The Role of Tetraspanin CD63 in Antigen Presentation to $CD4^+$ T Cells

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Abstract

CD4+ T cells play a key role in orchestrating adaptive immunity. Their activation requires antigen presentation via MHC II proteins on antigen presenting cells (APC). Exosomes are membrane vesicles released by various cell types including APCs. APC-derived exosomes are MHC class II-positive and can induce CD4+ T cell responses. MHC II delivery to the cell surface and/or exosomes might be influenced by tetraspanins, a family of transmembrane proteins.

We have prepared exosomes derived from Epstein-Barr virus (EBV)-infected human B lymphoblastoid cell lines (LCLs) and shown by Western blotting and immunoelectron microscopy that they contain MHC class II and tetraspanins including CD63, CD81 and CD82.

Such LCLs as well as LCL-derived exosomes can mediate immunologically specific recognition by MHC class II matched EBV antigen-specific CD4+ T cell clones when directly added to the T cells. Using shRNA, we have decreased CD63 expression in LCLs and had been studying the effect of such downregulation on LCL as well as LCL-derived exosome mediated antigen presentation. Despite an unaltered level of MHC II, CD63low LCLs showed to be hyperstimulatory. In spite of a similar depletion of CD63 in exosomes derived from CD63low LCLs, the CD4+ T cell stimulation by these exosomes was unaltered. In search for the mechanism of this phenomenon we found a higher level of exosome secretion by CD63low LCLs. We speculate that CD63 may influence T cell stimulation by exosome trafficking as well as exosome release.

To my parents who stood right behind me all the way,

for their love and encouragement

and for supporting me whatever path I chose to go.

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Abbreviations

α	Alpha
APC	Antigen presenting cell
Arp	Actin-related protein
B	Bone marrow
β	Beta
BCR	B cell receptor
BL	Burkitt's lymphoma
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLIP	Class II-associated Ii peptide
CpG	Cytosine-phosphate-Guanine
CSA	Cyclosporin A
CTL	Cytotoxic T cell
DEX	Dendritic cell-derived exosomes
DMSO	Dimethyl sulphoxide
DC	Dendritic cell
DNA	Desoxyribonucleic acid
EBNA	Epstein-Barr virus nuclear antigen

EC	Extracellular loop
ELISA	Enzyme linked immunosorbant assay
ER	Endoplasmatic reticulum
ESCRT	Endosomal sorting complex required for transport
FasL	Fas ligand
FBS	Foetal bovine serum
Foxp	Forkhead box protein
γ	Gamma
HD	Hodgkin's disease
HLA	Human leukocyte antigen
HS	Human serum
Hsc	Heat shock cognate
Hsp	Heat shock protein
ICAM	Inter cellular adhesion molecule
IFNγ	Interferon gamma
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IM	Infectious mononucleosis
IS	Immunological synapse

LCL	Lymphoblastoid cell line
LFA	Leukocyte Function-Associated Antigen
LMP	Latent membrane protein
m	Milli
M	Molar
MIIC	Major Histocompatibility complex II loading compartment
mAb	Monoclonal Antibody
MART	Melanoma antigen recognized by T-cells
MDA	Human breast adenocarcinoma cell line
MFG	Milk fat globule elongation factor
MHC	Major Histocompatibility Complex
MTOC	Microtubule Organising Centre
MVB	Multi vesicular body
mRNA	Messenger ribonucleic acid
µ	Micro
ND	Not determined
NK	Natural killer
NPC	Nasopharyngeal carcinoma
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline

PRR	Pattern recognition receptor
PTLD	Post transplant lymphoproliferative disease
rpm	Rounds per minute
RT	Room temperature
shRNA	Small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
T	Thymus
TAA	Tumour associated antigen
TAP	Transporter of antigen presentation
TCR	T cell receptor
TAE	Tris-base, acetic acid, EDTA
TERM	Tetraspanin enriched microdomain
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
Ub	Ubiquination

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1 Introduction

1.1 Immune system

1.1.1 Background

The immune system is comprised of the innate, evolutionary old and primitive and the adaptive, more advanced immune response. Both these parts of the immune system are either cellular or humoral and protect against disease by identifying and eliminating pathogens. The innate immune response involves macrophages, neutrophils, eosinophils, granulocytes and dendritic cells. The adaptive immune response is antigen specific and involves T lymphocytes and antigen presenting cells (APC) like dendritic cells (DC), B lymphocytes and macrophages. Evolved in the first jawed vertebrates the adaptive immune system is based on somatic hypermutation and recombination enabling the immune cells to generate a vast number of different antigen receptors. This variability provides a highly adaptable cellular response to pathogens of different kind such as viruses. The adaptive immune system includes primary response, occurring during first encounter to a pathogen, and secondary response, performed by B and T cells. These B and T cells differentiate to memory cells after clearing the pathogen and provide long term immunity to this pathogen.

1.1.2 Antigen presenting cells

APCs are able to recognise pathogens by a set of pattern recognition receptors (PRR) like the Toll like receptors (TLR) and are among the first cells of the immune system to respond to invading pathogens. Once activated, APCs increase the expression of various co-stimulatory molecules which allow these cells to induce a T cell response.

1.1.3 Dendritic cells

DCs provide a link between the innate and adaptive immune system by enabling primary as well as secondary immune responses. Deriving from the bone marrow, progenitors of these cells enter the circulatory system and eventually home to different tissues. Stimulated by the uptake of pathogens or the recognition of pathogenic determinants the progenitors become mature DCs, upregulating levels of stimulating molecules on their cell surface, and migrate to secondary lymphoid tissues. Here they are responsible for T cell activation (Tsai et al. 1989).

1.1.4 B lymphocytes

B cells are activated by antigen binding to B cell receptors (BCR), a membrane bound immunoglobulin, or by T cell engagement. B cell-activation induces their development to antibody secreting plasma cells. Hence B cells major role in the immune system is to produce antibodies specific to invading pathogens and cause their destruction.

In addition to secreting antibodies, B cells can function as APC. Receptor mediated endocytosis leads to antigen internalisation, reprocessing of the antigen and finally presentation of antigen fragments on major histocompatibility complex (MHC) class II molecules. These complexes are recognised by specific T cells leading to the initiation of signalling cascades and activation of both cell types (Bonilla and Oettgen 2010).

1.1.5 T lymphocytes

T lymphocyte progenitors originate in the bone marrow and migrate to the thymus for selection and maturation. After leaving the thymus T lymphocytes re-circulate through the lymphoid organs. After activation by encountering specific antigens presented by APCs, T lymphocytes develop to effector cells with different cell type specific functions. The two major T cell types are CD8+ and CD4+ cells expressing specific T cell receptors (TCRs) which recognise antigen derived peptides presented by specific MHC molecules (Rammensee 1995).

1.1.6 CD8+ T cells

CD8+ T cells (cytotoxic T cells) recognise and respond to foreign peptides presented by MHC class I on the surface of virtually all nucleated cells. Cytoplasmic antigens, or in the case of DCs exogenous antigens, are processed intracellularly by proteases and transported into the rough endoplasmatic reticulum (RER) where MHC class I loading occurs (Fig.1.1) (Groothuis and Neefjes 2005).

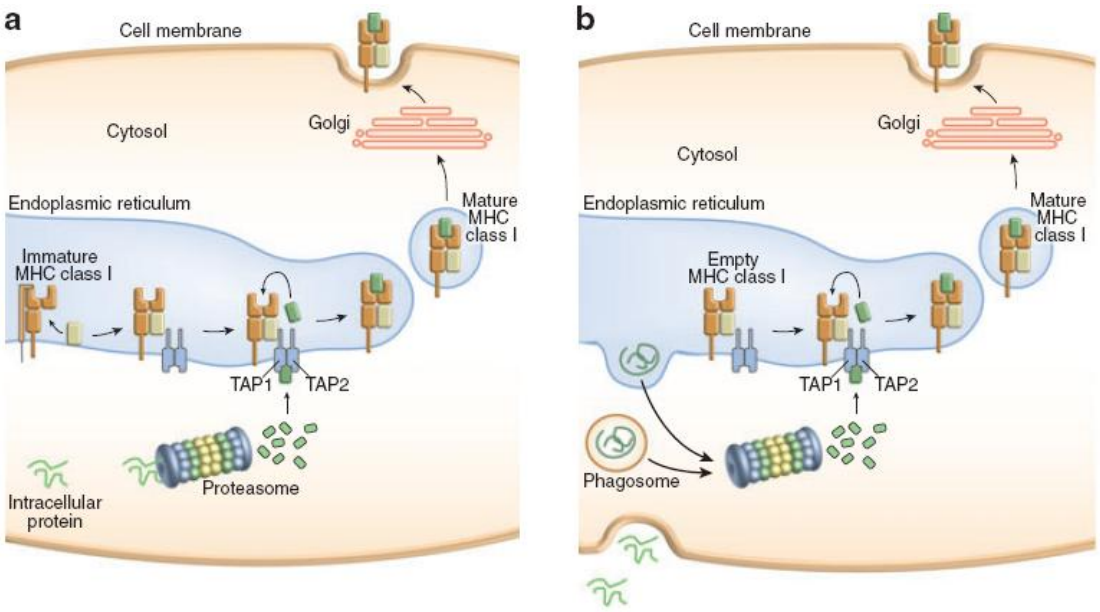


Figure 1.1: Antigen processing and presentation pathway for MHC class I
Antigens presented by MHC class I are derived from intracellular proteins. a) These proteins are degraded by the proteasome into short peptide fragments that are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) at the (ER). Peptide binding to MHC class I is followed by the transportation of this complex through the Golgi to the surface membrane. b) Alternatively extracellular or even ER-derived proteins can exit the endosomal pathway to the cytosol and enter the normal MHC class I loading pathway. Reproduced from: (Andersen et al. 2006)

MHC class I molecules derive from human leukocyte antigen (HLA)-A, -B, and -C loci where multiple allelic variants of the major loci ensure that a wide variety of peptides can be bound by the peptide binding groove. The structure of the peptide binding groove determines the antigen binding specificity of each molecule (Matsumura et al. 1992).

Upon recognition of an epitope bound to MHC class I, naïve CD8+ T cells undergo clonal expansion and differentiation into effector cells (Andersen, Schrama, Thor, & Becker 2006;Pfeifer, Wick, Roberts, Findlay, Normark, & Harding 1993). Even though an interaction of the TCR with the appropriate peptide-MHC complexes is crucial for T cell activation, clonal expansion and differentiation, further co-stimulatory signals are required. The best characterised for example is the interaction between T cell CD28 and CD80 and CD86 on the APC. When activated, CD8+ T

cells can kill their target cells in different ways. On one hand, they release cytotoxic effector molecules like perforin and granzymes which form pores in the plasma membrane of target cells, thereby compromising the membrane's integrity on one side, and activating proteinases called caspases, on the other. These events will result in death of target cell. On the other hand, CD8+ T cells can induce apoptosis by expressing Fas-ligand (FasL) which binds to Fas on the target cell (Chavez-Galan et al. 2009).

1.1.7 CD4+ T cells

CD4+ T cells (T helper cells) play a key role in orchestrating adaptive immunity by regulating cellular and humoral immune responses (Khanolkar et al. 2004;Zajac et al. 1998). They have been historically divided into two different lineages based on their cytokine production. T helper cells 1 (Th1 cells) are characterised by their production of Interferon (IFN)-γ, Interleukin (IL)-2 and Tumour necrosis factor (TNF)-α and evolved to enhance eradication of intracellular pathogens. Th2 cells, which instead evolved to enhance elimination of extracellular parasitic infections, produce the cytokines IL-4, IL-5 and IL-13 which are potent activators of immunoglobulin production and facilitate recruitment of eosinophils, a particular kind of granulocytes. In recent studies, Th17 cells which are so called because of their production of IL-17 have been further characterised (Park et al. 2005). They seem to be highly proinflammatory and involved in autoimmune disorders (Bettelli et al. 2007;Langrish et al. 2005).

Another subset of CD4+ T cells is regulatory T cells (Tregs) which can be divided into two different types. Natural occurring Tregs develop in the Thymus and go to the

periphery with a functional suppressive phenotype where they make up to 5-10% of the peripheral CD4+ T cell population (Wing et al. 2006). They express the transcription factor Forkhead box protein 3 (Foxp3) (Fontenot et al. 2003;Hori et al. 2003) and seem to mediate the suppression in a contact dependent way. They need TCR interaction to become suppressive but once activated they suppress all T cells independently of their antigen specificity (Wing et al. 2006). Adaptive Treg cells (also known as Tr1 cells or Th3 cells) may originate during a normal immune response from mature T-cell populations under certain conditions of cytokine- and antigenic stimulation (Barrat et al. 2002;Maloy and Powrie 2001). So, adaptive Treg cells are distinguished from natural Treg cells not by their origin (the thymus), but rather by their requirement for further differentiation as a consequence of exposure to antigen in a distinct immunological context.

1.1.7.1 Pathways of antigen presentation through MHC class II

CD4+ T cells are stimulated by pathogen-derived exogenous peptides, presented on MHC class II molecules, encoded by HLA-DR, -DQ and –DP loci and expressed on specific APCs. After encountering antigen, APCs can internalise antigens in three different ways: by phagocytosis, fluid phase pinocytosis or receptor mediated endocytosis (Thery and Amigorena 2001). The antigen then is proteolytically processed into small peptide fragments and loaded onto MHC class II molecules which occurs in special compartments called MHC class II enriched compartments (MIICs) (Fig.1.2). In the ER, MHC II α and β-chains assemble into heterodimers forming a binding groove capable of binding antigenic peptides. Yet in the early stages, this binding groove is occupied by a short peptide called CLIP from the

transmembrane chaperon called invariant chain (Ii) (Roche and Cresswell 1990). CLIP prevents MHC II from premature peptide binding. From here these molecule complexes can either be transported directly to the cell surface via vesicles derived from the Trans Golgi Network (TGN) or a sorting motif in the cytoplasmic portion of li directs MHC II complexes to MIICs (Bakke and Dobberstein 1990;Roche et al. 1993). Within MIICs low pH, protease activity and a chaperone called HLA-DM facilitate the exchange of CLIP for antigenic peptides. Also other proteins on the membranes of MIICs may interact with MHC II, HLA-DM (Hammond et al. 1998) and play a role in efficient peptide loading. Ultimately, fusion of MIIC with the plasma membrane is one way MHC II complexes are delivered to the surface for antigen presentation (Raposo et al. 1996;Wubbolts et al. 1996). An alternative way is MHC class II recycling which occurs by internalisation of MHC class II-peptide complexes via ubiquitin mediated endocytosis from the plasma membrane. This way of entering the endosomal pathway eventually leads to reprocessing of these MHC class II-peptide complexes in MIICs. In this context, where cells (APCs) naturally express MHC class II antigens, CD4+ T lymphocytes could potentially act as direct effectors in their own right and kill infected or malignant cells (Merlo et al. 2010).

