.

5.2. CD9 has a minor presence within lipid rafts that is enhanced by CD9 clustering.

CD9 and CD81 were recently identified as major proteins of a CHAPS insoluble low buoyant density in kidney epithelial cells using nano-electrospray tandem mass spectrometry [135]. Although the fraction contained many transport vesicle proteins CD9 and CD81 were not associated with membranes containing transport proteins and presumably derive from plasma-membrane rafts. It is not known whether CD9 and CD81 are components of B cell

lipid rafts. To address this question we employed buoyant density ultra-centrifugation to separate the low-density buoyant fraction of B cell lysates. Cells were lysed in 1% CHAPS either before or after anti-CD9 mAb cross-linking with a goat anti-mouse IgG to promote clustering. Gradient ultracentrifugation was performed at 4°C for 4-18 hours and six fractions collected. Following SDS-PAGE and transferring to a nitrocellulose membrane, CD9 was detected by HRP conjugated anti-CD9 mAb 50H.19 by enhanced chemiluminescence (Fig.16). In the absence of crosslinking CD9, distributed throughout the gradient but was enriched in lowest buoyant fraction. Cross-linking surface recruited CD9 almost entirely into the lowest density fraction suggesting that CD9 responds to ligation by entering lipid rafts. In contrast β1 integrins, receptors that are known to associate with tetraspanins and CD9, did not co-localize with CD9 in the raft (Fig 16).

Unlike other cholesterol-binding reagents that incorporate into the membrane, methyl-β-cyclodextrin (MBCD) is strictly surface-acting and selectively extracts membrane cholesterol by including it in its central, non-polar cavity of cyclic oligomers of glucopyranoside in an α-1,4 glycoside linkage [177]. For this reason MBCD is widely used to probe the involvement of lipid rafts that are disrupted by lowering of membrane cholesterol. We examined the effects of MBCD on the buoyancy and detergent insolubility of CD9 in Raji/CD9 cells. Cells treated with 10 mM MBCD for 1 hour at 37°C and lysed in 1% cold CHAPS selectively lost CD9 from the two lowest buoyancy fractions implicating these two fractions in the formation of lipid rafts (Fig. 17). Whether

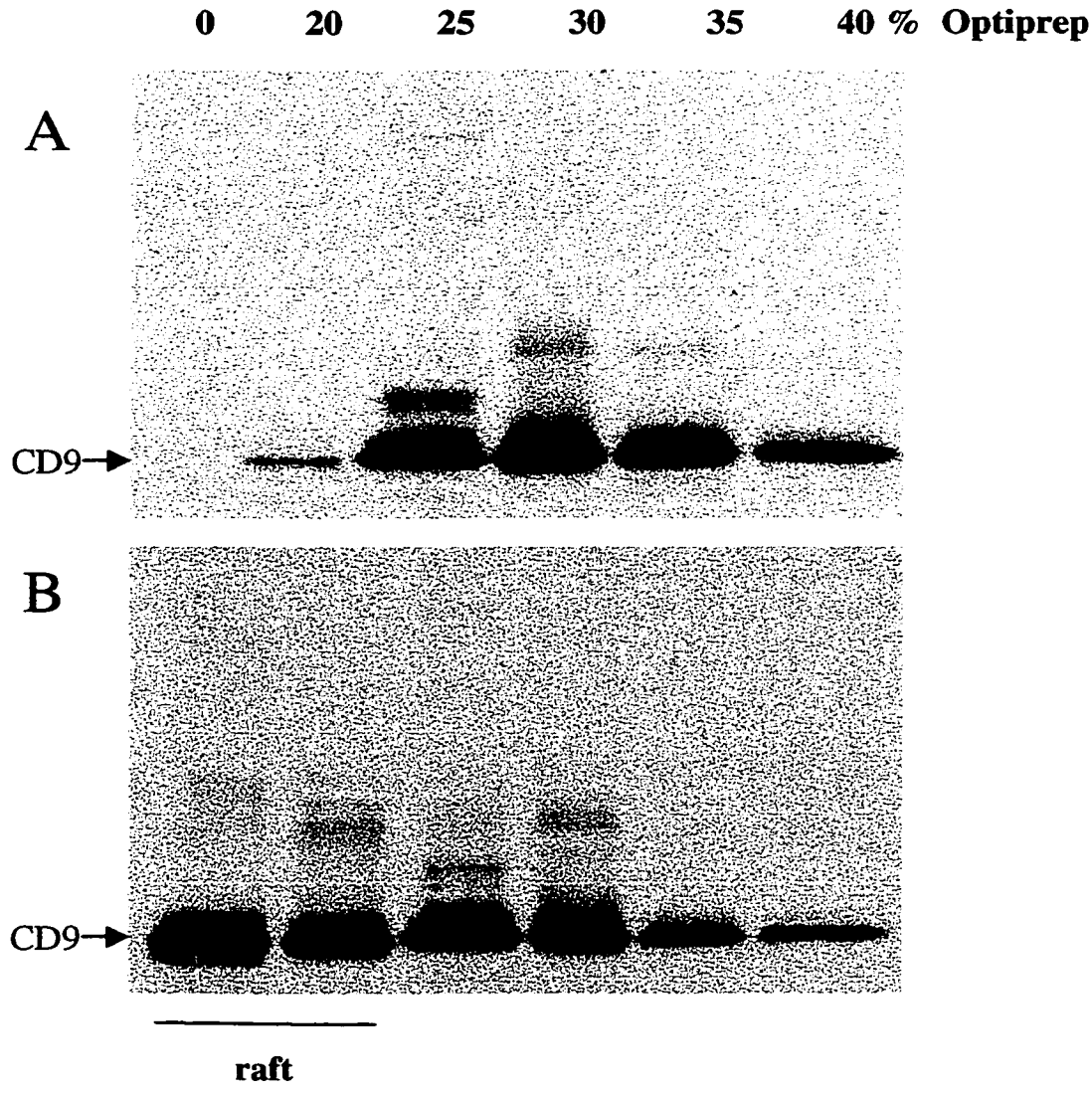


Fig. 17 *Cholesterol depletion results in loss of CD9 from the low buoyant-density fraction indicating that it is in lipid rafts.*

Raji/CD9 cells were treated with 10 mM methyl-β-cyclodextrin for 1 hour at 37C to deplete membrane cholesterol (A) or incubated without methyl-β-cyclodextrin to serve as a control (B). Cells were lysed in 1% CHAPS and fractionated on an Optiprep buoyant density gradient, resolved by SDS-PAGE and blotted with mAb 50H.10.HRP. Antibody was visualised by enhanced chemiluminescence.