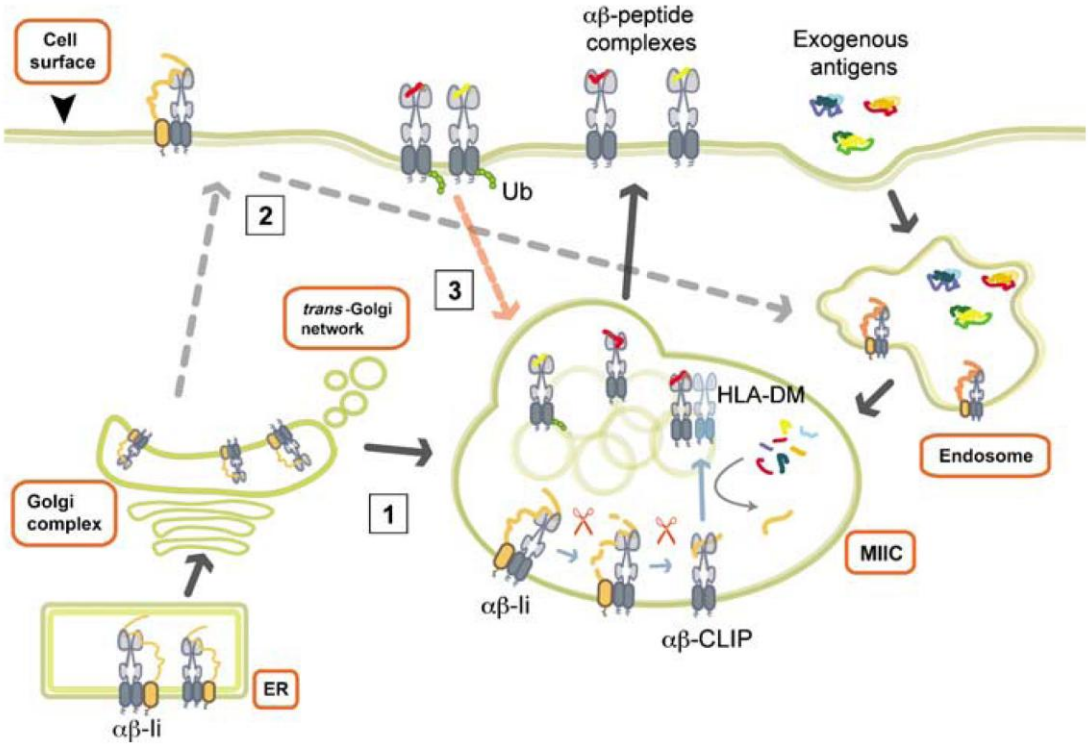


Figure 1.2: Antigen processing and presentation pathway for MHC class II
MHC class II heterodimers are assembled in the ER and transported by the invariant chain (li) through the Golgi (1) either directly to MIICs or (2) indirectly via the surface membrane where unbiquitination (Ub) results in re-internalisation and recycling of MHC molecules (3). In the MIIC, proteases degrade li leaving a small fragment called CLIP in the binding groove. HLA-DM later replaces CLIP with exogenous peptides before the MHC class II-peptide complex is integrated into the plasma membrane. Reproduced from: (Rocha and Neefjes 2008)

1.1.8 The immunological synapse (IS)

The adaptive immune response relies on T cell activation by their recognition of antigenic peptides presented by APCs which in turn necessitates the physical interaction of T cell and APC. This interaction is accompanied by pronounced changes in T cell morphology; these include a flattening of the T cell against the APC which requires changes in the cytoskeleton (Delon et al. 1998;Donnadieu et al. 1994). For T cells early research has shown that both, the microtubule organising centre (MTOC) and microfilaments orientate towards the APC (Geiger et al. 1982;Kupfer et

al. 1986). This T cell-APC interface is termed immunological synapse or immune synapse (IS) as it is thought to function in the communication between the two cells (Bromley et al. 2001). The IS is organised into distinct domains termed supramolecular activation clusters (SMACs). These clusters sequester receptor-ligand interactions and signalling molecules and facilitate adhesion and antigen presentation or recognition, respectively (Grakoui et al. 1999). The SMAC is canonically organised into concentric rings during antigen presentation. The orientation of molecules involved in antigen presentation and activation such as TCRs, MHCs and co-stimulatory molecules are located in the of the "Bull's eye" thermed central SMAC (cSMAC) within the IS; adhesion molecules such as LFA-1 and ICAM-1 are located within the peripheral SMAC (pSMAC) surrounding the centre (Monks et al. 1998a).

1.1.9 Cancer immune evasion

In the majority of cancer patients, tumours are not recognised by the immune system. This lack of natural induced immunity is not only due to the limited expression of tumour-associated antigens (TAAs) (Disis et al. 2000) but also to an active suppression of the adaptive immune activity by the tumour cells.

During the process of tumour growth, the tumour microenvironment, which is composed of immune cells, stromal cells and the extracellular matrix, is the main site of immune and cancer cell interaction allowing and supporting proliferation, survival and cell migration of tumour cells. Not only do malignant cells survive in these conditions but they are also capable of mimicking some of the signalling pathways of the immune system which enhances tumour immune tolerance and allow them to escape host immune response. The major influences are imbalances in the tumour

microenvironment including alterations in APC-subsets. This is caused by deregulation of the ratios of co-stimulatory and co-inhibitory molecules and of T cells and regulatory T cells (Zou 2005). Additionally, cancer cells can release surface antigens in order to evade detection of the immune system. This energy dependent process may involve secretion of a specific type of microvesicles called exosomes, which themselves have been shown to have a potential inhibitory effect on immune cells *in vitro* (Valenti et al. 2007;Whiteside 2005).

1.1.10 Cancer immunotherapy

The main strategy in cancer immunotherapy is to make use of the altered or de novo expression of particular proteins and to target these TAAs for host immune responses. Expression of most TAAs is not restricted to tumour cells but these antigens are either over-expressed in tumour cells or only occur naturally during the foetal development. Despite an existing shortage of well-defined tumour antigens, several immunotherapeutic approaches have been used in the treatment of various cancers. For example, patients could be vaccinated to a certain degree using tumour cell lysates and irradiated tumour cells (Mitchell et al. 2002). However, this treatment is costly and labour intensive and inoculation the entire repertoire of cellular proteins risks masking TAAs and potentially generates dangerous auto-reactive immune responses (Moingeon 2001). Monoclonal antibodies, unmodified, chimeric or armed with toxins or radionuclides, recognising particular TAAs have also been approved to treat cancer in several trials (Waldmann 2003). Compared to passive immunisation active immunotherapy mostly targets the direct enhancement or modulation of the function of APCs. Since DCs are considered most potent in initiating immune

responses, therapeutic approaches involve mature DCs either loaded with tumour antigens (Berger and Schultz 2003) or transduced with genes that encode relevant tumour antigens *in vitro* (Dyall et al. 2001). These DCs then are re-introduced to the patients to stimulate cytotoxic T cell response *in vivo*. Tumour-antigen specific cytotoxic T cells were also used directly to combat particular cancers. Similar to DCs they can be generated *ex vivo* to produce an anti-tumour response after re-administration to the patient (Yamaguchi et al. 2003). Recently Hunder et al. successfully treated a patient with refractory melanoma by isolating and expanding autologous tumour-antigen specific CD4+ T cells. They achieved a durable clinical remission of the tumours (Hunder et al. 2008). However, high costs, labour intensiveness and high specificity, limit the use of all methods involving *ex vivo* propagation and treatment of live cells in immunotherapies.

1.2 Exosomes

1.2.1 Definition and exosome formation

Exosomes are small membrane vesicles of endocytic origin, 30 – 100nm in size, which are secreted by a variety of different cell types including B cells (Raposo et al. 1996), T cells (Blanchard et al. 2002), DC (Zitvogel et al. 1998), neurons (Faure et al. 2006), epithelial cells (Van et al. 2001) and tumour cells (Andre et al. 2004b). Recent studies revealed the presence of exosomes in a number of body fluids such as plasma (Caby et al. 2005), urine (Pisitkun et al. 2004), synovial fluid (Skriner et al. 2006),

malignant effusions (Andre et al. 2002), epididymal fluid (Gatti et al. 2005) and seminal plasma (Ronquist 2007).

Exosomes are thought to originate from late endosomal compartments called mulitvesicular bodies (MVBs). They are formed by fusion of endosomes with one another and subsequent inward budding of the limiting endosomal membrane. MVBs either transport proteins for degradation to lysosomes, release their intraluminal vesicles into the cytoplasm as a result of a process called back fusion or fuse with the plasma membrane releasing its inner vesicles now called exosomes (Pan et al. 1985;Raposo et al. 1996;Simons and Raposo 2009). The intracellular transport of vesicles and sorting of cargo into MVBs is orchestrated among others by Rab proteins and Ca^{2+} (Savina et al. 2002;Savina et al. 2003;Savina et al. 2005), by the endosomal sorting complexes required for transport (ESCRT) (De et al. 2004;Tamai et al. 2010), lipid rafts (De et al. 2003;Mincheva-Nilsson and Baranov 2010) and ubiquitination (Longva et al. 2002;Pisitkun et al. 2004).

1.2.2 Exosome composition

The composition of exosomes varies between cell types but contains some common components such as MHC class I, Rabs, Arp2/3, annexins (Thery et al. 2001;Wubbolts et al. 2003a) and tetraspanins (Escola et al. 1998) as well as heat shock proteins (Clayton et al. 2005;Thery et al. 1999), cytoskeleton proteins like actin and moesin, ESCRT proteins, and CD55 and CD59, both of which have been shown to protect exosomes from complement lysis (Clayton et al. 2003) (Table 1.1). In APCs, MIICs, which represent a morphologically distinct class of MVBs, are the major sites for antigen loading of MHC class II molecules. Consequently, APC

derived exosomes bear peptide loaded MHC class II-peptide complexes in addition to co-stimulatory proteins such as CD54 (ICAM-1), CD80 (B7-1) and CD86 (B7-2) (Clayton et al. 2001;Escola et al. 1998;Lamparski et al. 2002).

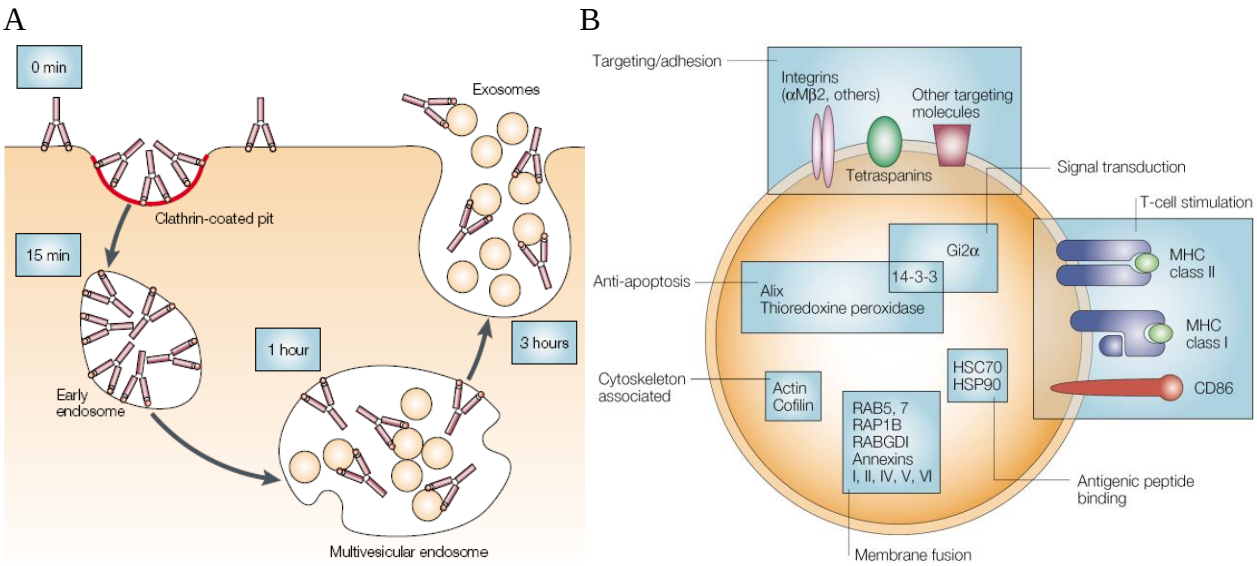


Figure 1.3: Exosome formation and protein composition
A) Tracking an anti-transferrin-receptor antibody reveals the formation of exosomes. Firstly clathrin coated pits are observed which quickly lead to the generation of early endosomes. These fuse with one another and form intraluminal vesicles by invagination leading to MVBs. After fusion of these MVBs with the plasma membrane the intraluminal vesicles are released as exosomes to the extracellular environment. B) According to their origin and development exosomes carry several cytosolic as well as specific and general membrane proteins. Reproduced from: (Thery et al. 2002b)

Table 1.1: protein expression of exosomes according to their cell type origin
modified from (Thery et al. 2002b)

Exosomes protein	*Cell type*
Antigen presentation	
MHC class I	B cells, Dendritic cells, Enterocytes, Tumours, T cells
MHC class II	B cells, Dendritic cells, Enterocytes (IFNγ-treated), Mastocytes, T cells
Integrins	
α4β1	Reticulocytes
αMβ2	Dendritic cells
β2	T cells
αLβ2	Mastocytes
Immunoglobulin-family members	
ICAM-1/CD54	B cells, Dendritic cells, Mastocytes
P-selectin	Platelets
A33 antigen	Enterocytes
Cell surface peptidases	
Dipeptidylpeptidase IV/CD26	Enterocytes
Aminopeptidase n/CD13	Mastocytes
Tetraspanins	
CD63	B cells, Dendritic cells, Enterocytes, Platelets, T cells, Mastocytes
CD37, CD53, CD81, CD82	B cells
CD9	Dendritic cells
Heat-shock proteins	
HSC70	Reticulocytes, Dendritic cells, Tumours
HSP84/90	Dendritic cells, Enterocytes
Cytoskeletal proteins	
Actin	Dendritic cells, Enterocytes, Mastocytes
Actin-binding protein (cofilin)	Dendritic cells
Tubulin	Dendritic cells, Enterocytes
Membrane transport and fusion	
Annexins I, II, IV, V, VI	Dendritic cells
Annexin VI	Mastocytes
RAB7/RAP1B/RABGDI	Dendritic cells
Signal transduction	
GI2α/14-3-3	Dendritic cells
CBL/LCK	T cells
miRNA	T cells (Mittelbrunn et al. 2011)
Metabolic enzymes	
Enolase 1	Enterocytes
Thioredoxine peroxidase	Dendritic cells

1.2.3 Exosome function in APCs

As well as being present on the cell surface MHC molecules and tetraspanins are released from cells on exosomes. These micro-vesicles were initially discovered studying reticulocytes which use exosomal secretion as a pathway to remove obsolete proteins such as the transferrin receptor during their maturation from reticulocytes to erythrocytes (Pan et al. 1983). Exosomes from APCs are shown to be involved in T cell stimulation both in vitro (Hwang et al. 2003;Raposo et al. 1996;Utsugi-Kobukai et al. 2003;Vincent-Schneider et al. 2002) and in vivo (Segura et al. 2005;Thery et al. 2002a). There is still a debate regarding how this stimulation occurs, with some studies showing exosomes being able to stimulate T cells directly without the presence of APCs (Hwang et al. 2003;Raposo et al. 1996) while others demonstrating that exosomes need APCs to exert their effect (Thery et al. 2002a;Vincent-Schneider et al. 2002). Different parameters may play a role in the efficiency of T cell stimulation by exosomes. Some of them are the phenotype of exosomes and of the T cells used, the affinity of the antigens or the doses or quality of the exosomes used. Hwang et al. proved that CD54 together with B7 and MHC class I/peptide complex on exosomes is strongly immunogenic (Hwang et al. 2003). Segura et al. showed that CD54 together with MHC class II is required to prime naïve T cells (Segura et al. 2005). Referring to the exosome's donor cell type, it is shown that exosomes from mature DCs are more potent inducing antigen specific T cell stimulation as compared to exosomes from other APCs (Segura et al. 2005;Utsugi-Kobukai et al. 2003) which may be due to the higher expression of the proteins CD54 and MHC II.

Another function for exosomes might be to serve as a vehicle to transport molecules from one cell to another. MHC/peptide complexes are transferred between DCs

enabling recipient cells to efficiently stimulate T cells in an antigen specific manner (Andre et al. 2004a;Hsu et al. 2003). Furthermore, exosomes can be internalised by DCs and sorted to the endosomal compartments for reprocessing of exosomal proteins, which will lead to presenting their peptides on MHC class II molecules and stimulation of CD4+ T cells. The targeting to DCs is mediated by a number of exosomal proteins including milk fat globule elongation factor 8 (MFG-E8), CD11a, CD54, phosphatidylserine and the tetraspanins CD9 and CD81 (Morelli et al. 2004). Moreover, *in vivo* experiments demonstrated that exosome like vesicles bearing MHC class II can be attached to follicular DCs. As these cells do not express MHC class II themselves the vesicles probably derive from another cell type and may be involved in modulating the immune response (Denzer et al. 2000). Additionally, B cell derived exosomes were shown to express integrin molecules which enable them to bind to extracellular matrix proteins like collagen and fibronectin as well as the surface of activated fibroblasts giving evidence for exosomes' ability to transfer signals at distances beyond the direct cell-to-cell contact (Clayton et al. 2004). Also exosomes secreted by mast cells are shown to have an immunological effect. They are able to stimulate maturation of DCs, which respond with up-regulation of MHC class II, CD40, CD80 and CD86. In addition, when co-incubated with splenocytes mast cell-derived exosomes induced blast-formation, proliferation and IL-2 and IFNγ production (Skokos et al. 2003). More recent *in vitro* studies demonstrated that exosomes could serve as a tool for a cell-free vaccine for immunoprophylaxis against leishmaniasis and other infectious diseases (Schnitzer et al. 2010).

1.2.4 Exosomes from tumour cells

During the last few years much effort has been invested to study exosomes in the context of tumour biology and cancer treatment. Tumour cell-derived exosomes were able to protect mice against establishment of the tumour (Wolfers et al. 2001). Later, exosomes bearing MHC class I, heat shock proteins (Hsp), CD81 and tumour antigens such as melanoma antigen recognised by T cells (MART1) could be purified from malignant effusions from cancer patients (Andre et al. 2002). In both studies these exosomes were able to transfer tumour specific antigens to DCs which made them capable to induce CD8+ T cell response. Although these features suggest tumour-derived exosomes as a potential tool in cancer immunotherapy, in some other studies they were also depicted to be immunosuppressive and promote cancer tolerance. These exosomes were shown to inhibit CD8+ T cell cytotoxic killing (Clayton and Tabi 2005) and promote apoptosis of CD8+ T cells by FasL-Fas interaction (Abusamra et al. 2005). Likewise, tumour-derived exosomes can inhibit the cytotoxicity of natural killer cells (NK) by reducing their perforin release (Liu et al. 2006) and activate differentiation of myeloid-derived suppressor cells (MDSC) which are shown to promote tumour progression (Xiang et al. 2008). Exosomes secreted by EBV-associated nasopharyngeal carcinoma cells were shown to express Galectin-9 *in vivo*. Galectin-9 binds to TIM (T cell, immunoglobulin domain and mucin domain)-3, an apoptosis inducing membrane receptor on mature CD4+ T cells. NPC exosomes induced significant apoptosis of EBV-specific CD4+ T cells and indicate their CD4+ T cell suppressive effect and a role in tumour tolerance (Klibi et al. 2008). Additionally, exosomes are proven to be involved in tumour angiogenesis by the expression of the tetraspanin D6.1A/CO-029 (Tspan8) (Gesierich et al. 2006) or

Tspan8-CD49d complex (Nazarenko et al. 2010a), proteins shown to be strong inducers of angiogenesis.

1.2.5 Exosomes in cancer immunotherapy

Even though tumour derived exosomes have been shown to exhibit inhibitory effects on immunity (Abusamra et al. 2005;Clayton & Tabi 2005;Liu et al. 2006) they are still an attractive target for immunotherapy as they represent a natural source of tumour antigens (Wolfers et al. 2001).

First evidence for the anti-tumour effect was shown for tumour-peptide pulsed DC-derived exosomes (DEX). Injected into mice they suppressed established tumour growth (Zitvogel et al. 1998). Since then many studies focussed on exosomes as a potential tool in immunotherapy. Different approaches examined either exosomes from tumour cells or exosomes from DCs loaded with tumour peptides as mentioned above (Altieri et al. 2004;Chaput et al. 2004;Hao et al. 2006b). Comparison of these two different potential cancer vaccines revealed tumour peptide loaded exosomes derived from DCs as an efficient vehicle in inducing an anti-tumour immunity (Hao et al. 2006a). Different approaches to improve the immunogenicity of exosomes have been utilised. For example, heat shocked lymphoma cells led to secretion of exosomes with a higher level of both, heat-shock proteins and lymphocyte proteins such as MHC class II, CD40 and CD86. These exosomes were more potent in inducing T cell responses and anti-tumour immunity when compared to exosomes from untreated lymphoma cells (Chen et al. 2006). Furthermore, an increase in exosomal immunogenicity could be achieved by tumour cells genetically modified to express IL-2. Their exosomes contain IL-2 which made them more efficient in inhibiting

tumour growth when used for immunotherapeutic experiments (Yang et al. 2007). Additionally, the supplemental treatment with adjuvants like CpG was shown to have positive effects on tumour rejection (Chaput et al. 2004). Autologous DEX loaded with tumour peptides were used in phase 1 clinical trials for vaccination of lung cancer and melanoma patients. Low toxicity of the exosome treatment could be shown (Escudier et al. 2005;Morse et al. 2005) which allowed going to phase II clinical trial (Mignot et al. 2006).

1.3 Tetraspanins

1.3.1 Background

Tetraspanins are ubiquitous surface proteins, firstly described as an distinct protein family in 1994 (Wright and Tomlinson 1994). Subsequent work has revealed much more about their structure and functions. Tetraspanins are known to be involved in various cellular processes including motility, invasion, fusion, signalling, maintaining cell morphology, immune response and even in pathogenesis of pathogenic diseases (Boucheix and Rubinstein 2001;Chambrion and Le 2010;Hemler 2003a;Romanska and Berditchevski 2010;Tarrant et al. 2003;van Spriel and Figdor 2010a).

1.3.2 Tetraspanin structure

All members of the tetraspanin family have a characteristic structure (Fig. 1.5) consisting of four transmembrane domains giving rise to a small and a large extracellular loop (termed EC1 and EC2, respectively), a small interconnecting cytoplasmic loop and a short C- and N-terminal cytoplasmic tails. A distinctive feature of tetraspanins compared to other four-transmembrane proteins is the conserved Cys-Cys-Gly motif in EC2 which stabilises the three dimensional structure of this loop. Intracellular juxtramembrane cysteins are palmitoylated which is thought to facilitate tetraspanin-tetraspanin binding and together with palmitoylation of specific integrins it contributes to formation of tetraspanin microdomains (Berditchevski et al. 2002;Charrin et al. 2002;Sharma et al. 2008). It has been proposed that the main tertiary structure of the protein is partly stabilised by polar residues in the transmembrane regions (Seigneuret 2006;Stipp et al. 2003b).

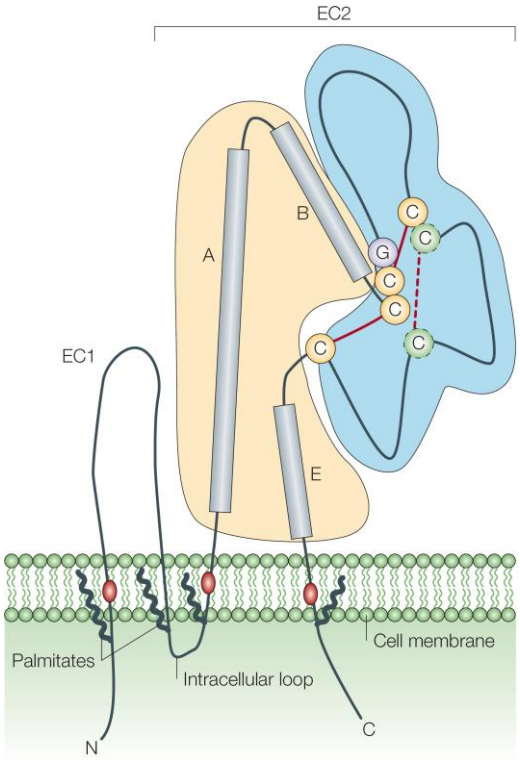


Figure 1.4: Tetraspanin structure
Tetraspanins are comprised of four transmembrane domains, an N- and C-terminal cytosolic tail, two extracellular loops (EC1 and EC2) and a short intracellular loop. EC2 can be separated into a constant (yellow) and a variable region (blue) where conserved cysteins, including the CCG motif, lead to sulphide bonds which are crucial for the correct folding of EC2 (Hemler 2005). Reproduced from: Hemler, 2005

1.3.3 Binding partners

The most important feature of tetraspanins is their ability to interact with one another as well as with other molecules in the cell membrane. Homophilic tetraspanin-tetraspanin interactions (e.g. CD9-CD9) are more common than the formation of heterophilic complexes (e.g. CD9-CD151, CD81-CD151) (Kovalenko et al. 2004). Non-tetraspanin associations involve several transmembrane and also some cytosolic molecules including integrins, growth factor receptors, peptidases, immunoglobulin superfamily members, G-protein coupled receptors and their associated G-proteins

and cytosolic signal transduction molecules (Table 2) (Andre et al. 2006;Berditchevski 2001;Hemler 2005;Levy and Shoham 2005a).

Apart from these direct interactions, creating protein-oligomers, tetraspanins associate with cholesterol and gangliosides (Charrin et al. 2003;Miura et al. 2004;Odintsova et al. 2006), enabling these small protein complexes to group together to form higher order protein aggregates, the tetraspanin-enriched microdomains (TERMs) (Hemler 2003b;Hemler 2005).

Table 1.2: known tetraspanin partners
Reproduced from M. Yunta, P. Lazo, 2002 and M. Yáñez-Mó et al. 2009

		CD9	*CD37*	*CD53*	*CD63*	*CD81*	*CD82*	*CD151*
Integrins	α3β1	+	+	+	+	+	+	+
	α4β1	+	nd	+	+	+	+	nd
	α6β1	+	nd	+	+	+	+	+
	α5β1	+	nd	nd	+	+	+	+
	α6β1	+	nd	nd	nd	nd	nd	+
	α7β1	+	nd	nd	nd	nd	nd	+
	Precursor β1	+	nd	nd	-	-	-	nd
	αLβ2	nd	nd	nd	+	nd	+	nd
	αMβ2	nd	nd	nd	+	nd	nd	nd
	αIIbβ3	+	nd	nd	-	-	-	-
	α3β4	+	+	+	+	+	+	+
	αvβ4	nd	nd	nd	nd	+	nd	nd
	CD11/CD18	nd	nd	nd	+	nd	nd	nd
MHC	HLA-DR	+	+	+	+	+	+	+
	HLA-DM	+	nd	nd	+	+	+	nd
	HLA-DQ	+	+	+	+	+	+	+
	HLA-DO	+	nd	nd	+	+	+	nd
	MHC I	nd	nd	+	+	+	+	nd
Growth factor receptor	EGF-R	nd	nd	nd	nd	nd	+	nd
	TGF-α	+	nd	nd	nd	nd	nd	nd
	HB-EGF	+	nd	nd	nd	nd	nd	nd
Immunoglobulin superfamily	FRPP	+	nd	nd	nd	+	nd	nd
	EWI-2	+	nd	nd	nd	+	nd	nd
	EWI-F	+	nd	nd	nd	+	nd	nd
	CD2	+	nd	+	nd	nd	nd	nd
	CD4	nd	nd	nd	nd	+	+	nd
	CD8	nd	nd	nd	nd	+	+	nd
	CD19	+	nd	nd	nd	+	+	nd
	CD20	nd	nd	+	nd	+	+	nd
	CD54 (ICAM-1)	+	nd	nd	nd	nd	nd	nd
	CD106 (VCAM-1)	nd	nd	nd	nd	nd	nd	+
Other membrane proteins	CD36	+	nd	nd	nd	nd	nd	nd
	CD46	+	nd	nd	nd	+	+	+
	CD21/CD19/Leu13	nd	nd	nd	nd	+	nd	nd
	E-cadherin	nd	nd	nd	nd	nd	nd	+
	L6	nd	nd	nd	nd	nd	nd	+
	Dectin-1	nd	+	nd	+	nd	nd	nd
Signalling molecules	PKC	+	-	+	-	+	+	+
	PI 4K	+	nd	-	+	+	-	+

nd: not determined

1.3.4 Tetraspanins and immune responses

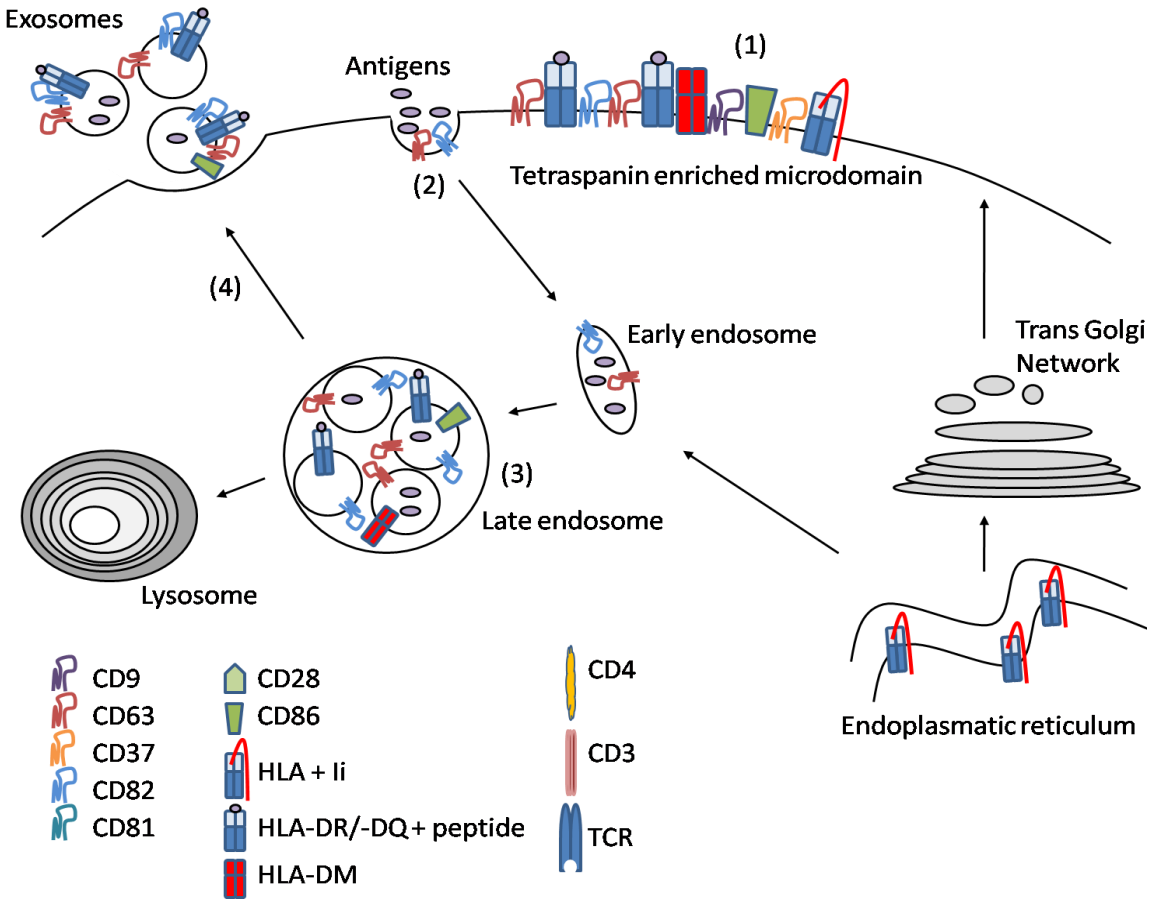
Tetraspanins function via modulating the functions of associated proteins rather than exhibiting direct enzymatic activities. Tetraspanins sequester partner molecules into

TERMs where these molecules are influenced by the composition of the microdomains as well as the surrounding tissue and the maturation state of the cell. For example, tetraspanins influence cell adhesion, migration and spreading predominantly by compartmentalisation, clustering, internalisation, recycling of integrins and regulating integrin-dependent signalling (Berditchevski 2001;Hemler 2005;Levy and Shoham 2005b;Stipp et al. 2003b). Since all cells of the immune system express tetraspanins (Tarrant et al. 2003) they also have been implicated in various aspects of host immunity (Table 1.3). Tetraspanins and associated molecules are involved in intercellular interactions involving immune cells as well as immune cell migration, adhesion, proliferation and signalling (Rubinstein et al. 1996). At the cellular level tetraspanins contribute to synapse formation (Mittelbrunn et al. 2002) and intracellular transport (i.e. endocytosis and exocytosis) of key immune receptors and associated effector proteins (Pols and Klumperman 2008) (Fig. 1.5 A). Tetraspanins are shown to interact with fungal pattern-recognition receptors (F-PRR) and may regulate their function in the response to fungal antigens by recruitment of F-PRRs into TERMs (Figdor and van Spriel 2010). Moreover, tetraspanins are suggested to play a role in the defense to viral, bacterial and parasitic infections (van Spriel & Figdor 2010a).