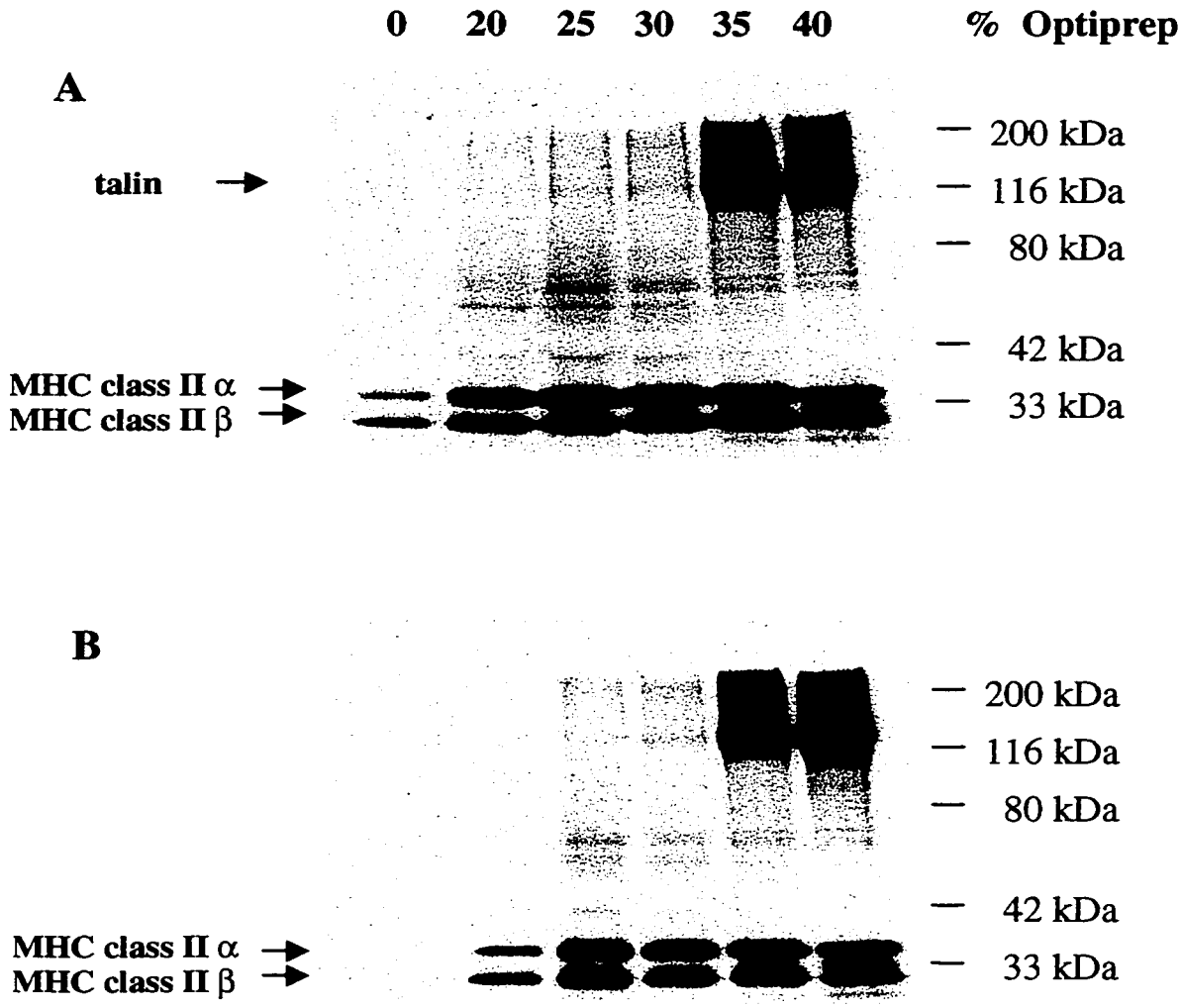


Fig. 18 *Cholesterol depletion results in loss of MHC class II from the low buoyant density fraction indicating that it is in lipid rafts.*

Raji/CD9 cells were incubated at 37°C for 1 h alone (A) or with 10 mM methyl-β-cyclodextrin to deplete membrane cholesterol (B). The cells were lysed in 1% CHAPS, and fractionated on an Optiprep buoyant density gradient. Proteins were resolved by SDS-PAGE and blotted with an anti-class MHC class II polyclonal antiserum against heavy and light chains followed by HRP conjugated anti-rabbit IgG.HRP and anti-talin mAb followed by HRP conjugated anti-mouse IgG.

CD9 is selective extracted from the lipid rafts or just simply dispersed into the non-raft region of the cell surface is not known.

5. 3. MHC class II has a minor presence in lipid rafts that is enhanced by clustering

It has been known for some time that a small proportion of MHC class II molecules are detergent insoluble and it was assumed that they associate with the actin cytoskeleton. It has been estimated that as much as 20% of MHC class II molecules exhibit this property [178]. Truncating the cytoplasmic tail of either MHC class II α or β did not affect detergent insolubility indicating that lateral interactions with other proteins are involved in the association [179]. We employed buoyant density centrifugation to ask whether MHC class II was present in a cholesterol-sensitive low buoyant density fraction in B cells. We observed that MHC class II extracted into CHAPS detergent distributed over the entire buoyant density gradient. In contrast the actin-binding protein talin was only present in the two highest buoyant density fractions that include the actin cytoskeleton (Fig. 18). Methyl-β-cyclodextrin selectively removed MHC class II from the two lowest buoyant density fractions but did not affect MHC class II in the higher density fractions. The selective sensitivity of MHC class II and CD9 to cholesterol depletion in the lowest density fractions indicates that a fraction of both MHC class II and CD9 are constitutively present within cholesterol-enriched microdomains where they could potentially interact. We had observed that crosslinking MHC class II at the cell surface promotes not only capping of MHC class II but also co-capping of CD9, and that MHC class II and CD9 form complexes in CHAPS

detergent. We therefore wondered whether MHC class II/CD9 complexes translocate in tandem into lipid rafts on MHC class II cross-linking.

5.4. CD9-MHC class II complexes translocate into lipid raft on antibody cross-linking

Re-organization of lipid rafts may underlie the phenomenon of capping or supramolecular assembly. To investigate whether MHC class II cross-linking would enhance the association of MHC class II with lipid rafts and whether CD9 would co-translocate with clustered MHC class II Raji/CD9 cells were incubated with the anti-MHC class II mAb 7H.3-biotin at 4°C followed by incubation for 1 hour at 37C with or without goat anti-mouse IgG cross-linking. Cells were lysed in 1% CHAPS cells fractionated on Optiprep buoyant density gradients, resolved on SDS-PAGE and blotted with mAb 50H.19-HRP to detect CD9 or streptavidin-HRP to detect the biotinylated anti-MHC class antibody respectively (Fig. 19). The position of MHC class II molecules in the gradient was indicated by the distribution of anti-MHC class II mAb. MHC class II molecules were distributed throughout the gradient with the highest levels in the four lowest density fractions. CD9 was also found throughout the gradient, with its highest levels in the four lowest density fractions. However, the distribution patterns of MHC class II and CD9 between those four fractions were dissimilar. Cross-linking MHC class II produced a dramatic shift to the low density fractions and was accompanied by partial redistribution of CD9 in the same direction. Since only a proportion of MHC class II and

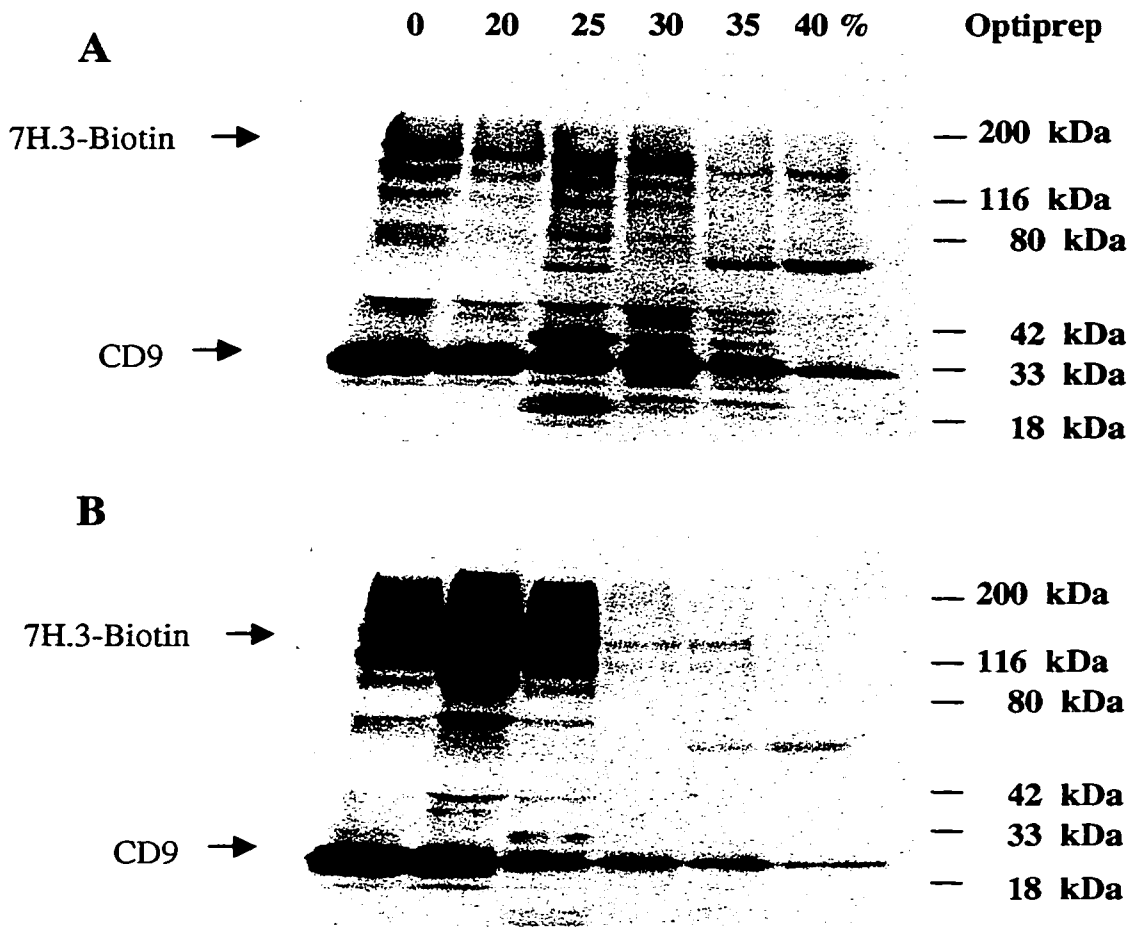


Fig. 19 *MHC class II cross-linking redistributes CD9 into glycolipid rafts.*

MHC class II molecules in Raji/CD9 cells were ligated by anti-MHC class II mAb 7H.3-biotin alone (A) or with mAb 7H.3 followed by cross-linking with goat anti-mouse IgG at 37°C (B). Proteins were extracted into 1% CHAPS and 10% sucrose for 1h at 4°C and fractionated on an Optiprep buoyant density gradient. Proteins were resolved by SDS-PAGE and antibodies detected with 50H.19-HRP and streptavidin-HRP by enhanced chemiluminescence.