In the context of tetraspanins and their role in antigen presentation via MHC II, tetraspanins CD9, CD37, CD53, CD81 and CD82 are documented to interact on the surface with MHC class II complexes (Engering et al. 2003;Kijimoto-Ochiai et al. 2004;Schick and Levy 1993a) (Fig. 1.5 B). It was also described that tetraspanins, which are abundant on MIIC can interact with HLA-DM and HLA-DO (Hammond et al. 1998). Given emerging roles of tetraspanin microdomains in sorting and trafficking of the associated proteins, it has been suggested that tetraspanins may play

a role in MHC transport (Vyas et al. 2007) or peptide loading (Rocha & Neefjes 2008) (Fig. 1.5 A). It is thought that peptide presentation by MHC molecules does not occur randomly but instead is a well organised process which brings MHC-peptide complexes into functionally potent clusters on the APC's surface (Unternaehrer et al. 2007). Tetraspanins are likely contributors in this process as particular multi protein surface complexes were shown to incorporate CD82, CD9, CD81 together with MHC II, CD86 and HLA-DM. Kropshofer et al. demonstrated that tetraspanin microdomains sequester particular subsets of MHC II molecules carrying the same antigenic peptide. In this way TERMs facilitate an optimal T cell stimulation by bringing these MHC-peptide complexes in juxtaposition to each other and generating stimulatory units (Kropshofer et al. 2002a). Interestingly, dendritic cells from mice deficient in CD37 or CD151 were hyperstimulatory (Sheng et al. 2009) (Table 1.4). Whilst CD151's influence appears to be purely due to an elevated co-stimulatory activity, the knockout of CD37 seems to change MHC II-peptide driven activation signals. These data clearly indicate that CD37 has an inhibitory effect in antigen presentation. The authors suggested that one of the possible mechanisms may involve CD37-dependent sequestration of MHC II away from the clusters required for effective stimulation of TCR. Further underlying the versatile regulatory roles of tetraspanins in the immune system, Gartlan et al. showed that *in vitro* DCs deficient in the expression of CD37 and Tssc6 induced hyperproliferation of T cells. In vivo however, the absence of both these tetraspanins leads to a poor immune response in mice (Gartlan et al. 2010). This observation is suggested to be due to a deregulation of cytokine production *in vivo* caused by a simultaneous T cell hyperstimulation and hyperproliferation (Gartlan et al. 2010).

A



B

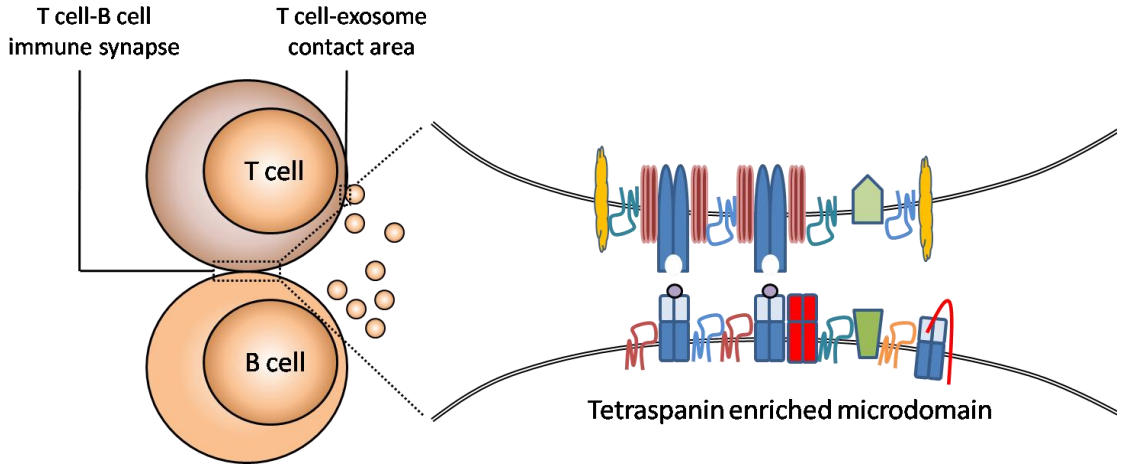


Figure 1.5: Model of tetraspanin functions and associated microdomains on MHC class II biology?

A) HLA class II molecules are incorporated into TERMs on the cell surface (1) and can get internalised during endocytosis (2). Particularly CD63 and CD82 have the potential to recycle between the cell surface and MIICs (termed here as late endosome, (3)). Once HLA-DM-mediated peptide loading in MIICs occurs, CD63 and CD82 also appear to keep together HLA class II clusters uniformly loaded with

peptide and may regulate the transport of HLA class II molecules to the surface and into exosomes (4). At the cell surface, mainly CD81, CD9 and to a lesser extent CD82 and CD63 aid in preserving the clustered configuration of HLA class II–peptide complexes. This configuration may be maintained in the synapse after TCR engagement. B) In APCs (illustrated here as a B cell), tetraspanins such as CD37, CD63 or CD81 associate with MHC class II molecules. Similarly, exosomes (shown as secreted vesicles in orange), which are enriched in tetraspanins, are involved in antigen presentation. In T cells, CD81 and CD82 associate with CD3 and CD4. CD81 is also reported to be distributed to the cSMAC.

Table 1.3: Tetraspanins known to be expressed in blood cells and their functions
Modified and updated from Tarrant et al. 2003

Tetraspanin	***Expression***	***Function***
CD9	B cells, activated T cells, Granulocytes, Monocytes, DCs, Megakaryocytes, Platelets, blood progenitors, widespread	T cell activation, Antigen presentation, B cell β1-integrin adhesion
CD37	B cells, T cells, Monocytes, DCs, Granulocytes	B cell-T cell interaction, T cell activation, antigen presentation, receptor-trafficking, IgA secretion
CD53	B cells, T cells, DCs, Monocytes, NK cells, Granulocytes	Monocyte and neutrophil activation, T cell activation, T cell positive selection, B cell β2-integrin adhesion
CD63	B cells, T cells, DCs, Monocytes, Platelets, widespread	Antigen presentation, β2-integrin adhesion, MHC II trafficking
CD81	B cells, T cells, NK cells, DCs, Monocytes, blood progenitors, widespread	B cell co-receptor, B cell-T cell activation, B cell-T cell interaction, Th2 response, β1 and β2-integrin adhesion
CD82	B cells, T cells, Granulocytes, Monocytes, CD34+ progenitors, widespread	T cell co-stimulation, β2-integrin adhesion, antigen presentation
CD151	B cells, T cells, Granulocytes, Megacaryocytes, Platelets, erythroleuceia and T cell lines, widespread	Neutrophil motility, co-stimulation
Tssc6	B cells, T cells, Granulocytes, Monocytes, DCs, erythroid cells, blood progenitors	T cell activation, T cell proliferation

Table 1.4: immunological phenotypes of various tetraspanin knockdowns in mice

Tetraspanin	*Immune cell phenotype upon knockdown*	*Reference*
CD9	No significant phenotype on immune cells (Infertility due to defective fusion capacity of eggs), decreased exosome release in DCs	(Chairoungdua et al. 2010;Miyado et al. 2000)
CD81	Hyperproliferation of T cells, deficiency in T cell dependent IgG production and Th2 cytokine secretion, enhanced B cell activation	(Maecker and Levy 1997;Sanyal et al. 2009)
CD9, CD81	Enhanced cell fusion of alveolar macrophages and bone marrow cells after *in vivo* and *in vitro* stimulation, less motile macrophages and increased production of matrix metalloproteinase (MMP)-2, MMP9	(Takeda et al. 2003;Takeda et al. 2008)
CD37	Hyperproliferation of T cells, impaired T cell-B cell interaction under sub-optimal co-stimulatory conditions, antigen presentation via MHC II	(Knobeloch et al. 2000)
Tssc6	Hyperproliferation of T cells due to increased IL2 production, impaired Platelet function	(Goschnick et al. 2006;Sheng et al. 2009;Tarrant et al. 2002)
CD37, Tssc6	*in vitro*: Hyperproliferation of T cells, increased stimulatory capacity of DCs *in vivo*: reduced ability to produce influence-specific T cells, mice suffer from hyperparasitemia	(Gartlan et al. 2010)
CD151	Hyperproliferation of T cells in response to *in vitro* mitogenic stimulation, increased co-stimulatory capacity of DCs	(Sheng et al. 2009;Wright et al. 2004)
CD63	Minor differences in platelet function	(Schroder et al. 2009)

1.4 Model System designed for this project

I am using Epstein-Barr virus (EBV) antigens as a model system to explore the role of tetraspanin CD63 in antigen presentation to CD4+ T cells. In the human body, EBV infected B cells, MHC class I and II positive, establish a lifelong latent infection that is controlled by an anti-viral T cell response. *In vitro*, EBV infection of B cells leads to the generation of so called lymphoblastoid cell lines (LCLs) which here serve as APCs for EBV-antigen specific CD4+ T cells. This model was chosen since LCLs express a variety of tetraspanins including CD63 and EBV-derived antigens which are shown to be recognised by CD8+ as well as CD4+ T cells (Long et. al 2005). Furthermore, EBV-infection of B cells stimulates exosome-secretion (McLellan et al.

2009). Therefore, this system allows me to investigate the role of CD63 in the sorting of protein complexes and their compartmentalisation, especially in respect of MHC class II, and in the formation and trafficking of exosomes as well as the translocation of proteins into the endosomal pathway.

1.5 Epstein-Barr virus

Epstein-Barr virus (EBV), a γ1-herpesvirus and one of eight known human herpesviruses, is associated with several diseases and malignancies. EBV was discovered by Anthony Epstein, Yvonne Barr and Bert Achong in 1964. They grew cells cultured from the tumour specimens sent from Uganda by Denis Burkitt who described a novel childhood lymphoma endemic, now known as Burkitt's lymphoma (BL), throughout equatorial Africa (Burkitt and Wright 1966). Subsequently, EBV was identified as the causative agent of infectious mononucleosis (IM) and has been implicated in the pathogenesis of an increasing number of human malignancies like BL, nasopharyngeal carcinoma (NPC), post transplant lymphoproliferative disease (PTLD), Hodgkin's disease (HD), some cases of gastric carcinomas and T cell lymphoma (Rickinson and Moss 1997;Young and Rickinson 2004).

1.5.1 EBV infection cycle

EBV is widespread in all human populations all over the world and is carried by the vast majority of individuals as a life-long asymptomatic infection. Primary infection usually occurs during early childhood and is almost always asymptomatic. However,

in industrialised Western countries primary infection is becoming increasingly delayed and up to 50% of serologically confirmed primary EBV infection in adolescence and early adulthood result in IM (Khanna et al. 1995a).

The mechanism of infection and persistence of the virus is not yet fully understood but basically viral transmission occurs orally followed by infection, replication and expression of the full range of lytic cycle genes. There is still debate about which cells exactly are targeted by the initial infection but it is likely that, apart from B cells, the oropharyngeal epithelium plays a role in it. Later, infected B cells either enter the lytic cycle, where EBV particles are reproduced and infect again other mucosal cells and lymphocytes, or enter the latent cycle, where B cells migrate back into the lymphoid tissue. There, B cells start undergoing proliferation in which a considerable percentage of these lymphocytes is eliminated by cytotoxic T cells. This leads to infected B cells becoming resting memory B cells and going into a latency programme avoiding the expression of antigens which induces CD8+ T cell response. EBV-infected memory B cells still keep the ability to occasionally replicate the virus and release it into saliva. Some of them differentiate into plasma cells which may enter the lytic cycle whilst migrating into peripheral tissues. These lytic cycle-derived EBVs are thought to be responsible for EBV-related epithelial neoplasms such as gastric and nasopharyngeal carcinoma (NPC), whereas EBV-associated Burkitt lymphoma appears to derive from germinal-centre B-cell blasts, which remain proliferative due to activated *c-myc* oncogene. EBV-associated Hodgkin's lymphoma however is considered to arise from EBV-infected B cells carrying a cellular mutation. In immunocompromised patients, the absence of CD8+ T cells does possibly lead to lymphoproliferative disease (Maeda et al. 2009).

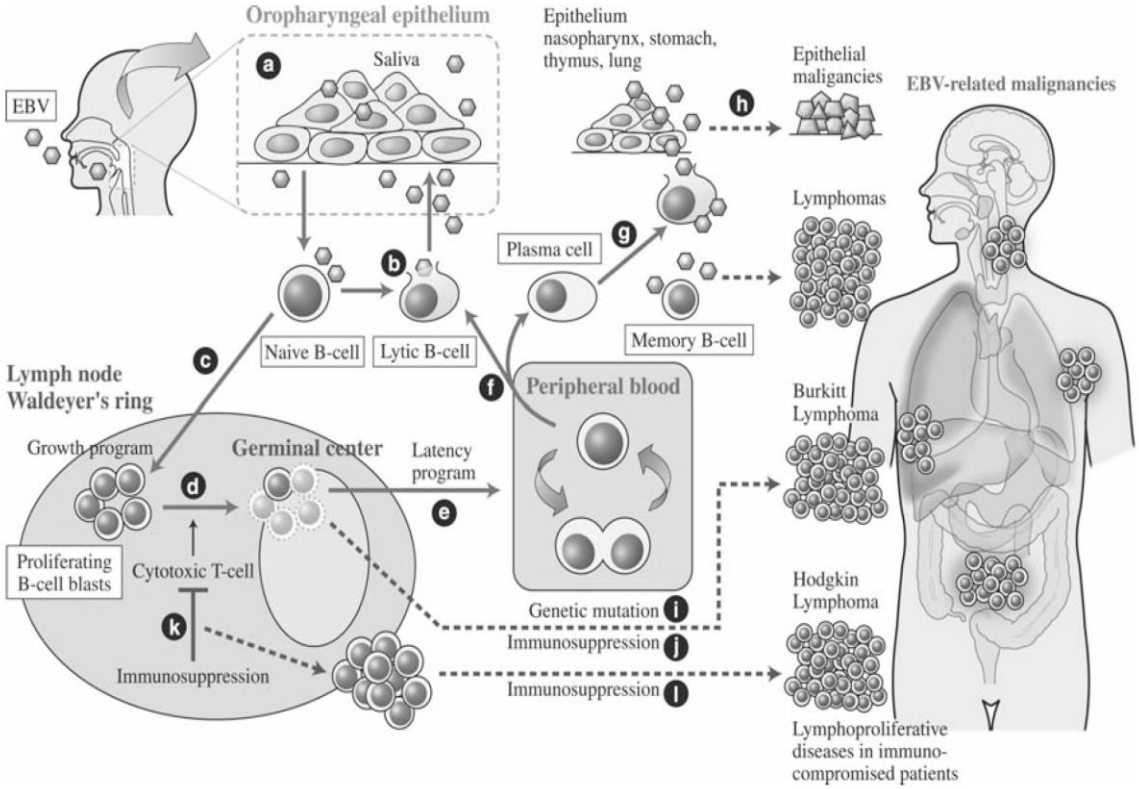


Figure 1.6: EBV infection cycle and its influence on development of certain cancer types
EBV transmission occurs via saliva followed by infection of probably oropharyngeal epithelium and/or naïve B cells (a). Infected B cells enter the lytic cycle and infect again other mucosal cells and lymphocytes (b), or the latent cycle and migrate back into the lymphoid tissue (c). Cytotoxic T cells respond to proliferating infected B cells (d). Infected B cells turn into resting memory B cells with a latency expression programme avoiding CD8+ T cell response (e). EBV-infected memory B cells replicate the virus and release it into saliva (f). Some of them differentiate into plasma cells and may enter the lytic cycle whilst migrating into peripheral tissues (g). This is thought to be the source for EBV-related epithelial neoplasms such as gastric carcinoma and NPC (h). EBV-associated BL appears to derive from germinal-centre B-cells (i). EBV-associated HL may arise from EBV-infected B cells carrying a cellular mutation (j). The absence of CD8+ T cells following immunosuppression (k) does possibly lead to lymphoproliferative disease (l). Reproduced from: (Maeda et al. 2009)

1.5.2 EBV latent infection

Primary human B cells infected with EBV *in vitro* transform into a proliferating and immortalised lymphoblastoid cell line (LCL) where the virus is represented by multiple copies of episomes (Nilsson et al. 1971). This infection by a non-productive but replication-competent virus is described as latency. Four types of latent EBV

infections are characterised by expression of different combinations of up to nine latent genes (Table 1.5). LCLs exhibit the latent state known as latency III which is essentially characterised by the expression of six EBV nuclear antigens, EBNA 1, 2, 3A, 3B, 3C and LP (leader protein), three latent membrane proteins, LMP1, 2A and 2B and two small non-polyadenylated RNAs termed EBV-encoded RNA (EBER)1 and EBER2. Other EBV-associated malignancies show different latencies as for example BL-cells only express EBNA1 and EBERs which represents latency I.

Table 1.5: EBV gene latency programmes, adapted from (Kuppers 2003)

Latency programme	***EBV genes expressed***						***Occurrence***
	EBERs	**EBNA1**	**LMP1**	**LMP2A**	**EBNA2**	**EBNA3s, EBNA-LP**	
0	+	nd	-	+	-	-	Memory B cells in peripheral blood
I	+	+	-	-	-	-	Burkitt lymphoma, PEL
II	+	+	+	+	-	-	Hodgkin lymphoma
III	+	+	+	+	+	+	PTLD
IV?	nd	nd	-	nd	+	nd	Infectious mononucleosis, PTLD

nd: not determined

1.5.3 The immune response to EBV

Primary infection predominantly occurs in early childhood and with no clinically important symptoms. However, if infection is delayed into adolescence or beyond, it may result in acute IM. IM is a self limiting disease, which is generally characterised by a huge expansion of T cells and usually resolved through generation of a strong

CD8+ T cell response. In healthy carriers, EBV persists in B cells as a latent infection that periodically reactivates into lytic cycle. The absolute frequency of infected B cells lies within the normal range of 1-50 per 10^6 peripheral blood mononuclear cells (PBMC) (Khan et al. 1996). Components of the EBV-specific immune system continually act to control the viral infection and enable a life-long, disease free co-existence of host and pathogen in the vast majority of individuals.

1.5.3.1 CD8+ T cell immune response to EBV

During the acute phase of EBV infection CD8+ T cells expand dramatically and represent the main source of adaptive immune response (Moss et al. 1988;Steven et al. 1997). EBV-specific CD8+ T cells generated, mainly recognise lytic antigens rather than latent antigens, of which 22-50% may be directed towards individual EBV lytic antigens in certain IM patients (Callan et al. 1998). But following resolution of the primary infection, lytic and latent antigen-specific CD8+ T cells may be detected in healthy EBV-seropositive individuals (Tan et al. 1999).

1.5.3.2 CD4+ T cell recognition of EBV-transformed cells

Although CD4+ T cells are crucial for priming and maintenance of CD8+ T cell responses, the role of CD4+ T cells has not been studied carefully until recently and little is known about this facet of the EBV-response. The frequency of EBV-specific CD4+ T cells during primary infection is only 1.4% in average of the total CD4+ population and drops to even to 0.34% during persistent infection (Amyes et al.

2003). CD4+ T cells came into focus of recent research due to their helper role they play in CD8-mediated virus-specific immunity and the fact that the latent EBV-infection occurs in cells in which the HLA class II pathway of antigen presentation is active. CD4+ T lymphocytes, additionally to their classical functions in the immune system, could potentially act as direct effectors in their own right and kill infected or malignant cells, when these naturally express MHC class II proteins (Merlo et al. 2010). It is known for endogenously expressed antigens to gain entry into the HLA class II processing pathway in LCLs, which applies to non-EBV-related antigens (Chen and Wu 1998;Malnati et al. 1993;Nimmerjahn et al. 2003) as well as EBV latent cycle antigens (Long et al. 2005a). First observations of polyclonal CD4+ T cell lines being able to directly recognise and lyse LCLs (Misko et al. 1984;Moss et al. 1988) did years later lead to the demonstration of *in vitro*-primed CD4+ T cell lines, generated from virus naïve individuals, to kill autologous LCLs (Savoldo et al. 2002). Identified as components of LCL-reactivated memory T cell preparations, the first CD4+ T cell clones to EBV latent proteins are specific for an EBNA-1 or an EBNA-2 derived epitope, respectively (Khanna et al. 1995b;Khanna et al. 1997). Only the EBNA-2 specific clone however seems to be capable of killing LCLs in vitro (Khanna et al. 1997). Since that time, more CD4+ T cell clones recognising latent-cycle epitopes have been generated by various groups (Leen et al. 2001;Omiya et al. 2002;Steigerwald-Mullen et al. 2000;Voo et al. 2002). However, only in 2005 the first clones which could be shown to reliably and reduceably recognise LCLs naturally expressing cognate antigens from the EBV genome were published (Long et al. 2005b). This work provides CD4 epitope maps for the EBV latent-cycle antigens EBNA1, -2,-3A and 3C and establishes CD4+ T cell clones to 12 selected epitopes

from these antigens. Two of these clones recognising epitopes from the antigens EBNA2 or EBNA3C, respectively were used as model cell lines in this project.

2 Materials and Methods

2.1 Cell culture

DMEM

Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine purchased from GIBCO or Sigma was used for culture of all adherent cells used in this study.

RPMI 1640

RPMI 1640 supplemented with L-glutamine (2.05mM) purchased from GIBCO or Sigma was used for culture of all lymphocyte cell lines.

AIM-V

Serum-free AIM-V media (GIBCO) containing L-glutamine, 50μg/ml streptomycin sulphate and 10μg/ml gentamycin sulphate was used to grow lymphocyte cells prior to purifying exosomes from the cell supernatant. This medium enables to grow cells without any bovine serum thus avoiding any contamination of growth media with exosomes or cell material of bovine origin.

Foetal calf serum (FCS)

Virus- and mycoplasma-free FCS (purchased from GIBCO) was filtered through 0.2μm pore membrane prior to aliquoting into 50ml volumes and storing at -20°C until use.

Human Serum (HS)

Sterile male HS of type AB (virus and mycoplasma screened) was purchased from HD supplies, UK and stored in 25ml aliquots at -20°C until use.

Penicillin-Streptomycin

A combined solution of the antibiotics Penicillin (10^4 units/ml) and Streptomycin (10mg/ml) in 0.9% NaCl was purchased from GIBCO. The solution was stored at -20°C until use.

Trypsin

0.5% Trypsin EDTA was purchased from GIBCO diluted 1:10 in EDTA solution (0.5mM), aliquoted in 5ml volumes and stored at -20°C until use.

MLA

Supernatant of the Gibbon cell line MLA-144, which spontaneously releases IL-2, was used as a source of growth stimulus to supplement T cell media. Cells were cultured in RPMI 1640, 10% FCS without further feeding. After 2 weeks the cell supernatant was harvested by centrifugation at 1600rpm for 10min, followed by filtration through a 146mm 0.2µm membrane (PALL). MLA supernatant was stored at -20°C in 60ml aliquots until use.

IL-2

Lyophilised powder (Peprotech) was reconstituted in water at concentration of 10^5 IU/ml, filtered through 0.2µm membrane and stored at -20°C in 500µl aliquots.

Cyclosporin A (CSA)

CSA (Sandimmun, Sandoz) was stored in -20°C and reconstituted in RPMI 1640 to a stock concentration of 10µg/ml.

Phosphate buffered saline (PBS)

10 tablets (OXOID) were dissolved per litre sterile water and autoclaved if required sterile (NaCl 8g/l; KCl 0.2g/l; Na_2HPO_4 1.44g/l; KH_2PO_4 0.24g/l; pH 7.4).

CFSE

Carboxyfluorescein succinimidyl ester, stored at -20°C (5µM, 20µM or 40µM, respectively)

2.1.1 Cryopreservation and recovery of cryopreserved cells

Cell to be cryopreserved were centrifuged at 1200-1500 rpm for 10min and resuspended in freezing media: RPMI 1640 containing 20% FCS and 10% DMSO. The cells were transferred into sterile plastic cryovials (Nunc) in 1ml aliquots and placed in a -70°C freezer overnight in a Mr. Frosty (Nalgene) which allows gradual lowering the temperature of the cell suspension. The following day the frozen cells were transferred into a freezer containing liquid nitrogen for long-term storage. Cell were recovered from such storage by fast-thawing at 37°C in a water bath and transferred to a 15ml tube (Sarstedt) filled with 10ml RPMI 1640 plus 10% FCS using a 10ml (Corning) pipette. After allowing cell suspension to stand for 1min, they were pelleted by centrifugation at 1200-1500rpm and resuspended in the appropriate growth medium for culture.

2.1.2 Mycoplasma Test

Cells were routinely tested for mycoplasma infection using MycoAlert Mycoplasma Detection Kit (Lonza). In cases of contamination cells were either discarded or treated using Mycoplasma Removal Agent (MP Biomedicals) according to the protocol supplied by the manufacturer.

2.1.3 Transfection of eukaryotic cells by electroporation

For the standard procedure for electroporation, the relevant cells, including LCLs, were resuspended in 400 μL of complete medium with DNA or RNA in a cuvette. After 10-min incubation at 25 °C, the culture was pulsed with 230 V at 975 μF using a Bio-Rad Gene Pulser II. Transfected cells were cultured in complete medium with 10% FBS for 4–5 days. Every 5–8 days, viable cell numbers were determined by hemocytometry based on trypan blue exclusion. Cultures were then split, and total viable cell numbers were calculated relative to the initial culture.

2.1.4 Blood donors

Blood was donated by healthy laboratory personnel, the EBV status of whom was identified by serological staining for IgG antibodies to the viral capsid antigen. Additionally, buffy coats were obtained from Birmingham Blood Transfusion Service, Vincent Drive, Edgbaston, Birmingham.

2.1.5 Generation of lymphoblastoid cell lines

LCLs were generated by in vitro transformation of the B cells from donors who had previously donated blood in the past. 5-10x10^6 PBMCs were centrifuged at 1600rpm to remove all media then resuspended in a 500µl aliquot of the prototype 1 B95.8 or the prototype 2 strain Ag876 virus and incubated at 37°C with 5% CO_2, flicking every 15min. After 1hr the cells were washed and resuspended in a 48-well plate (Iwaki) in RPMI 1640 with L-glutamine, 10% FCS and penicillin/streptomycin, with the addition of 0.1µg/ml cyclosporine A for the first 2 weeks to suppress T cell activity. LCL cultures were subsequently split and re-fed with medium twice weekly. According to its individual donor, each LCL is labelled with "PER" standing for person followed by an anonymous three digit code.

CD4+ T cell clones specific for defined epitopes within EBNA 2 or 3C were used. Overall, the experiments involved CD4+ T cell clones to two different EBNA-derived epitopes identified along with their HLA class II-restricting alleles in Table I.

Table 2.1: T cell clones with their EBV derived antigens and HLA restriction used in these experiments.

EBV Antigen	*Epitope Coordinates*	*Epitope Sequence*	*HLA Restriction*	*Clone*
EBNA2	**276-295**	**PRSTVFYNIPPMPLPPSQL**	**DR52b**	**c93 (CD4+)**
EBNA3C	**386-400**	**SDDELPYIDPNMEPV**	**DQ5**	**c38 (CD4+)**
BMLF1	**259-267**	**GLCTLVAML**	**A2**	**c6 (CD8+)**
LMP2	340-349	SSCSSCPLSK	A11	c24 (CD8+)
EBNA1	77-96	GLRALLARSHVERTTDEGTW	DR1	c4 (CD4+)

bold: T cell clones capable of recognising LCL PER241

2.1.6 Lentiviral transduction of LCLs

2.1.6.1 Vectors

The vectors pLV-THM, psPAX2 and pMD2.G were provided by D. Trono (Geneva, Switzerland); the vector pLV-THM allows for direct cloning of shRNAs (http://tronolab.epfl.ch/).

The oligonucleotides containing the shRNAs sequences (Table 2.6.1) were cloned into the plasmid pSuperior puro purchased from OligoEngine (Seattle, WA). pSuperior puro is under the control of the H1 PolIII promoter and enables to use the EcoRI-ClaI sites to replace the H1 promoter in pLVTHM with the H1-shRNA cassette from pSuperior puro.

Table 2.2: **used shRNA constructs**

target protein	*Target sequences*
CD63 a	5′-GGTTTTTCAATTAAACGGA
CD63 b	5′-GGAGAACTATTGTCTTATG
CD81	5’-ATCTGGAGCTGGGAGACAA
CD82	5’-TGTAGTCTTCGGAATGGACGTGC

2.1.6.2 Verification of RNA Interference

Efficacy of RNA Interference (RNAi) was verified by transfection of Hela cells using electroporation. Hela cells were detached and electroporated with a single pulse at 250V and 250µF using GenePulser Xcell electroporation system (BioRad) and 7-10µg of pSuperior including the respective shRNA fragment. The efficacy of knocking–down was assessed 48 hours later by flow cytometry.

2.1.6.3 Lentivirus production

We used VSV-G pseudotyped lentivirus to deliver gene–specific shRNAs. For silencing, lentiviral particles were produced by transfecting (FuGene6, Roche Diagnostics, Indianapolis, IN) 30% confluent 10cm^2 tissue culture dish of HEK293T cells with a plasmid mix containing the vector shRNA-pLVTHM encoding the specific shRNAs, the packaging construct psPAX2 and the envelope plasmid pMD2G-VSVG (DNA-ratio: 49:37:14). Fresh medium was added 16 hours after transfection. Virus-containing supernatants were harvested every 24 hours and filtered through a 0.45µm filter (Millipore, Billerica, MA). For control purposes we generated an LCL transduced with the lentivirus which does not carry any shRNA construct.

2.1.6.4 Target cell transduction

Virus-containing supernatant was pooled to an amount of up to 12ml and ultracentrifuged at 19500rpm (SW40Ti, Beckman Coulter) for 2hrs at 4°C.

Meanwhile target LCLs were plated into a 24 well plate at a density of $5x10^5$ cells/well. After removing as much medium from the cells as possible the resuspended virus was mixed with the cells in the presence of polybrene (8µg/ml, Sigma) and incubated overnight until fresh medium was added.

2.2 Assays on living or fixed cells

Triton X-100 permeabilisation buffer

For cell permeabilisation in immunofluorescence experiments 0.1% Triton X-100 (Sigma) was prepared in Tris-buffered saline (TBS) (10 mM Tris, pH 7.5/150 mM NaCl/1 mM KCl).

CHAPS permeabilisation buffer

CHAPS (Sigma) was solubilised to 1% in 30 mM Tris-Cl pH 7.5, 150 mM NaCl and diluted dependent on the purpose. For permeabilisation in immunofluorescence experiments a 0.1% solution in PBS was used.

Brij 98

1% Brij 98 detergent was diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4 + 40µM $CaCl_2$, 100µM $MgCl_2$). The exact procedure includes melting Brij 98 in the water bath and adding 5ml Brij 98 into 500ml buffer of above composition.

Human IFNγ

Human IFNγ (2000 pg/ml) was purchased from R&D Systems.

Wash buffer (ELISA)

PBS/0.05% Tween (0.5ml in 1l)

Coating buffer (ELISA)

0.1M Na_2HPO_4, adjusted to pH9 with 0.1M NaH_2PO_4

Blocking buffer (ELISA)

1% BSA/PBS filtered and then Tween added (50µ/100ml)

Stopping-solution (ELISA)

1M HCl

Peptides

Synthetic 20mer and 15mer peptides were synthesised using 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham), and dissolved in dimethylsulfoxide (DMSO, Sigma Aldrich). Peptides used in this study covered epitopes of the Epstein Barr Virus Nuclear Antigens EBNA2 or EBNA3c, respectively.