CD9 molecules associate with each other the partial redistribution of CD9 on crosslinking MHC class II suggests that MHC class II and CD9 may transfer as a complex into lipid rafts. This would be compatible with the extensive co-localization of MHC class II and CD9 observed on crosslinking either protein at the cell surface (Fig. 2 and Fig. 4) and with the hypothesis that lipid rafts play a role in stabilizing protein-protein interactions at sites of MHC class II engagement.

At steady-state lipid rafts are thought to be small and highly dispersed [180]. Clustering of membrane components that have an affinity for lipid rafts will promote dispersed lipid microdomains to coalesce and thereby increase the affinity for the clustered protein within the raft. These changes in the structure of raft domains may serve as a starting point for the generation of transmembrane signals by gathering signaling molecules and their targets into a relative compact area and overcoming limiting concentrations of reactants [180]. This is supported by the finding that TCR engagement is accompanied by an increased presence in lipid rafts and by enhanced phosphotyrosine signaling [105, 106]. Similar mechanisms may exist in B cells because cross-linking the Raji cell surface protein CD20, a membrane protein bearing four transmembrane domains but not a tetraspanin family member, causes CD20 to rapidly redistribute into lipid raft and phosphorylate a unique 50 kDa protein [181]. Cross-linking MHC class II induces extensive signaling events including calcium flux [182-184], protein tyrosine phosphorylation [176, 184] and apopotosis [185]. Whether MHC class II signaling in B cells requires association of MHC class II with lipid rafts and whether MHC class II signaling is influenced by tetraspanin expression are questions that should now be addressed.

CHAPTER 6: DISCUSSION

In this study we demonstrate that MHC class II associates with CD9 on the cell surface by immunoprecipitation and by co-capping, and that ectopic expression of CD9 in a B cell line inhibits the spontaneous recycling of MHC class II. CD9, a B cell activation antigen, may therefore impose new conditions on the trafficking and function of MHC class II in B cells following cognate interaction. An obvious consequence of CD9 expression would be that recycling of MHC class II into peptide-loading compartments would be reduced thereby increasing retention of immunogenic peptide on the cell surface and decreasing the possibility it would be displaced from MHC class II in a peptide-loading compartment. How CD9 alters the trafficking of MHC class II is an interesting question. Association of integral membrane proteins with lipid rafts could enhance their signaling potential by promoting interactions with non-receptor kinases [106], and with the actin cytoskeleton [100], but at the same time would diminish accessibility to sites of endocytosis in clathrin-coated pits that lie outside lipid rafts [135]. We showed that in CHAPS detergent we could identify cholesterol low-density domains that have the characteristics of lipid rafts and contain both MHC class II and CD9. Since clustering MHC class II did translocate both MHC class II and CD9 into low-density membrane domains co-translocation of CD9 could affect MHC class II function. This appears to be the case since confocal microscopic analysis of fluorescently labeled cross-linked MHC class II demonstrated reduced internalization of antibody-labeled MHC class II in CD9 transfectants. Whether CD9 enhances association with the actin cytoskeleton, or inhibits endocytosis, or both, are interesting questions. Although CD82 and CD63 contain a C-terminal YSKV endocytic targeting motif there is no comparable

targeting motif in CD9. In our confocal studies we did not observe internalization of CD9 despite significant internalization of MHC class II. It seems likely therefore that CD9 performs a function on the cell surface that opposes rather than facilitates MHC class II recycling (Fig. 20). Although CD9 reduced both spontaneous MHC class II re-cycling and internalization of capped and clustered MHC class II, the kinetics of internalization were quite different. More MHC class II internalized spontaneously than following clustering as might be expected if clustering MHC class II increased its interaction with the actin cytoskeleton. A recent report that MHC class II clustering in a gamma interferon-induced monocytic cell line involves partitioning within a lipid raft but not association with the actin cytoskeleton [176] would also be compatible a reduction in endocytosis. It seems likely, however, that clustering MHC class II in lipid rafts would anticipate interactions with the actin cytoskeleton in cells in which MHC class II is constitutively expressed.

Even though the precise functions of tetraspanins are unknown, their similarity and conservation of primary structure suggests they could perform variations on a closely related theme. Tetraspanins have been proposed to function as molecular facilitators [116] or molecular organizers [122] interconnecting surface molecules. Possibly tetraspanins lower the threshold for signal transduction by facilitating formation of stable, functionally active signal transduction complexes [116]. Many recent findings support this hypothesis. An anti-mouse CD9 mAb, together with suboptimal concentrations of anti-CD3 mAb, was an effective co-stimulator of T cells [186]. We have shown that CD9 can also lower the threshold for motility-induction by β1 integrin [130]. CD81-null mice contain normal numbers of B cells but produce weaker antibody responses suggesting that

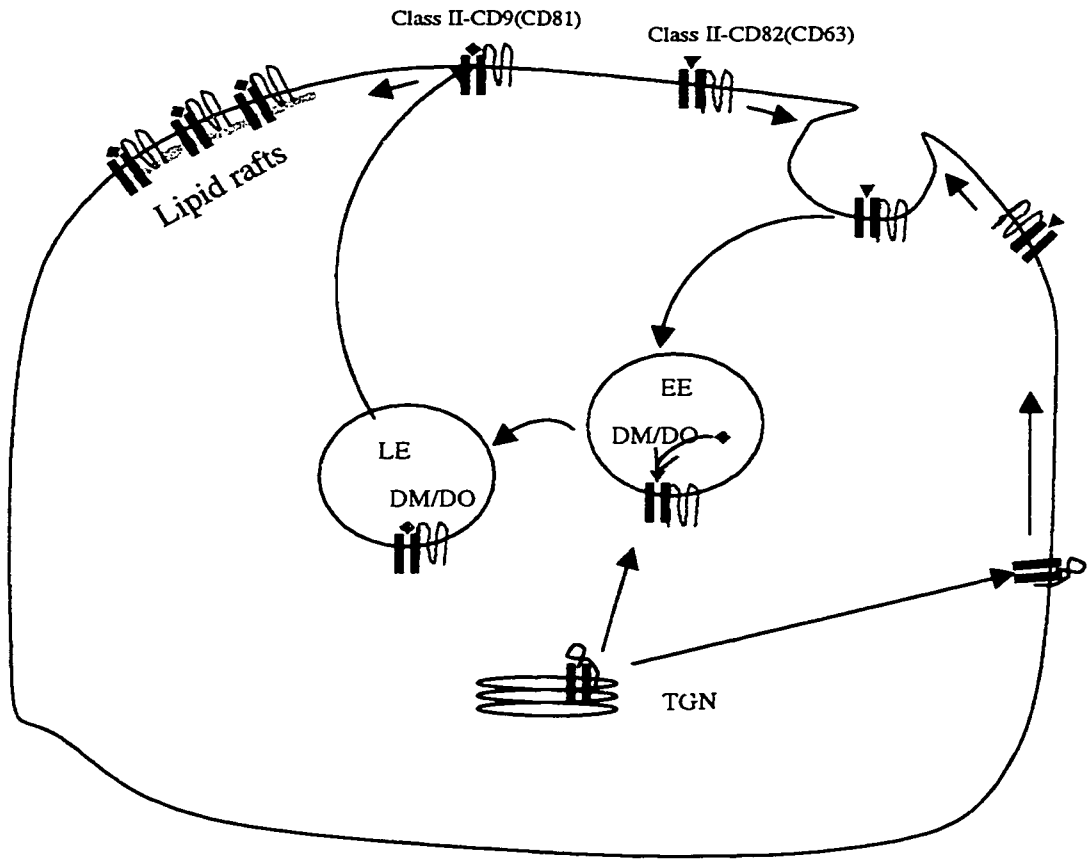


Fig. 20. *Proposed model for tetraspanins regulate MHC class II trafficking and antigen presentation*

MHC class II molecules reach endocytic pathway by two routes: 1) Newly synthesized class Ii molecules associate with invariant chain (Ii) in the ER and are transported from the TGN to the late endosomes (LE) either directly or via the plasma membrane. 2) MHC class II molecules on the cell surface may internalize and recycle via clathrin-coated vesicles. The internalization of surface-exposed MHC class II is facilitated by class II- CD82/CD63 association for loading and exchanging antigen peptides. CD9/CD81 reduce surface MHC class II-peptide complex internalization by driving the complex into lipid rafts and away from clathrin-coated pits. This lipid rafts association may also stabilize class II-peptide binding and facilitate antigen presentation within a mechanically rigid region of the cell membrane. Finally lipid rafts may assist the transduction of signals to the B cell receptor.

CD81 may lower the threshold for primary stimulation of the B cell [187]. The CD19/CD21/CD81/Leu-13 B cell complex augments triggering of B cells by reducing the number of engaged B cell receptors necessary to achieve a signaling threshold, as defined by cell activation and proliferation [188]. Similarly when B cells present antigens in the presence of anti-CD81 or anti-HLA-DR mAb, IL-4 and IL-10 synthesis by CD4+ T cells is enhanced significantly [189]. Our data suggests one way in which tetraspanins might act as facilitators is by increasing the residency of their proximal proteins with lipid rafts.

Our finding that cholesterol depletion dramatically decreases the distribution of CD9 in the top two fractions of the buoyant density gradient strongly suggests that the detergent insolubility of CD9 is due to partitioning of CD9 into a sphingolipid-cholesterol-enriched raft. The best characterised raft targeting sequences contain dual saturated acyl chains. These are glycosylphosphatidylinositol (GPI) anchors that contain predominantly saturated fatty acids [86, 87] and an N-terminal Met-Gly-Cys motif in which Gly is myristoylated and Cys is palmitoylated [88, 91]. However, recently a tandem palmitoylated Cys residue was identified that targets GAP-43, a protein of axonal growth cones, into detergent insoluble lipid domains [190]. Because no GPI anchor and palmitoylation or myristoylation sites are present within MHC class II molecules, possible targeting signals for MHC class II-CD9 complexes may be located in CD9. CD9 can be both palmitoylated and myristoylated [118, 119]. CD9 contains vicinal cysteines within the cytoplasmic loop that may be palmitoylation sites constituting a raft targeting motif.

The general function of rafts may be to concentrate receptors for interaction with ligands and effectors on both sides of the membrane, thus speeding up binding during signaling and preventing inappropriate crosstalk between pathways. Importantly, the

association of proteins with rafts are modulated. Here we propose that CD9 could reduce the threshold for T-B reaction by facilitating recruitment of MHC class II molecules into lipid raft microdomains on the antigen presenting cell surface and hence into an environment optimized for cell-cell communication (Fig. 21). This may occur for three reasons: 1. Lipid rafts are signal molecule-rich microdomains. Targeting a protein into lipid raft will therefore lead to interaction with signal-transduction proteins. This is supported by the recent observations that TCR recruitment into lipid raft initiated by cross-linking CD3 induces strong tyrosine phosphorylation signal and tyrosine kinase Lck activation [105]. 2. The increased local concentration of MHC class II molecules may facilitate formation of an MHC class II tetramer important for presenting antigen to T cells [191]. 3. Lipid rafts may increase the association rate and decrease the dissociation rate of MHC class II-antigen peptide binding and MHC class II/peptide-TCR/CD3 associations. Small changes in these rates can produce dramatic changes in cell responses.

The recent finding of specific intracellular tetraspanin-MHC class II associations in multivesicular bodies suggests that tetraspanins may also be involved in the later stages of MHC class II maturation. Based on the membrane transport studies, Simons suggested that there are two routes for membrane traffic, which connect the Golgi complex with the cell surface. One route transports membrane proteins that have sorting signals such as a clathrin associated motif in their cytoplasmic domains, the other handles glycosphingolipid-cholesterol rafts and raft proteins [109]. Different tetraspanin associations with MHC class II may therefore provide mechanisms to direct MHC class II trafficking through different pathways of peptide acquisition and surveillance. A final possibility for tetraspanin association is that tetraspanins may link associative molecules

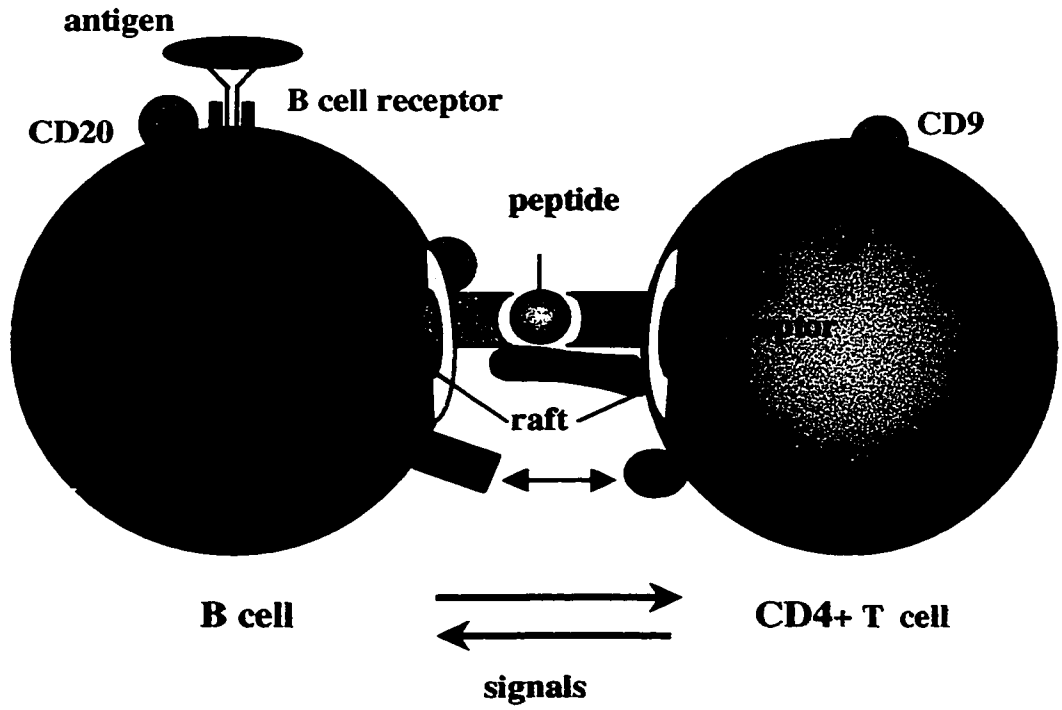


Fig. 21. *Antigen presentation by B cell*

Antigen presentation to T-cell receptor (TCR) by MHC class II may be regulated by CD9-class II association and lipid raft translocation. CD9 could reduce the threshold for T-B reaction by facilitating recruitment of MHC class II molecules into lipid raft microdomains on the antigen presenting cell surface and hence into an environment optimized for cell-cell communication. Lipid rafts formation at cell contact area on both T and B cell may integral cell activation signals including tyrosine kinase signal and/or second lipid messenger.

to specific signal effectors. This possibility is made more attractive by the finding that B cell receptor signaling increases not only the efficiency of MHC class II-mediated antigen presentation but also an increase in phosphoproteins and GTP binding proteins within peptide-loading compartments [192]. MHC class II-tetraspanin interaction therefore provide an ideal opportunity to investigate both the diversity of tetraspanin function and the principles of MHC class II trafficking and signaling.

Exactly how tetraspanins regulate peptide acquisition and presentation will require detailed genetic studies involving tetraspanin knockouts and mutants coupled with careful quantitative and qualitative studies. For now we can speculate that the tetraspanin web has both two-dimensional structure within the lateral plane of the plasma membrane connecting with a 3 dimensional structure that reaches into the cell through the endosomal/loading pathways. Tetraspanins form a multicomponent network containing MHC class I and MHC class II molecules that regulates trafficking of MHC proteins between peptide-loading compartments and the cell membrane. Within this network heterotypic MHC class II DR-DM-DO complexes may be embedded in supramolecular peptide-loading complexes supported by a framework of tetraspanins. Possibly tetraspanins stabilize multicomponent complexes containing MHC class II both during their formation in MIIC's but also during their transport to the cell surface. CD63 and CD82 might participate not only in the assembly of complexes containing DR, DM and DO, but also in transport to the cell membrane. MHC class II clusters stabilized by CD9 and CD81 may therefore stabilize peptides for effective presentation at the cell surface while CD63 and CD82 may assist recycling of MHC class II antigens through early endosomes. The differential association of CD82 and CD63 with MHC class II in MIIC-like structures but not the cell surface could

reflect the rapid internalization of MHC class II through endosomal targeting sequences. Possibly tetraspanins serve to retain MHC class II-peptide complexes of similar subcellular origin in close proximity so that cells present arrays of clustered subsets of MHC class II molecules carrying a particular peptide epitope that may impose unique properties on T cell triggering. Surface DM is thought to act as a peptide editor leading to replacement of peptides of low affinity with those of high affinity within early endosomes of an alternative antigen processing pathway. Such a pathway would enable cells to display protease-sensitive epitopes in newly endocytosed proteins that would be destroyed in lysosomal compartments. If DM/DO interactions favor more stable MHC class II peptide complexes then CD9, by reducing DM-dependent MHC class II recycling, may favor the acquisition of less stable interactions and hence drive diversity. CD9, a late stage activation antigen, may therefore confer the ability to present intrinsically less stable peptide complexes in a more efficient manner from within lipid rafts thereby driving antigenic diversity in the later phases of the immune response.