2.2.1 IFNγ ELISA

Known numbers of T cells (500, 2500, 5000) were incubated in V-bottom wells of a microtest plate (Nunc) with either standard numbers of autologous, HLA-matched or HLA-mismatched LCLs. In other experiments T cells were incubated either with LCLs premixed with previously purified LCL-derived exosomes or with exosomes

alone. In all settings LCLs were prepulsed for 1hr with 5μM peptide or an equivalent concentration of DMSO solvent as a control and then washed six times with washing buffer. In blocking assays, LCLs or exosomes were preincubated with different concentration of monoclonal antibodies (MAbs) specific for HLA-DR (L243; ATCC clone HB-55), HLA-DQ (SPV-L3; Serotec) for 1 h before addition of T-cells to the assay.

The supernatant medium harvested approximately after 18hrs was assayed for IFNγ by ELISA (Endogen) following the manufacturer's recommended protocol. 96-well Maxisorp plates (Nunc) were coated with 50μl of a 0.75μg/ml solution of a mouse IgG_{1K} anti-human IFNγ mAb (Pierce, Endogen) overnight at 4°C. The following morning the antibody was flicked out of the wells which were subsequently blocked at RT for 1hr with 1% BSA, 0.05% Tween 20 in PBS. The plates were then washed 6 times in washing buffer (0.05% Tween 20 in PBS) and 50μl of neat (or diluted) supernatant from the T cell assay was added to each well for 2-4 hrs at RT. After incubation the supernatant was discarded, plates washed 6 times in washing buffer, and a biotinylated mouse IgG_{1K} anti-human IFNγ mAb (Pierce, Endogen) was added at 0.375μg/ml and left at RT for 1-2hrs. The wells were washed and supplemented with an Extravidin peroxidise conjugate (Sigma) for a further 30min. After a final 10 washes of the plate in washing buffer, 100μl of the developing TMB (3,5',5,5'-tetramethyl benzidine, Tebu-Bio laboratories) solution was added and left at RT for 20min to allow the colour to develop. 100μl 1M HCl was added to each well to stop the reaction and the absorbencies were read at 450nm using an automated spectrophotometer (Wallac Victor 1420 Multilabel Counter). All results represent the mean of duplicate or triplicate wells.

2.2.2 Analysis of co-stimulatory activity of LCLs

Nunc C96 MicroWell™ Plates (Maxisorp, cat# 430341) were coated overnight with an α-CD3 mAb antibody (OKT3) in the concentration 0, 0.5, 1 and 2mg/ml in coating buffer. These plates were then used to co-culture CD4+ T cells with either CD63low a, b or the control LCLs for 18 hours at 37°C. After this incubation period supernatants were harvested and directly analysed for IFNγ by ELISA assays.

2.2.3 Flow cytometry

Cells (~$1x10^6$) were transferred to U-bottom 96-well plate and incubated with a control or a primary antibody for 1hr at 4°C. The cells were washed three time with PBS and were incubated with either fluorescein isothiocyanate- (FITC) or phycoerytrin- (PE) conjugated secondary antibody (polyclonal Goat anti-mouse Immunoglobulins, Dako) diluted 1:300 in BSA/PBS for 1hr at 4°C in the dark. Cells were washed several times in PBS and resuspended in 500µl PBS for analysis on EPICS XL Flow Cytometer (Coulter) using System II software (Coulter). The mean fluorescence intensity was calculated by subtraction of the average intensity of the control antibody (non-specific binding, α-rat PtdIns 4-kinase (4C5G) IgG1) from the target antibody value.

2.2.4 Peptide binding assay

Cells ($3x10^5$) in 50µl complete medium were added to 150µl PBS containing biotinylated peptide SDD or PRS (50µM final concentration, Alta Bioscience) and incubated at 37°C for 4 hours. Subsequently the cells were incubated PE-conjugated streptavidin (Sigma) in a dilution 1:300 for up to 1 hour at 4°C. As greater sensitivity was desired, staining of cells via incubating with biotinylated peptide was conducted as described above. But after adding PE-conjugated streptavidin, incubation with biotinylated α-PE antibody (BD Biosciences) did occur, which finally allowed us to add an additional layer of PE-conjugated streptavidin.

2.2.5 Immunofluorescence microscopy

Cells or LCL-T cell conjugates were cytospun onto polylysine-coated slides (SurgiPath Europe) and then immediately fixed with 4% paraformaldeyde in PBS for 10 min and permeabilised with either 1% Triton X-100, CHAPS (0.05%) or Brij 98 for 5 min. Cells were then blocked with 10% heat inactivated goat serum in PBS for 1-2 hours and stained with primary antibody and subsequently after washing 3 times with PBS fluorochrome-conjugated secondary antibodies at room temperature for 1 hour each time. After washing 3 times with PBS cells were stained with Hoechst 33342 for 5 min and slides were mounted with Slow Fade®, Antifade kit (Invitrogen) and examined using a Nikon E600 microscope.

2.2.6 Confocal Microscopy to analyse immune synapses

LCLs and T cells were washed twice in PBS and mixed 1:1 in PBS before spinning them down, discarding the supernatant and resuspending the pellet in the remaining amount of supernatant (app. 20µl). The cells were then incubated at 37ºC for 30 min to allow conjugate formation and then gently resuspended in PBS to achieve a concentration of $1x10^5$ cells/ml. After being cytospun onto slides (500 rpm) and fixed and permeabilised as described above, cells stained and mounted as described above. Confocal images were acquired using a Carl Zeiss LSM510 microscope with a 63× oil-immersion objective (Plan-APOCHROMAT, Zeiss) and analyzed using LSM Image software.

2.3 Protein analysis

10x Running buffer

To prepare 10x running buffer for SDS-PAGE 144g glycine, 30.3g Tris (tris(hydroxymethyl)aminomethane) and 10g SDS (sodium dodecyl sulphate) were dissolved in 1L dH_2O.

10x Transfer buffer

To prepare 10x transfer buffer for Western blotting 200ml of 10x Tris/Glycine (0.25M Tris, 1.92M glycine, Geneflow) was mixed with 400ml methanol and topped up to 2L with dH_2O.

Triton X-100 buffer

Triton X-100 lysis buffer was prepared by mixing 250µl $CaCl_2$ (1mM), 250µl $MgCl_2$ (2mM) and 5ml Triton X-100 (Sigma) and topping up to 500ml with 1x PBS.

Laemmli buffer

Solution contains 5% w/v SDS, 40% v/v glycerol, 0.005% w/v bromphenol blue and 0.25 M w/v Tris HCl, pH6.8; additionally 5% v/v 2-mercaptoethanol if reduced conditions were required

Acrylamide

Purchased from Geneflow

Bio-Rad D_c protein assay kit

Bio-Rad D_c protein assay Reagent A, B, S purchased from Bio-Rad Laboratories

MES

2-(N-Morpholino)ethanesulfonic acid hemisodium salt, 4-Morpholineethanesulfonic acid hemisodium salt ($C_6H_{13}NO_4S \cdot 0.5Na$); purchased from Sigma-Aldrich

2.3.1 Determining protein concentration

Protein concentration of lysate samples or exosomes was determined using a DC protein assay kit (Biorad) according to the manufacturers operating recommendations. BSA was used to prepare protein standards at serial dilutions from 2mg/ml to 32.5µg/ml.

2.3.2 Preparation of protein extracts

Preparing cell lysates, cells were spun down and washed twice in PBS before transferred to ice cold Triton X-100 lysis buffer. The suspension was rotated at 4°C for a few hours until the remaining cell debris was pelleted at 13,000rpm and the supernatant used for further analysis. Exosomes were simply resuspended in PBS, mixed with the appropriate amount of Laemmli buffer and prepared for further analysis.

2.3.3 SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

Cell lysates and exosome samples were mixed 1:3 (v/v) with 4x Laemmli buffer and boiled at 95°C for 5min prior to loading onto a SDS-PAGE. Proteins were then separated using a Biorad minigel system. The acrylamide concentration of the stacking gel was 5% whereas the concentration of the running gel depended on the particular protein of interest and its molecular weight. Prestained molecular weight markers (6-175kDa, NEB) were run alongside the protein samples. Electrophoresis was conducted at 15-22mA in running buffer.

2.3.4 Western blotting

After electrophoresis the gel was equilibrated in transfer buffer and laid onto nitrocellulose membrane (Amersham). Both were wrapped between two layers of

Whatman paper and all these layers together placed into a blotting cassette. After the transfer performed at 60V for 2hrs, the membrane was blocked for 1hr in 5% milk at room temperature on the shaker and washed three times for approximately 10min in PBS-Tween-80 followed by incubation with the first antibody (commonly overnight at 4°C). The next morning the antibody solution was taken off, the membrane washed again in PBS-Tween and incubated with the secondary antibody (e.g. goat anti-mouse IgG HRP-conjugated, 1:20000, Sigma) for 1hr at RT. The membrane was washed the last time before detecting the protein using a chemiluminescence kit (Western Lighting^{TM}, Perkin Elmer Life Sciences).

2.3.5 Analysis of HLA class II dimers using immunoblotting

Cells were lysed at 5 x 10^6/ml in 1% Trion X-100 buffer, pH 7.2, 1.0 mM PMSF, 1.0 µg/ml leupeptin, and 1.0 µg/ml pepstatin for 30 min at 4°C. To detect HLA-DR αβ-dimers, total cell lysate was mixed with the appropriate volume of 4x non-reducing SDS laemmli buffer and incubated for 30 min at room temperature. To detect HLA-DR β-monomers, total cell lysate was mixed with an appropriate volume of 4x reducing SDS sample buffer and boiled for 10 min. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and subsequently transferred to nitrocellulose. Immunoblotting was carried out as described above. Following monoclonal antibodies were used to detect HLA class II: DA6-14, SPC-L3 or L243.

2.3.6 Fractionation in sucrose density gradient

Cells centrifuged at 200 x g for 3 min, and washed once with PBS. Sodium carbonate (pH 11.0) buffer (100 mM) supplemented with 10µg/ml aprotinin, 10µg/ml leupeptin and 2mM PMSF was mixed with the pelleted cells. Lysates were prepared using the detergents Triton X-100, CHAPS or Brij98 or were homogenised using Dounce homogenizer (15 strokes) and a sonicator (4×20 s bursts, 1 min interval, power 10). The resulting homogenate was mixed with equal volume of 90% (w/v) sucrose prepared in 25 mM MES buffer (pH 6.5), and overlaid with 2 vol. of 35% (w/v) sucrose and followed by 1 vol. of 5% sucrose (w/v; both in MES). Samples were centrifuged at 55000 x g for 16–18 h at 4 °C. Then, 400 µl aliquots were collected from the top of the tube. The pellet was dissolved in Laemmli buffer supplemented with protease inhibitors. Equal amounts of each fraction were mixed with 4× Laemmli loading buffer and the distribution of proteins was analysed by SDS/PAGE followed by Western blotting. Protein concentration has been measured by Bio-Rad kit.

2.4 Exosome purification

2.4.1 Exosome depletion of growth medium

Exosomes present in FCS were removed by centrifugation at 100,000xg overnight. The next morning the exosomes free supernatant was harvested, sterile filtered through a 22µm syringe membrane filter, diluted to 10% FCS and stored at 4°C until use.

2.4.2 Differential centrifugation

LCLs growing in complete media were counted, span down and plated to 150cm^2 flasks to enrich secreted exosomes for 48hrs. Cells were grown either in complete media depleted from bovine serum's exosomes (above) or in serum free AIMV medium at a starting cell number of 4x10^5 cells/ml. The supernatant was centrifuged at 4°C by optimised sequential centrifugation (Fig. 2.1): 1) to remove living cells at 200xg for 4min (twice); 2) dead cells at 500xg for 8min; 3) debris at 2000xg for 10min; 4) cell membranes at 10,000xg for 45min; 5) to pellet exosomes at 70,000xg for 2hrs. The pellet was resuspended in 12ml ice cold PBS and centrifuged again at 70,000xg for 1hr. The final exosomes pellet was resuspended in PBS or medium depending on the procedure whether the exosomes were further purified by sucrose cushion centrifugation or directly used for functional assays. For sucrose cushion centrifugation, exosomes were resuspended in 10ml PBS and layered onto 2ml of sucrose/D_2O (99.9% Sigma) (1.2g/ml). After spinning at 100,000xg overnight the cushion was carefully taken, diluted with 10ml fresh ice cold PBS and the exosomes sedimented a last time at 70,000xg for 1hr. This final pellet was resuspended in 40-80µl and stored in -70°C or used immediately.

2.4.3 Sucrose gradient flotation of exosomes

In order to further define the grade of purification, the flotation density of LCL-derived exosomes was determined by flotation on a sucrose gradient. Following the second ultracentrifugation step (see 2.6.2), the pellet was resuspended in 2.4ml of a 2.5M sucrose/D_2O, 20mM HEPES (Sigma), pH7.2 solution and filled into a SW60

centrifuge tube (Beckman Coulter). This solution was overlaid with 8 successive fractions of 200µl with concentrations ranging from 2 to 0.25mM. After centrifugation at 100,000xg overnight, each fraction was carefully harvested without disturbing underlying fractions, diluted in up to 2ml PBS, span again and the resulting pellet analysed by WB for exosomal marker proteins.

2.4.4 Sucrose cushion flotation of exosomes

In order to assure that the final pellet contains exosomes, a subsequent purification was conducted by flotation on a sucrose cushion. Using a centrifuge tube for SW40Ti (Beckman) a density cushion of 1.2g/ml, composed of 20 mM Tris/30% sucrose/deuterium oxide (D_2O) pH 7.4 (2 ml) was overlaid with the PBS-resuspended exosome pellet from 2.6.2 forming a visible interphase. The samples were ultracentrifuged at 100,000×*g* and 4 °C for 75 min in a SW-40 swinging bucket rotor (Beckman).

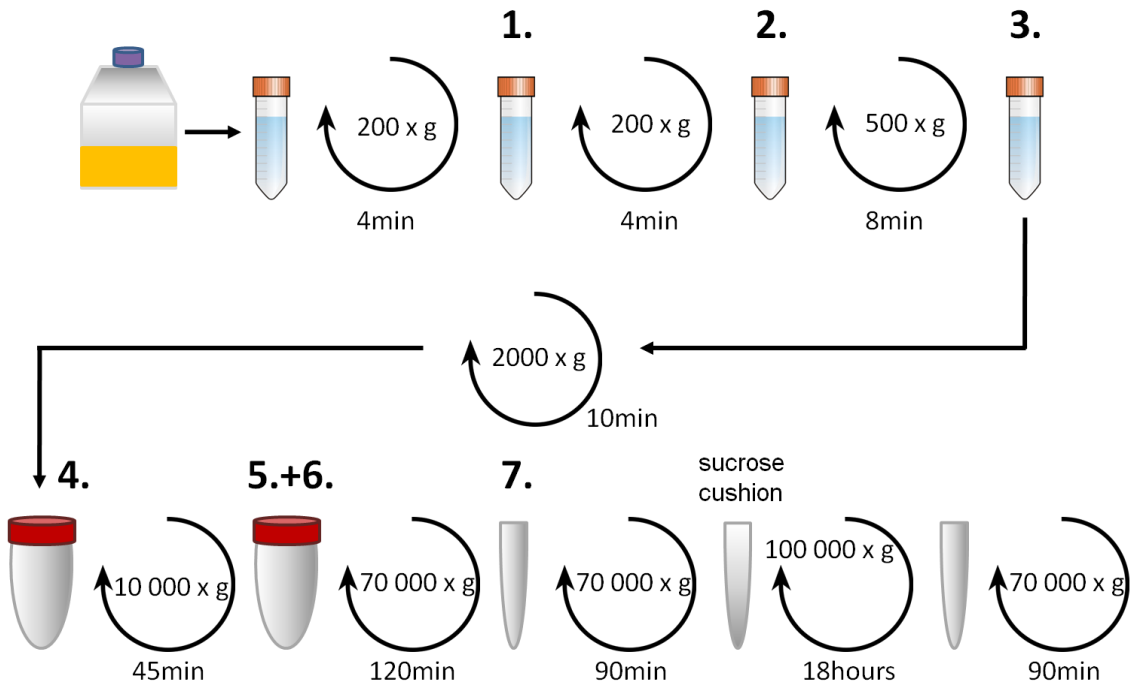


Figure 2.1: Exosome purification by differential centrifugation and flotation on a sucrose cushion
Exosomes were separated from the LCL-supernatant and concentrated by differential centrifugation in six single steps which, depending on the purity required, was followed by centrifugation on a sucrose cushion (density 1.2g/ml). Each pellet after each centrifugation step (1 to 7, whereas #6 represents a sample of the supernatant after spin #5) was analysed for protein content by Western blotting in the initial experiments performed to optimise the purification protocol.