BIBLIOGRAPHY

1. Germain, R.N., MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell, 1994. **76**(2): p. 287-99.
2. Bjorkman, P.J., et al., Structure of the human class I histocompatibility antigen, HLA-A2. Nature, 1987. **329**(6139): p. 506-12.
3. Kaufman, J.F., et al., The class II molecules of the human and murine major histocompatibility complex. Cell, 1984. **36**(1): p. 1-13.
4. Brown, J.H., et al., Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature, 1993. **364**(6432): p. 33-9.
5. Fremont, D.H., et al., Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. Science, 1992. **257**(5072): p. 919-27.
6. Stern, L.J., et al., Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature, 1994. **368**(6468): p. 215-21.
7. Madden, D.R., et al., The three-dimensional structure of HLA-B27 at 2.1 A resolution suggests a general mechanism for tight peptide binding to MHC. Cell, 1992. **70**(6): p. 1035-48.
8. Chicz, R.M., et al., Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature, 1992. **358**(6389): p. 764-8.
9. Cresswell, P., Invariant chain structure and MHC class II function. Cell, 1996. **84**(4): p. 505-7.
10. Anderson, K.S. and P. Cresswell, A role for calnexin (IP90) in the assembly of class II MHC molecules. Embo J, 1994. **13**(3): p. 675-82.

11. Anderson, M.S. and J. Miller, Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. Proc Natl Acad Sci U S A, 1992. **89**(6): p. 2282-6.

12. Claesson-Welsh, L. and P.A. Peterson, Implications of the invariant gamma-chain on the intracellular transport of class II histocompatibility antigens. J Immunol, 1985. **135**(5): p. 3551-7.

13. Lotteau, V., et al., Intracellular transport of class II MHC molecules directed by invariant chain. Nature, 1990. **348**(6302): p. 600-5.

14. Layet, C. and R.N. Germain, Invariant chain promotes egress of poorly expressed, haplotype- mismatched class II major histocompatibility complex A alpha A beta dimers from the endoplasmic reticulum/cis-Golgi compartment. Proc Natl Acad Sci U S A, 1991. **88**(6): p. 2346-50.

15. Roche, P.A. and P. Cresswell, Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature, 1990. **345**(6276): p. 615-8.

16. Peters, P.J., et al., Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. Nature, 1991. **349**(6311): p. 669-76.

17. Roche, P.A., et al., Cell surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalization. Proc Natl Acad Sci U S A, 1993. **90**(18): p. 8581-5.

18. Warmerdam, P.A., E.O. Long, and P.A. Roche, Isoforms of the invariant chain regulate transport of MHC class II molecules to antigen processing compartments. J Cell Biol, 1996. **133**(2): p. 281-91.

19. Roche, P.A. and P. Cresswell, Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. Proc Natl Acad Sci U S A, 1991. **88**(8): p. 3150-4.

20. Blum, J.S. and P. Cresswell, Role for intracellular proteases in the processing and transport of class II HLA antigens. Proc Natl Acad Sci U S A, 1988. **85**(11): p. 3975-9.

21. Maric, M.A., M.D. Taylor, and J.S. Blum, Endosomal aspartic proteinases are required for invariant-chain processing. Proc Natl Acad Sci U S A, 1994. **91**(6): p. 2171-5.

22. Ghosh, P., et al., The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. Nature, 1995. **378**(6556): p. 457-62.

23. Sanderson, F., et al., Association between HLA-DM and HLA-DR in vivo. Immunity, 1996. **4**(1): p. 87-96.

24. Sherman, M.A., D.A. Weber, and P.E. Jensen, DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. Immunity, 1995. **3**(2): p. 197-205.

25. Sloan, V.S., et al., Mediation by HLA-DM of dissociation of peptides from HLA-DR. Nature, 1995. **375**(6534): p. 802-6.

26. Denzin, L.K. and P. Cresswell, HLA-DM induces CLIP dissociation from MHC class II alpha beta dimers and facilitates peptide loading. Cell, 1995. **82**(1): p. 155-65.

27. Roche, P.A., HLA-DM: an in vivo facilitator of MHC class II peptide loading. Immunity, 1995. **3**(3): p. 259-62.

28. Wolf, P.R. and H.L. Ploegh, How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. Annu Rev Cell Dev Biol, 1995. **11**: p. 267-306.

29. Denzin, L.K., et al., Negative regulation by HLA-DO of MHC class II-restricted antigen processing. Science, 1997. **278**(5335): p. 106-9.

30. Liljedahl, M., et al., HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. Embo J, 1996. **15**(18): p. 4817-24.

31. Jensen, P.E., Antigen processing: HLA-DO--a hitchhiking inhibitor of HLA-DM. Curr Biol, 1998. **8**(4): p. R128-31.

32. Tulp, A., et al., Isolation and characterization of the intracellular MHC class II compartment [see comments]. Nature, 1994. **369**(6476): p. 120-6.

33. West, M.A., J.M. Lucocq, and C. Watts, Antigen processing and class II MHC peptide-loading compartments in human B-lymphoblastoid cells [see comments]. Nature, 1994. **369**(6476): p. 147-51.

34. Qiu, Y., et al., Separation of subcellular compartments containing distinct functional forms of MHC class II. J Cell Biol, 1994. **125**(3): p. 595-605.

35. Harding, C.V. and H.J. Geuze, Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. J Cell Biol, 1992. **119**(3): p. 531-42.

36. Kleijmeer, M.J., G. Raposo, and H.J. Geuze, Characterization of MHC Class II Compartments by Immunoelectron Microscopy. Methods, 1996. **10**(2): p. 191-207.

37. Kleijmeer, M.J., et al., Major histocompatibility complex class II compartments in human and mouse B lymphoblasts represent conventional endocytic compartments. J Cell Biol, 1997. **139**(3): p. 639-49.

38. Peters, P.J., et al., Major histocompatibility complex class II compartments in human B lymphoblastoid cells are distinct from early endosomes. J Exp Med, 1995. **182**(2): p. 325-34.

39. Amigorena, S., et al., Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. Nature, 1994. **369**(6476): p. 113-20.

40. Amigorena, S., et al., Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. J Exp Med, 1995. **181**(5): p. 1729-41.

41. Ferrari, G., et al., Distinct intracellular compartments involved in invariant chain degradation and antigenic peptide loading of major histocompatibility complex (MHC) class II molecules. J Cell Biol, 1997. **139**(6): p. 1433-46.

42. Glickman, J.N., et al., The biogenesis of the MHC class II compartment in human I-cell disease B lymphoblasts. J Cell Biol, 1996. **132**(5): p. 769-85.

43. Castellino, F. and R.N. Germain, Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. Immunity, 1995. **2**(1): p. 73-88.

44. Sadegh-Nasseri, S., et al., MHC class II function preserved by low-affinity peptide interactions preceding stable binding. Nature, 1994. **370**(6491): p. 647-50.

45. Niebling, W.L. and S.K. Pierce, Antigen entry into early endosomes is insufficient for MHC class II processing. J Immunol, 1993. **150**(7): p. 2687-97.

46. Harding, C.V. and H.J. Geuze, Antigen processing and intracellular traffic of antigens and MHC molecules. Curr Opin Cell Biol, 1993. **5**(4): p. 596-605.

47. Raposo, G., et al., B lymphocytes secrete antigen-presenting vesicles. J Exp Med, 1996. **183**(3): p. 1161-72.

48. Escola, J.M., et al., Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B- lymphocytes. J Biol Chem, 1998. **273**(32): p. 20121-7.

49. Matsui, K., et al., Low affinity interaction of peptide-MHC complexes with T cell receptors. Science, 1991. **254**(5039): p. 1788-91.

50. Weber, S., et al., Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. Nature, 1992. **356**(6372): p. 793-6.

51. Kessler, B.M., et al., Effects of epitope modification on T cell receptor-ligand binding and antigen recognition by seven H-2Kd-restricted cytotoxic T lymphocyte clones specific for a photoreactive peptide derivative. J Exp Med, 1997. **185**(4): p. 629-40.

52. Luescher, I.F., et al., CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. Nature, 1995. **373**(6512): p. 353-6.

53. Corr, M., et al., T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. Science, 1994. **265**(5174): p. 946-9.

54. Matsui, K., et al., Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. Proc Natl Acad Sci U S A, 1994. **91**(26): p. 12862-6.

55. Margulies, D.H., Interactions of TCRs with MHC-peptide complexes: a quantitative basis for mechanistic models. Curr Opin Immunol, 1997. **9**(3): p. 390-5.

56. Davis, M.M., et al., Ligand recognition by alpha beta T cell receptors. Annu Rev Immunol, 1998. **16**: p. 523-44.

57. Shahinian, A., et al., Differential T cell costimulatory requirements in CD28-deficient mice. Science, 1993. **261**(5121): p. 609-12.

58. Kundig, T.M., et al., Duration of TCR stimulation determines costimulatory requirement of T cells. Immunity, 1996. **5**(1): p. 41-52.

59. Viola, A. and A. Lanzavecchia, T cell activation determined by T cell receptor number and tunable thresholds. Science, 1996. **273**(5271): p. 104-6.

60. Bachmann, M.F., et al., Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. Immunity, 1997. **7**(4): p. 549-57.

61. Linsley, P.S., et al., Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J Exp Med, 1991. **173**(3): p. 721-30.

62. Harlan, D.M., et al., Mice expressing both B7-1 and viral glycoprotein on pancreatic beta cells along with glycoprotein-specific transgenic T cells develop diabetes due to a breakdown of T-lymphocyte unresponsiveness. Proc Natl Acad Sci U S A, 1994. **91**(8): p. 3137-41.

63. Walunas, T.L., et al., CTLA-4 can function as a negative regulator of T cell activation. Immunity, 1994. **1**(5): p. 405-13.

64. Krummel, M.F. and J.P. Allison, CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med, 1995. **182**(2): p. 459-65.

65. Wu, Y., et al., Rapid induction of a novel costimulatory activity on B cells by CD40 ligand. Curr Biol, 1995. **5**(11): p. 1303-11.

66. Buhlmann, J.E., et al., In the absence of a CD40 signal, B cells are tolerogenic. Immunity, 1995. **2**(6): p. 645-53.

67. Ipsen, J.H., et al., Phase equilibria in the phosphatidylcholine-cholesterol system. Biochim Biophys Acta, 1987. **905**(1): p. 162-72.

68. Ipsen, J.H., O.G. Mouritsen, and M. Bloom, Relationships between lipid membrane area, hydrophobic thickness, and acyl-chain orientational order. The effects of cholesterol. Biophys J, 1990. **57**(3): p. 405-12.

69. Recktenwald, D.J. and H.M. McConnell, *Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol.* Biochemistry, 1981. **20**(15): p. 4505-10.

70. Mouritsen, O.G., Theoretical models of phospholipid phase transitions. Chem Phys Lipids, 1991. **57**(2-3): p. 179-94.

71. Davies, M.A., et al., Effects of cholesterol on conformational disorder in dipalmitoylphosphatidylcholine bilayers. A quantitative IR study of the depth dependence. Biochemistry, 1990. **29**(18): p. 4368-73.

72. Thompson, T.E. and T.W. Tillack, Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. Annu Rev Biophys Biophys Chem, 1985. **14**: p. 361-86.

73. Rietveld, A. and K. Simons, The differential miscibility of lipids as the basis for the formation of functional membrane rafts. Biochim Biophys Acta, 1998. **1376**(3): p. 467-79.

74. Ahmed, S.N., D.A. Brown, and E. London, On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. Biochemistry, 1997. **36**(36): p. 10944-53.

75. Schroeder, R.J., et al., Cholesterol and sphingolipid enhance the Triton X-100 insolubility of glycosylphosphatidylinositol-anchored proteins by promoting the

formation of detergent-insoluble ordered membrane domains. J Biol Chem, 1998. **273**(2): p. 1150-7.

76. Brown, D.A. and E. London, Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol, 1998. **14**: p. 111-36.
77. Maxfield, F.R. and S. Mayor, Cell surface dynamics of GPI-anchored proteins. Adv Exp Med Biol, 1997. **419**: p. 355-64.
78. Rock, P., et al., Organization of glycosphingolipids in phosphatidylcholine bilayers: use of antibody molecules and Fab fragments as morphologic markers. Biochemistry, 1990. **29**(36): p. 8484-90.
79. Mayor, S., K.G. Rothberg, and F.R. Maxfield, Sequestration of GPI-anchored proteins in caveolae triggered by cross- linking. Science, 1994. **264**(5167): p. 1948-51.
80. Harder, T., et al., Lipid domain structure of the plasma membrane revealed by patching of membrane components. J Cell Biol, 1998. **141**(4): p. 929-42.
81. Scheiffele, P., M.G. Roth, and K. Simons, Interaction of influenza virus haemagglutinin with sphingolipid- cholesterol membrane domains via its transmembrane domain. Embo J, 1997. **16**(18): p. 5501-8.
82. Varma, R. and S. Mayor, GPI-anchored proteins are organized in submicron domains at the cell surface. Nature, 1998. **394**(6695): p. 798-801.
83. Friedrichson, T. and T.V. Kurzchalia, Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. Nature, 1998. **394**(6695): p. 802-5.
84. Brown, D. and G.L. Waneck, Glycosyl-phosphatidylinositol-anchored membrane proteins. J Am Soc Nephrol, 1992. **3**(4): p. 895-906.

85. McConville, M.J. and M.A. Ferguson, The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J, 1993. **294**(Pt 2): p. 305-24.

86. Rodgers, W., B. Crise, and J.K. Rose, Signals determining protein tyrosine kinase and glycosyl- phosphatidylinositol-anchored protein targeting to a glycolipid- enriched membrane fraction. Mol Cell Biol, 1994. **14**(8): p. 5384-91.

87. Arreaza, G. and D.A. Brown, Sorting and intracellular trafficking of a glycosylphosphatidylinositol- anchored protein and two hybrid transmembrane proteins with the same ectodomain in Madin-Darby canine kidney epithelial cells. J Biol Chem, 1995. **270**(40): p. 23641-7.

88. Milligan, G., M. Parenti, and A.I. Magee, The dynamic role of palmitoylation in signal transduction. Trends Biochem Sci, 1995. **20**(5): p. 181-7.

89. Resh, M.D., Myristylation and palmitylation of Src family members: the fats of the matter. Cell, 1994. **76**(3): p. 411-3.

90. Robbins, S.M., N.A. Quintrell, and J.M. Bishop, Myristoylation and differential palmitoylation of the HCK protein- tyrosine kinases govern their attachment to membranes and association with caveolae. Mol Cell Biol, 1995. **15**(7): p. 3507-15.

91. Shenoy-Scaria, A.M., et al., Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. J Cell Biol, 1994. **126**(2): p. 353-63.

92. Brown, D.A. and E. London, Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem Biophys Res Commun, 1997. **240**(1): p. 1-7.

93. Maekawa, S., et al., Identification of NAP-22 and GAP-43 (neuromodulin) as major protein components in a Triton insoluble low density fraction of rat brain. Biochim Biophys Acta, 1997. **1323**(1): p. 1-5.

94. Arni, S., et al., Differential regulation of Src-family protein tyrosine kinases in GPI domains of T lymphocyte plasma membranes. Biochem Biophys Res Commun, 1996. **225**(3): p. 801-7.

95. Cerneus, D.P., et al., Detergent insolubility of alkaline phosphatase during biosynthetic transport and endocytosis. Role of cholesterol. J Biol Chem, 1993. **268**(5): p. 3150-5.

96. Hanada, K., et al., Both sphingolipids and cholesterol participate in the detergent insolubility of alkaline phosphatase, a glycosylphosphatidylinositol- anchored protein, in mammalian membranes. J Biol Chem, 1995. **270**(11): p. 6254-60.

97. Schroeder, R., E. London, and D. Brown, Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI- anchored proteins in liposomes and cells show similar behavior. Proc Natl Acad Sci U S A, 1994. **91**(25): p. 12130-4.

98. Robinson, P.J., Signal transduction by GPI-anchored membrane proteins. Cell Biol Int Rep, 1991. **15**(9): p. 761-7.

99. Brown, D., The tyrosine kinase connection: how GPI-anchored proteins activate T cells. Curr Opin Immunol, 1993. **5**(3): p. 349-54.

100. Harder, T. and K. Simons, Clusters of glycolipid and glycosylphosphatidylinositol-anchored proteins in lymphoid cells: accumulation of actin regulated by local tyrosine phosphorylation. Eur J Immunol, 1999. **29**(2): p. 556-62.