2.5 Electron microscopy

Phosphate buffer, 0.2M, pH 7.4

A solution of 0.2M Na_2HPO_4 and a solution of 0.2M NaH_2PO_4 are prepared separately. Prior to using, the two buffers are mixed in a ratio 81:19 whilst measuring the pH.

Paraformaldehyde 16%

16g of paraformaldehyde powder (Polysciences, Inc.) is dissolved in 90ml distilled H_2O by heating to 65°C whilst stirring. pH 7.4 is adjusted by titrating NaOH. After topping up to 100ml with distilled H_2O, small aliquots were stored in -20°C.

Grids

Formvar/Carbon coated copper grids, 200 mesh, Agar Scientific, catalogue number S162

Methyl cellulose

For a final volume of 200ml, 196ml of dH_2O was heated to 90°C and 4g of methyl cellulose (Sigma, 25 centipoises) were added while stirring. The solution was rapidly cooled down on ice to 10°C while stirring. Slow stirring was continued overnight at 4°C and then stopped to let the solution "ripen" for 3 days at 4°C.

Methyl cellulose/uranyl acetate, ph4

90ml of methyl cellulose solution was mixed with 10ml of 4% uranyl acetate. This solution was centrifuged for 95min at 29,000rpm (4°C) and the supernatant stored at 4°C in the dark until use.

Protein A Gold (PAG)

Protein A coated Gold particles (10 or 15nm) were purchased from Cell Microscopy Center (CMC), Utrecht (www.cmc-utrecht.nl)

2.5.1 Preparation of exosome samples

Following sucrose cushion centrifugation, 5µl from a freshly prepared exosome pellet resuspended in up to 40µl PBS was put on a formvar carbon-coated grid (Agar Scientific) and left for 20-40min. The liquid was soaked up and the sample attached to the grid was fixed by adding a drop of 2% PFA (Park Scientific) in 0.1M phosphate buffer (0.081M Na_2HPO_4, 0.019M NaH_2PO_4) for 15min. The PFA was washed off by dipping the grid once onto a drop of 0.15% glycine in phosphate buffer and once onto 1% BSA. Specific labelling was done by adding a drop with the first antibody (2-20µg/ml in 1% BSA/PBS) for more than 20min onto the grid. After rinsing the grids, labelling with a bridging antibody (1:250 in 0.1% BSA/PBS, polycl. rabbit α-mouse immunoglobulins, Dako Cytomation) was conducted if necessary (Protein A binds only mouse IgG2a, 2b). Then onto the rinsed grids a drop of Protein A-Gold (PAG, CMC-Utrecht) diluted in 0.1% BSA/PBS according to company's recommendation was added and left for at least 20min. The grids had to be rinsed with plain PBS before fixation for 5min with 1% glutaraldehyde (Sigma) needed to be done. To contrast the grids had to be rinsed in dH_2O and put onto a drop of methylcellulose/uranylacetate. If double labelling was done, the specimen was taken after glutaraldehyde fixation and the procedure started again from the step of dipping the grid onto 0.15% glycine and 1% BSA.

Table 2.3: antibodies used for electron microscopy

Antibody	*Source*	*Type*	*MW of protein (kDa)*	*Dilution*
CD63 (6H1)	(Berditchevski et al. 1995)	mc	40-70	20-50μg/ml
CD81 (M38)	kindly provided by Dr. O. Yoshi	mc	20	20-50μg /ml
CD82 (M104)	kindly provided by Dr. O. Yoshi	mc	60	20-50μg/ml
MHC I (W6/32) (1mg/ml)	kindly provided by Prof. M. Rowe	mc	45	20-50μg/ml
MHC II – DQ (SPV-L3) (1mg/ml)	kindly provided by Dr. G. Taylor (Spits et al. 1983)	mc	30	20-50μg/ml
MHC II – DR (L243) (1mg/ml)	kindly provided by Dr. G. Taylor (Fong et al. 1981)	mc	30	20-50ug/ml
Rabbit anti-mouse Ig	Dako	pc		1:250

mc: monoclonal; pc: polyclonal

2.6 Details of cell lines and T cell clones used in this study

Table 2.4: cell lines used in this study

Cell line	*Origin*	*Supplier/donor*	*Complete media*
HEK 293T	Embryonic Kidney	CRUK	DMEM
HeLa	Cervical cancer	CRUK	DMEM
HeLa CIITA	Cervical cancer	1*	DMEM
KEM	Burkitt lymphoma	CRUK	RPMI
Ramos	Burkitt lymphoma	CRUK	RPMI
LCL, PER141	B lymphoplasts	PER141	RPMI
LCL, PER149	B lymphoplasts	PER149	RPMI
LCL, PER213	B lymphoplasts	PER213	RPMI
LCL, PER241	B lymphoplasts	PER241	RPMI
LCL, PER255	B lymphoplasts	PER255	RPMI
LCL, PER279	B lymphoplasts	PER279	RPMI
LCL, PER282	B lymphoplasts	PER282	RPMI
T cell, clone 38 (SDD)	CD4+	PER141	T cell medium
T cell, clone 98 (PRS)	CD4+	PER141	T cell medium
T cell, clone 4 (GLR)	CD4+	PER253	T cell medium
T cell, clone 6 (GLC)	CD8+	PER141	T cell medium
T cell, clone 24 (SSC)	CD8+	PER050	T cell medium

1*: kindly provided by Dr. M. Pegtel

Table 2.5: HLA-types of LCLs and HeLa cells used in this study

LCL donor	*MHC class I*		*MHC class II*	
	HLA-A	**HLA-B**	**HLA-DR**	**HLA-DQ**
LCL, PER241	2	27, 44	1, 11, 52b	5, 7
LCL, PER213	1	8, 57	7, 17, 52a	2, 9
LCL, PER141	1, 2	16, 40	1, 13, 52b	5, 7
LCL, PER149	1, 2	44, 57	4, 7, 53	7, 9
LCL, PER255	2, 24	27, 35	4, 53	8
LCL, PER279	1, 3	8, 35	1, 17, 52a	2, 5
LCL, PER282	1, 24	27, 35	9, 15, 51, 53	6, 9
HeLa cells	68	15	1	5

LCLs were grown in tissue culture vented flasks (Iwaki) in complete RPMI media in a humidified atmosphere at 37°C in 5% CO_2. Cells were passaged by removal of approximately 2/3 of the old media and either splitting the cells into multiple new flasks or discarding a part of the cells and topping up the remaining cells with fresh complete media. Adherent cells used in this study were grown in complete DMEM under same conditions like LCLs. In order to passage cells, media was removed, cells

were washed twice with PBS and detached using Trypsin-EDTA (GIBCO). Cells were then resuspended in fresh media and replaced at a ratio of 1:3 to 1:10.

Polyclonal T cells were grown in 24 well plates and maintained in T cell specific RPMI media (see above).

2.7 Antibodies used in this study

Table 2.6: antibodies used for Western blotting and flow cytometry

Antibody	*Source*	*Type*	*MW of protein (kDa)*	*Dilution*	*Reduced*
PtdIns 4-kinase (4C5G)	(Endemann et al. 1991)	mc	45	serum supernatant (FC)	no
CD37 (MCA 483S)	Serotec	mc	40-52	1:100	no
CD53 (MCA 723G)	Serotec	mc	32-40	1:100	no
CD63 (6H1)	(Berditchevski et al. 1995)	mc	40-70	serum supernatant (FC)	no
CD63 (MX-49.129.5)	Santa Cruz Bio.	mc	40-70	1:1000 (WB)	no
CD81 (M38)	kindly provided by Dr. O. Yoshi	mc	20	serum supernatant	no
CD82 (M104)	kindly provided by Dr. O. Yoshi	mc	60	serum supernatant (FC)	no
CD82 (TS82b)	kindly provided by Dr. E. Rubinstein	mc	60	serum supernatant (WB)	no
CD151 (5C11)	(Berditchevski et al. 1997)	mc	29	serum supernatant	no
HSP90 (SPA830)	Stressgen	mc	90	1:1000 (WB)	yes
CD19	Cell Signaling	mc	95	1:1000 (WB)	yes
CD19 (HIB19)	BD Pharmingen	mc	95	1:500 (FC)	Nd
MHC I (W6/32)	kindly provided by Prof. M. Rowe	mc	45	1:800 (FC) 1:250 (WB)	yes
MHC II (DA6-14)	kindly provided by Prof. M. Rowe (Lehner and Jones 1984)	mc	30	1:250 (WB)	yes
MHC II – DQ (SPV-L3)	kindly provided by Dr. G. Taylor (Spits et al. 1983)	mc	30	1:800 (FC/EM)	Nd
MHC II – DR (L243)	kindly provided by Dr. G. Taylor (Fong et al. 1981)	mc	30	1:800 (FC/EM)	Nd
MHC I (ILA88, α-bovine)	kindly provided by Dr. S. Ellis	mc	45	1:10000 (5ng/ml, WB)	yes
Calnexin (AF-8)	(Hochstenbach et al. 1992)	mc	90	1:50000	yes

Antibody	*Source*	*Type*	*MW of protein (kDa)*	*Dilution*	*Reduced*
ICAM-1 (CD54)	CR UK	mc	90	1:100	yes
Talin (8D4)	Sigma	mc	230	1:2000	yes
LMP-1 (CSI-4)	CR UK, kindly provided by Prof. M. Rowe	mc	63	1:50	yes
LMP-2a (14B7)	Santa Cruz, kindly provided by Prof. M. Rowe	mc	65	1:500	yes
EBNA2 (PE2)	kindly provided by Prof. M. Rowe	mc	86	1:50	yes
EBNA3c (E3C)	Santa Cruz, kindly provided by Prof. M. Rowe	mc	170	1:50	yes
CD3 (OKT-3)	kindly provided by Dr. S. Lee	mc	23	0-2ug/ml	Nd
α-PE	e-Bioscience	mc, biotin-labelled	240	1:40	Nd
Syntenin	Abnova	mc	32	1:500	yes
Goat anti-mouse Ig-HRP conjugated	Dako	pc HRP-conjugated		1:25000	Nd
Goat anti-rabbit Ig	Dako	pc HRP-conjugated		1:25000	Nd
Mouse anti-human CLIP	BD Pharmingen	mc		1:50	Nd
Mouse anti-human α-tubulin	kindly provided by Dr. S. Roberts	mc		1:1000	Nd
AlexaFluor 594-phalloidin	invitrogen			1:100	Nd

mc: monoclonal; pc: polyclonal; Nd: not determined; WB: Western blotting; FC: Flow cytometry

2.8 Molecular Biology

Luria Broth (LB)

LB was prepared by dissolving 5g Peptone, 2.5g yeast extract and 5g NaCl in 500ml sterile water. The solution was autoclaved for 20min at 15psi and 121°C.

LB agar

LB agar was prepared by addition of 7.5g agar to 500ml LB before autoclaving.

Antibiotics

Bacteria containing plasmids were seleted using ampicillin. Concentrated stocks of ampicillin (100mg/ml) were prepared in sterile water and stored in aliquots at - 20°C. Ampicillin was added to autoclaved media directly before use to make a final concentration of 100µg/ml.

Tris/acetate buffered EDTA (TAE)

500ml of a concentrated stock of TAE (50x) was prepared by dissolving of 121g Tris base in H_2O and adding 28.6ml glacial acetic and 5ml 0.5M EDTA.

Competent cells

NEB 5-alpha F'I^q Competent *E.coli;* stored at -80°C

DNA extraction kit

QIAprep Spin Miniprep Kit, Qiagen Germany

QIAGEN Plasmid Maxi Kit, Qiagen Germany

2.8.1 DNA digestion with endonuclease enzymes

Plasmid DNA was diluted in sterile water and dependent on the particular purpose an aliquot of 0.5-5ug was taken to perform a DNA digestion by adding 5-20 Units of particular endonuclease restriction enzymes (Roche) and appropriate amounts of 10x enzyme buffers.

2.8.2 Agarose gel electrophoresis

Separation of DNA fragments generated by restriction enzyme digestion was performed by agarose gel electrophoresis containing ethidium bromide to allow visualisation of the DNA in a ultra-violet (UV) transilluminator. To make the gel, the particular amount of agarose was added to H_2O and heated until boiling point in a microwave oven. Once the agarose solution had cooled slightly, the appropriate volume of 10xTAE and ethidium bromide was added and the gel was cast into a gel former (Geneflow). When set, the gel was submerged in an electrophoresis tank (Geneflow) in 1 x TAE. DNA samples were diluted in a 5x sample loading buffer alongside standard size markers (NEB 1kb or 100bp ladder mix). The samples were run at 100V and electrophoresis was stopped when the dye front migrated a desired distance.

2.8.3 DNA extraction from an agarose gel

DNA fragments separated by agarose gel electrophoresis were visualised and photographed. DNA fragments requiring purification were excised from the gel into a sterile 1.5ml eppendorf tube under a UV transilluminator using a clean scalpel blade. The excised pieces of gel were weighed and purified using a Quiagen QIAquick Gel Extraction Kit according to the manufacturer's instructions.

2.8.4 DNA ligation

The ligation of enzyme digested DNA fragments (generated from vector and insert DNAs) was performed using T4 Ligase (NEB) in 10x ligation buffer. 3:1 molar ratios of purified insert and vector DNA were mixed with 1µl 10x ligation buffer and topped up to 10µl with sterile dH_2O. The mixture was either incubated at RT for several hours or at 16°C overnight. Ligated DNA was either used directly for transformation of competent bacteria or stored at -20°C until use.

2.8.5 Transformation of competent bacteria

Competent *E.coli* cells were thawed on ice. An aliquot of 14µl was transferred to a sterile eppendorf tube, mixed with 1µl of the ligation mixture and incubated for 30min on ice. After this the cells were made receptive for DNA-uptake by exposing them to 42°C for 30sec and immediately returning to ice for 5min incubation. 200µl SOC medium was added to the bacteria followed by incubation for 1hr at 37°C in a shaker.

The cells were plated onto LB agar plates containing ampicillin (100µg/ml) for selection of the transformed cells and incubated at 37°C overnight.

2.8.6 Preparation of plasmid DNA from transformed bacteria

All procedures were conducted according to "QIAprep® Miniprep Handbook" as briefly described:

Mini-prep

Colonies of transformed bacteria were picked into 5ml of LB medium supplemented with 100µg/ml ampicillin and grown overnight in a shaker at 37°C. 1.5ml of the culture was centrifuged in a 1.5ml eppendorf tube at 5000rpm for 3min in a bench top centrifuge. The supernatant was discarded and the bacteria-pellet resuspended in 250µl P1-buffer. The suspension was mixed with 250µl P2-buffer and incubated for max 5min in order to lyse the bacterial cells. 350µl of buffer N3 was added to the lysate to neutralise the solution and precipitate insoluble bacterial material. After the precipitate was sedimented at 13.000rpm for 10min (bench-top centrifuge), the supernatant was transferred into a QIAprep spin column and centrifuged for 1min. The remaining DNA was washed once with 0.5ml buffer PB and once with 0.75ml buffer PE before eluted in 50µl sterile, deionised water.

Maxi-prep

200-300µl of an overnight bacterial culture was transferred into 200ml of LB medium supplemented with 100µg/ml ampicillin and grown overnight in a shaker at 37°C. The bacteria were pelleted and resuspended in 10ml buffer P1. 10ml buffer P2 was added

to bacterial suspension to lyse the cells for 5min at room temperature followed by neutralisation with 10ml buffer P3. After incubation for 20min on ice the precipitate was spun down and the supernatant was transferred into a buffer QBT-equilibrated QIAGEN-tip 500. The tip was washed twice with 30ml buffer QC before the DNA was eluted into a 50ml centrifuge tube by adding 15ml buffer QF. Thereupon, the DNA was precipitated with isopropanol and the remaining pellet washed with 1ml 70% ethanol before dissolved in the required amount of sterile deionised water.