101. Field, K.A., D. Holowka, and B. Baird, Fc epsilon RI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling. Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9201-5.

102. Stauffer, T.P., et al., Inhibition of Lyn function in mast cell activation by SH3 domain binding peptides. Biochemistry, 1997. **36**(31): p. 9388-94.

103. Gouy, H., P. Debre, and G. Bismuth, Triggering of a sustained calcium response through a p56lck-dependent pathway by exogenous ganglioside GM1 in human T lymphocytes. J Immunol, 1995. **155**(11): p. 5160-6.

104. Bijlmakers, M.J., et al., Intrinsic signals in the unique domain target p56(lck) to the plasma membrane independently of CD4. J Cell Biol, 1997. **137**(5): p. 1029-40.

105. Montixi, C., et al., Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. Embo J, 1998. **17**(18): p. 5334-48.

106. Viola, A., et al., T lymphocyte costimulation mediated by reorganization of membrane microdomains [see comments]. Science, 1999. **283**(5402): p. 680-2.

107. Rodriguez-Boulan, E. and S.K. Powell, Polarity of epithelial and neuronal cells. Annu Rev Cell Biol, 1992. **8**: p. 395-427.

108. Matter, K., E.M. Yamamoto, and I. Mellman, Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. J Cell Biol, 1994. **126**(4): p. 991-1004.

109. Simons, K. and E. Ikonen, Functional rafts in cell membranes. Nature, 1997. **387**(6633): p. 569-72.

110. Brown, D.A. and J.K. Rose, Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell, 1992. **68**(3): p. 533-44.

111. Bretscher, M.S. and S. Munro, Cholesterol and the Golgi apparatus. Science, 1993. **261**(5126): p. 1280-1.

112. Fiedler, K., et al., Glycosphingolipid-enriched, detergent-insoluble complexes in protein sorting in epithelial cells. Biochemistry, 1993. **32**(25): p. 6365-73.

113. Futerman, A.H., et al., Vesicle transport during cell growth and in the maintenance of cell polarity. Biochem Soc Trans, 1995. **23**(3): p. 530-4.

114. Keller, P. and K. Simons, Cholesterol is required for surface transport of influenza virus hemagglutinin. J Cell Biol, 1998. **140**(6): p. 1357-67.

115. Ledesma, M.D., K. Simons, and C.G. Dotti, Neuronal polarity: essential role of protein-lipid complexes in axonal sorting. Proc Natl Acad Sci U S A, 1998. **95**(7): p. 3966-71.

116. Maecker, H.T., S.C. Todd, and S. Levy, The tetraspanin superfamily: molecular facilitators. Faseb J, 1997. **11**(6): p. 428-42.

117. Horejsi, V. and C. Vlcek, Novel structurally distinct family of leucocyte surface glycoproteins including CD9, CD37, CD53 and CD63. FEBS Lett, 1991. **288**(1-2): p. 1-4.

118. Seehafer, J.G., et al., The functional glycoprotein CD9 is variably acylated: localization of the variably acylated region to a membrane-associated peptide containing the binding site for the agonistic monoclonal antibody 50H.19. Biochim Biophys Acta, 1988. **957**(3): p. 399-410.

119. Seehafer, J.G., et al., The functional cell surface glycoprotein CD9 is distinguished by being the major fatty acid acylated and a major iodinated cell-surface component of the human platelet. Biochim Biophys Acta, 1988. **952**(1): p. 92-100.

120. Berditchevski, F., M.M. Zutter, and M.E. Hemler, Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). Mol Biol Cell, 1996. **7**(2): p. 193-207.

121. Imai, T., et al., Molecular analyses of the association of CD4 with two members of the transmembrane 4 superfamily, CD81 and CD82. J Immunol, 1995. **155**(3): p. 1229-39.

122. Rubinstein, E., et al., CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. Eur J Immunol, 1996. **26**(11): p. 2657-65.

123. Slupsky, J.R., et al., Evidence that monoclonal antibodies against CD9 antigen induce specific association between CD9 and the platelet glycoprotein IIb-IIIa complex. J Biol Chem, 1989. **264**(21): p. 12289-93.

124. Rubinstein, E., et al., CD9 antigen is an accessory subunit of the VLA integrin complexes. Eur J Immunol, 1994. **24**(12): p. 3005-13.

125. Imai, T. and O. Yoshie, C33 antigen and M38 antigen recognized by monoclonal antibodies inhibitory to syncytium formation by human T cell leukemia virus type 1 are both members of the transmembrane 4 superfamily and associate with each other and with CD4 or CD8 in T cells. J Immunol, 1993. **151**(11): p. 6470-81.

126. Angelisova, P., I. Hilgert, and V. Horejsi, Association of four antigens of the tetraspans family (CD37, CD53, TAPA- 1, and R2/C33) with MHC class II glycoproteins. Immunogenetics, 1994. **39**(4): p. 249-56.

127.Hammond, C., et al., The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. J Immunol, 1998. **161**(7): p. 3282-91.

128.Oren, R., et al., TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. Mol Cell Biol, 1990. **10**(8): p. 4007-15.

129.Skubitz, K.M., et al., CD63 associates with tyrosine kinase activity and CD11/CD18, and transmits an activation signal in neutrophils. J Immunol, 1996. **157**(8): p. 3617-26.

130.Shaw, A.R., et al., Ectopic expression of human and feline CD9 in a human B cell line confers beta 1 integrin-dependent motility on fibronectin and laminin substrates and enhanced tyrosine phosphorylation. J Biol Chem, 1995. **270**(41): p. 24092-9.

131.Levy, S., S.C. Todd, and H.T. Maecker, CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. Annu Rev Immunol, 1998. **16**: p. 89-109.

132.Lebel-Binay, S., et al., CD82, member of the tetra-span-transmembrane protein family, is a costimulatory protein for T cell activation. J Immunol, 1995. **155**(1): p. 101-10.

133.Todd, S.C., et al., CD81 expressed on human thymocytes mediates integrin activation and interleukin 2-dependent proliferation. J Exp Med, 1996. **184**(5): p. 2055-60.

134.Tai, X.G., et al., A role for CD9 molecules in T cell activation. **J** Exp Med, 1996. **184**(2): p. 753-8.

135.Shevchenko, A., et al., Identification of components of trans-Golgi network-derived transport vesicles and detergent-insoluble complexes by nanoelectrospray tandem mass spectrometry. Electrophoresis, 1997. **18**(14): p. 2591-600.

136. MacLean, G.D., et al., Antigenic heterogeneity of human colorectal cancer cell lines analyzed by a panel of monoclonal antibodies. I. Heterogeneous expression of Ia- like and HLA-like antigenic determinants. J Natl Cancer Inst, 1982. **69**(2): p. 357-64.

137. Hadjiargyrou, M., et al., Association of the tetraspan protein CD9 with integrins on the surface of S-16 Schwann cells. J Neurochem, 1996. **67**(6): p. 2505-13.

138. Mannion, B.A., et al., Transmembrane-4 superfamily proteins CD81 (TAPA-1), CD82, CD63, and CD53 specifically associated with integrin alpha 4 beta 1 (CD49d/CD29). J Immunol, 1996. **157**(5): p. 2039-47.

139. Rubinstein, E., et al., CD9, but not other tetraspans, associates with the beta1 integrin precursor. Eur J Immunol, 1997. **27**(8): p. 1919-27.

140. Nakamura, K., R. Iwamoto, and E. Mekada, Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF) and diphtheria toxin receptor-associated protein (DRAP27)/CD9 form a complex with integrin alpha 3 beta 1 at cell-cell contact sites. J Cell Biol, 1995. **129**(6): p. 1691-705.

141. Matsumoto, A.K., et al., Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes. J Exp Med, 1993. **178**(4): p. 1407-17.

142. Berditchevski, F., et al., A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. J Biol Chem, 1997. **272**(5): p. 2595-8.

143. Yauch, R.L., et al., Highly stoichiometric, stable, and specific association of integrin alpha3beta1 with CD151 provides a major link to phosphatidylinositol 4- kinase, and may regulate cell migration. Mol Biol Cell, 1998. **9**(10): p. 2751-65.

144.Serru, V., et al., Selective tetraspan-integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions. Biochem J, 1999. **340**(Pt 1): p. 103-11.

145.Schick, M.R. and S. Levy, The TAPA-1 molecule is associated on the surface of B cells with HLA-DR molecules. J Immunol, 1993. **151**(8): p. 4090-7.

146.Szollosi, J., et al., Supramolecular complexes of MHC class I, MHC class II, CD20, and tetraspan molecules (CD53, CD81, and CD82) at the surface of a B cell line JY. J Immunol, 1996. **157**(7): p. 2939-46.

147.Pozzan, T., et al., Cap formation by various ligands on lymphocytes shows the same dependence on high cellular ATP levels. Biochim Biophys Acta, 1980. **602**(3): p. 558-66.

148.Pavan, A., et al., Patching and capping of LFA-1 molecules on human lymphocytes. Histochemistry, 1992. **98**(4): p. 253-8.

149.Espinosa-Cantellano, M. and A. Martinez-Palomo, Entamoeba histolytica: mechanism of surface receptor capping. Exp Parasitol, 1994. **79**(3): p. 424-35.

150.Schreiner, G.F. and E.R. Unanue, Calcium-sensitive modulation of Ig capping: evidence supporting a cytoplasmic control of ligand-receptor complexes. J Exp Med, 1976. **143**(1): p. 15-31.

151.Klausner, R.D., et al., Model for capping derived from inhibition of surface receptor capping by free fatty acids. Proc Natl Acad Sci U S A, 1980. **77**(1): p. 437-41.

152.Hoover, R.L., et al., Effects of linoleic acid on capping, lectin mediated mitogenesis, surface antigen expression, and fluorescent polarization in lymphocytes and BHK cells. J Cell Physiol, 1980. **103**(3): p. 399-406.

153. Turner, C.E. and D.M. Shotton, Effects of capping on the non-ionic detergent solubility of rat thymocyte glycoproteins. Eur J Cell Biol, 1989. **50**(2): p. 324-32.

154. Kellie, S., et al., Capping of cholera toxin-ganglioside GM1 complexes on mouse lymphocytes is accompanied by co-capping of alpha-actinin. J Cell Biol, 1983. **97**(2): p. 447-54.

155. Salamero, J., et al., Mouse B lymphocyte specific endocytosis and recycling of MHC class II molecules. Embo J, 1990. **9**(11): p. 3489-96.

156. Reid, P.A. and C. Watts, Constitutive endocytosis and recycling of major histocompatibility complex class II glycoproteins in human B-lymphoblastoid cells. Immunology, 1992. **77**(4): p. 539-42.

157. Neefjes, J.J., et al., The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. Cell, 1990. **61**(1): p. 171-83.

158. Wraight, C.J., et al., Human major histocompatibility complex class II invariant chain is expressed on the cell surface. J Biol Chem, 1990. **265**(10): p. 5787-92.

159. Koch, N., et al., Rapid intracellular pathway gives rise to cell surface expression of the MHC class II-associated invariant chain (CD74). J Immunol, 1991. **147**(8): p. 2643-51.

160. Saudrais, C., et al., Intracellular pathway for the generation of functional MHC class II peptide complexes in immature human dendritic cells. J Immunol, 1998. **160**(6): p. 2597-607.

161. Pinet, V., et al., Antigen presentation mediated by recycling of surface HLA-DR molecules. Nature, 1995. **375**(6532): p. 603-6.

162. Pinet, V.M. and E.O. Long, Peptide loading onto recycling HLA-DR molecules occurs in early endosomes. Eur J Immunol, 1998. **28**(3): p. 799-804.

163. Swier, K. and J. Miller, Efficient internalization of MHC class II-invariant chain complexes is not sufficient for invariant chain proteolysis and class II antigen presentation. J Immunol, 1995. **155**(2): p. 630-43.

164. Zhong, G., P. Romagnoli, and R.N. Germain, Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. J Exp Med, 1997. **185**(3): p. 429-38.

165. Mellman, I., Endocytosis and molecular sorting. Annu Rev Cell Dev Biol, 1996. **12**: p. 575-625.

166. Schmid, S.L., Clathrin-coated vesicle formation and protein sorting: an integrated process. Annu Rev Biochem, 1997. **66**: p. 511-48.

167. Traub, L.M. and S. Kornfeld, The trans-Golgi network: a late secretory sorting station. Curr Opin Cell Biol, 1997. **9**(4): p. 527-33.

168. Kirchhausen, T., J.S. Bonifacino, and H. Riezman, Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Curr Opin Cell Biol, 1997. **9**(4): p. 488-95.

169. Robinson, M.S., The role of clathrin, adaptors and dynamin in endocytosis. Curr Opin Cell Biol, 1994. **6**(4): p. 538-44.

170. Kornfeld, S. and I. Mellman, The biogenesis of lysosomes. Annu Rev Cell Biol, 1989. **5**: p. 483-525.

171. Bogen, B. and S. Weiss, Processing and presentation of idiotypes to MHC-restricted T cells. Int Rev Immunol, 1993. **10**(4): p. 337-55.

172.Stulnig, T.M., et al., Signal transduction via glycosyl phosphatidylinositol-anchored proteins in T cells is inhibited by lowering cellular cholesterol. J Biol Chem, 1997. **272**(31): p. 19242-7.

173.Romagnoli, P. and C. Bron, Phosphatidylinositol-based glycolipid-anchored proteins enhance proximal TCR signaling events. J Immunol, 1997. **158**(12): p. 5757-64.

174.Moran, M. and M.C. Miceli, Engagement of GPI-linked CD48 contributes to TCR signals and cytoskeletal reorganization: a role for lipid rafts in T cell activation. Immunity, 1998. **9**(6): p. 787-96.

175.Germain, R.N., T-cell signaling: the importance of receptor clustering. Curr Biol, 1997. **7**(10): p. R640-4.

176.Huby, R.D., R.J. Dearman, and I. Kimber, Intracellular phosphotyrosine induction by major histocompatibility complex class II requires co-aggregation with membrane rafts. J Biol Chem, 1999. **274**(32): p. 22591-6.

177.Pitha, J., et al., Drug solubilizers to aid pharmacologists: amorphous cyclodextrin derivatives. Life Sci, 1988. **43**(6): p. 493-502.

178.Woda, B.A. and M.B. Woodin, The interaction of lymphocyte membrane proteins with the lymphocyte cytoskeletal matrix. J Immunol, 1984. **133**(5): p. 2767-72.

179.Chia, C.P., et al., MHC class II molecules that lack cytoplasmic domains are associated with the cytoskeleton. J Immunol, 1994. **153**(8): p. 3398-407.

180.Harder, T. and K. Simons, Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr Opin Cell Biol, 1997. **9**(4): p. 534-42.

181.Deans, J.P., et al., Rapid redistribution of CD20 to a low density detergent-insoluble membrane compartment. J Biol Chem, 1998. **273**(1): p. 344-8.

182.Mooney, N.A., et al., Early biochemical events after MHC class II-mediated signaling on human B lymphocytes. J Immunol, 1990. **145**(7): p. 2070-6.

183.Scott, D.W., et al., Lymphoma models for B cell activation and tolerance. V. Anti-Ig mediated growth inhibition is reversed by phorbol myristate acetate but does not involve changes in cytosolic free calcium. J Mol Cell Immunol, 1987. **3**(2): p. 109-20.

184.Morio, T., R.S. Geha, and T.A. Chatila, Engagement of MHC class II molecules by staphylococcal superantigens activates src-type protein tyrosine kinases. Eur J Immunol, 1994. **24**(3): p. 651-8.

185.Vidovic, D. and J.I. Toral, Selective apoptosis of neoplastic cells by the HLA-DR-specific monoclonal antibody. Cancer Lett, 1998. **128**(2): p. 127-35.

186.Tai, X.G., et al., CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. J Immunol, 1997. **159**(8): p. 3799-807.

187.Maecker, H.T. and S. Levy, Normal lymphocyte development but delayed humoral immune response in CD81-null mice. J Exp Med, 1997. **185**(8): p. 1505-10.

188.Bradbury, L.E., V.S. Goldmacher, and T.F. Tedder, The CD19 signal transduction complex of B lymphocytes. Deletion of the CD19 cytoplasmic domain alters signal transduction but not complex formation with TAPA-1 and Leu 13. J Immunol, 1993. **151**(6): p. 2915-27.

189.Secrist, H., et al., Ligation of TAPA-1 (CD81) or major histocompatibility complex class II in co-cultures of human B and T lymphocytes enhances interleukin-4 synthesis by antigen-specific CD4+ T cells. Eur J Immunol, 1996. **26**(7): p. 1435-42.

190.Arni, S., et al., Association of GAP-43 with detergent-resistant membranes requires two palmitoylated cysteine residues. J Biol Chem, 1998. **273**(43): p. 28478-85.

191. Schafer, P.H. and S.K. Pierce, Evidence for dimers of MHC class II molecules in B lymphocytes and their role in low affinity T cell responses. Immunity, 1994. **1**(8): p. 699-707.

192. Xu, X., et al., B cell antigen receptor signaling links biochemical changes in the class II peptide-loading compartment to enhanced processing. Int Immunol, 1996. **8**(12): p. 1867-76.