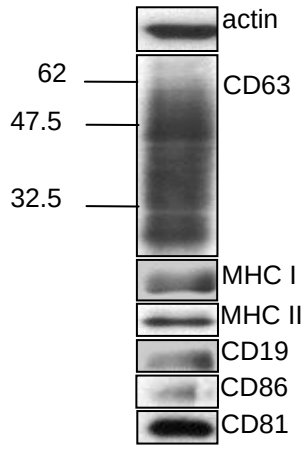
3 Results

3.1 Characterisation of the model system

3.1.1 Characterisation of LCLs

Prior to starting immunological assays, it was important to characterise the cell lines selected for use in this research. EBV-transformed LCLs serve in this project as a model antigen presenting cell. An LCL established from a healthy laboratory donor (PER241) was selected on the basis of possessing both HLA-DR52b and HLA-DQ5 and therefore able to present both of the EBV epitope peptides that were selected for the study. As expected, the LCL expressed high levels of MHC class I and class II molecules (Fig. 3.1A). The LCL also expressed the tetraspanins CD37, CD63, CD81, CD82 and CD151. Analysis by flow cytometry confirmed these tetraspanins were present at the LCL surface (Fig. 3.1B).

A



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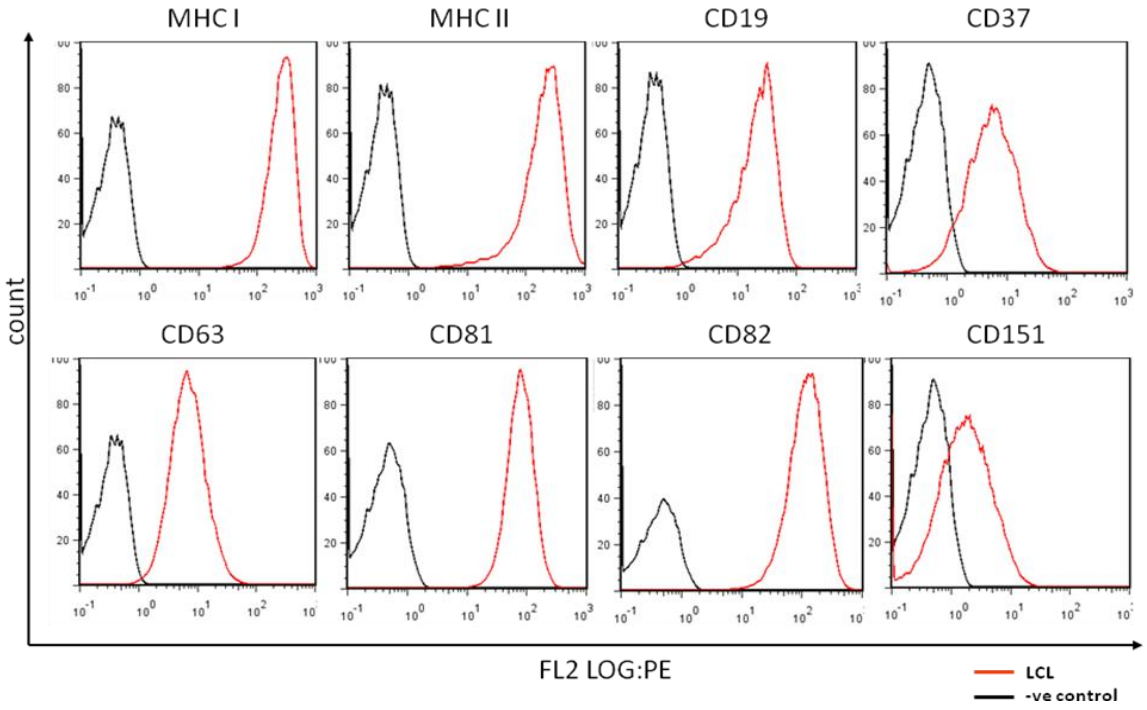


Figure 3.1: Cell surface expression of relevant membrane proteins by LCLs
A) Western blotting shows the total cell expression of common B cell proteins such as MHC I, MHC II, CD19, CD86 as well as the tetraspanins CD63 and CD81. The tetraspanin CD9 however, could not be detected (data not shown). B) Cell surface expression was confirmed by flow cytometry for the proteins MHC I, II, CD19, CD37, CD63, CD81, CD82 and CD151. Again an antibody specific for CD9 did not result in any detectable labelling (data not shown). LCLs were first incubated with antigen-specific primary antibodies or, as a negative control, an irrelevant primary antibody before labelling with the secondary antibody, a polyclonal PE-conjugated anti-mouse antibody.

3.1.2 Characterisation of T cells

The CD4+ T cell clones selected for use in this research were also tested for their expression of relevant proteins. As expected, the clones expressed CD4 and also the tetraspanins CD53, CD63, CD81, CD82 and CD151 but not CD9 and CD37 (Fig. 3.2).

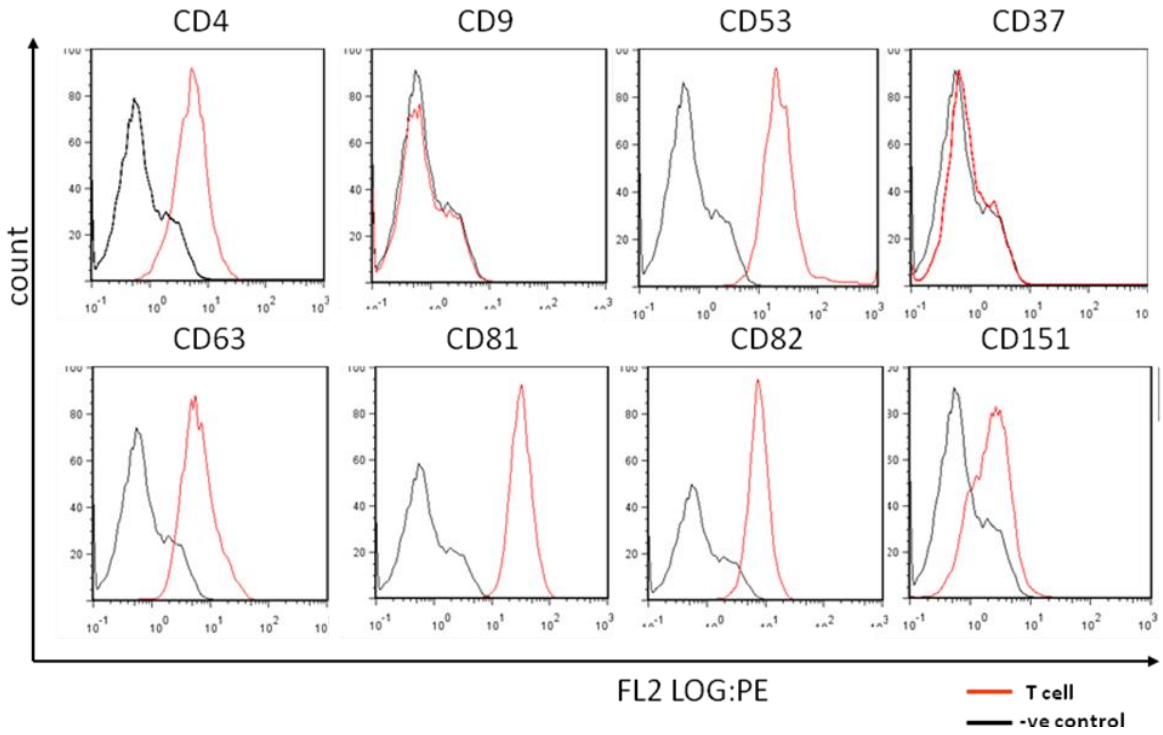


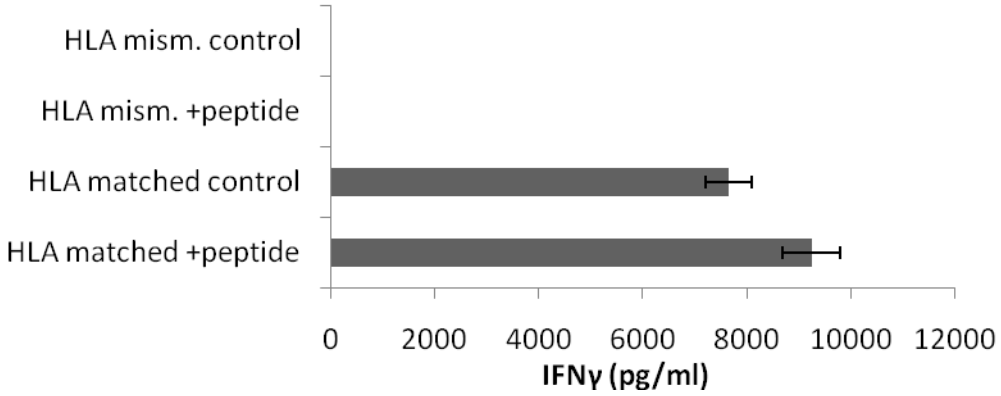
Figure 3.2: Cell surface expression of CD4 and tetraspanins by CD4+ T cell clone c38
Flow cytometry confirms CD4+ T cell clones (here: c38 (HLA-DQ5 restricted)) express CD4 and the tetraspanins CD53, CD63, CD81, CD82, CD151. The tetraspanins CD9 and CD37 were not detected. The same molecules were also expressed by the second CD4+ T cell clone c93 (HLA-DR52b restricted) (data not shown). Staining and controls are as described for Fig. 3.1.

3.1.3 Recognition of LCLs by CD4+ T cells

After characterising the expression of relevant proteins by the LCL and CD4+ T cell clones, the ability of the LCL to activate the CD4+ T cell clones was determined.

Using IFNγ to measure T cell activation revealed that only the HLA-matched LCL, established from PER241, was capable of stimulating both CD4+ T cell clones (Fig. 3.3). Note that recognition of the LCL by the c93 clone was relatively weak without peptide loading. A second HLA-mismatched LCL (PER213, see Table 2.4) did not stimulate the T cell clones, demonstrating the HLA specificity of T cell activation.

A



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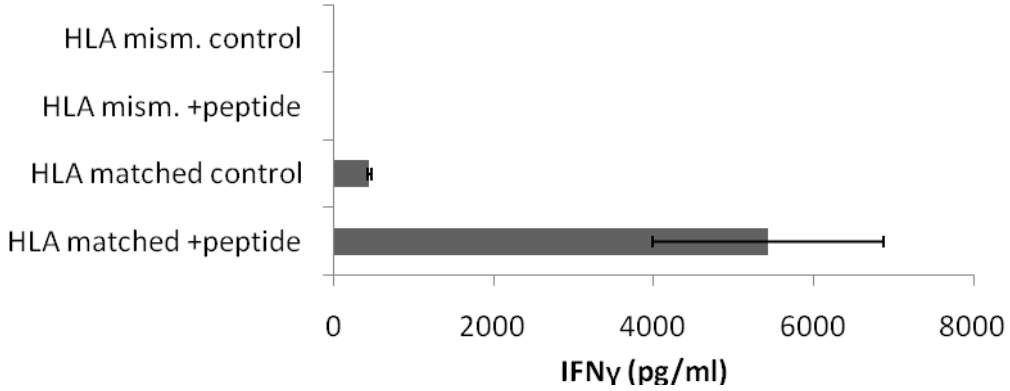


Figure 3.3: Activation of CD4+ T cell clones is HLA-restricted
The chosen HLA-matched LCL (PER241), either left unmanipulated (HLA matched, control) or prepulsed with epitope peptide ($1x10^{-5}$M) and then washed (HLA matched+peptide), was recognised by c38, a DQ5 restricted CD4+ T cell clone (A) and c93, a DR52b restricted clone (B). Note that the unmanipulated LCL activates the T cells and this is increased when the LCL is pre-treated with synthetic epitope peptide prior to co-culture with the T cells. An HLA-mismatched LCL (PER213), whether untreated or peptide pulsed, did not cause any activation. Shown is one representative result from three experiments. Error bars show the mean +/- standard deviation of triplicate ELISA-wells.

3.1.4 Depletion of tetraspanins in LCLs

3.1.4.1 Transient transfection of LCLs with siRNA

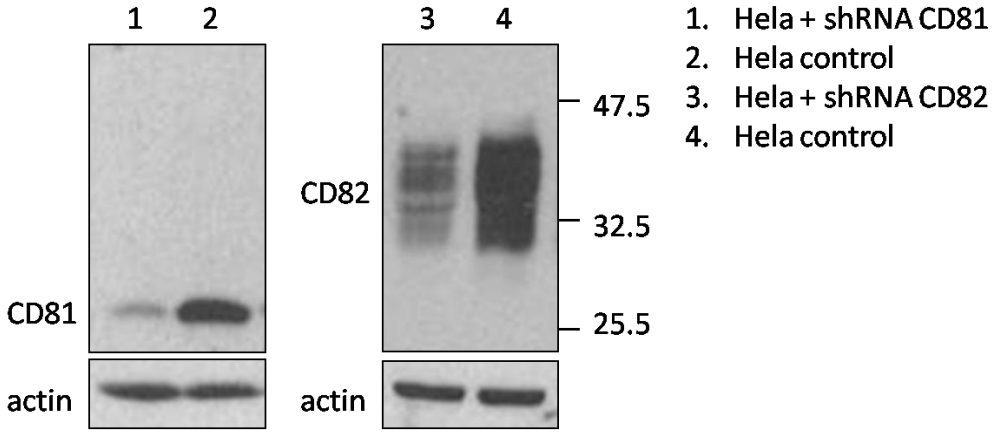
To knockdown the expression of tetraspanins in LCLs, an siRNA duplex targeting a sequence in the open reading frame of CD63 (5'-GGTTTTTCAATTAAACGGA-3') previously shown to cause a marked knockdown in other cell lines such as HeLa and MDA293 cells (data not shown) was first tested. A second positive control duplex known to knockdown maxGFP, a GFP variant encoded by a plasmid provided within the Nucleofector Kit (Lonza), was also included in the experiment. Both, a standard electroporation protocols (250V, 975µF, 4mm electrode gap) as well as commercially available transfection kits were tested. Cell line Nucleofector Kit (Lonza), siPORT NeoFX (ambion) and NanoJuice Transfection Reagent (Merck) were tested using a variety of conditions by changing settings for the electroporation, buffers and preset programs according to manufacturers' recommendations. None of these methods decreased expression of CD63 or, in cells transfected with pmaxGFP, did result in any noticeable expression of GFP which was planned to be knocked down subsequently. Since both siRNA duplexes had worked in other cell lines, this result suggested that LCLs are inherently much harder to transfect with siRNA.

3.1.4.2 Designing and cloning of shRNA constructs

Since siRNA supplied exogenously to the LCLs did not appear to work in LCLs, another strategy to knock down the tetraspanins was investigated, this time

endogenously expressing shRNA. A shRNA construct targeting the same sequence in CD63 was designed. Despite repeated attempts it was not possible to clone the annealed oligonucleotide duplex directly into the lentivirus vector (pLV-THM). A different strategy was therefore followed. Oligonucleotide primers were designed and synthesised to allow the same shRNA duplex to be cloned into a different vector, pSuperior which would serve as a platform to firstly, test the shRNA efficacy by transfection into HeLa cells and, secondly, to allow subcloning of shRNA sequences shown to knockdown their target genes into pLV-THM. In addition to the CD63-specific construct a further four duplexes targeting CD82, designed as described above, were also cloned into pSuperior. An additional pSuperior plasmid containing a duplex targeting CD81 (see 2.1.6.1 for target sequence) was kindly provided by Fedor Berditchevski (Baldwin et al. 2008). Transfection of the pSuperior plasmids into HeLa cells identified one plasmid able to knockdown CD81, one able to knockdown CD82 and a third able to knockdown CD63 (Fig 3.4). Note that, especially for the CD63 construct, a small population of the HeLa cells was transfected and these showed only a reduction in tetraspanin expression.

A



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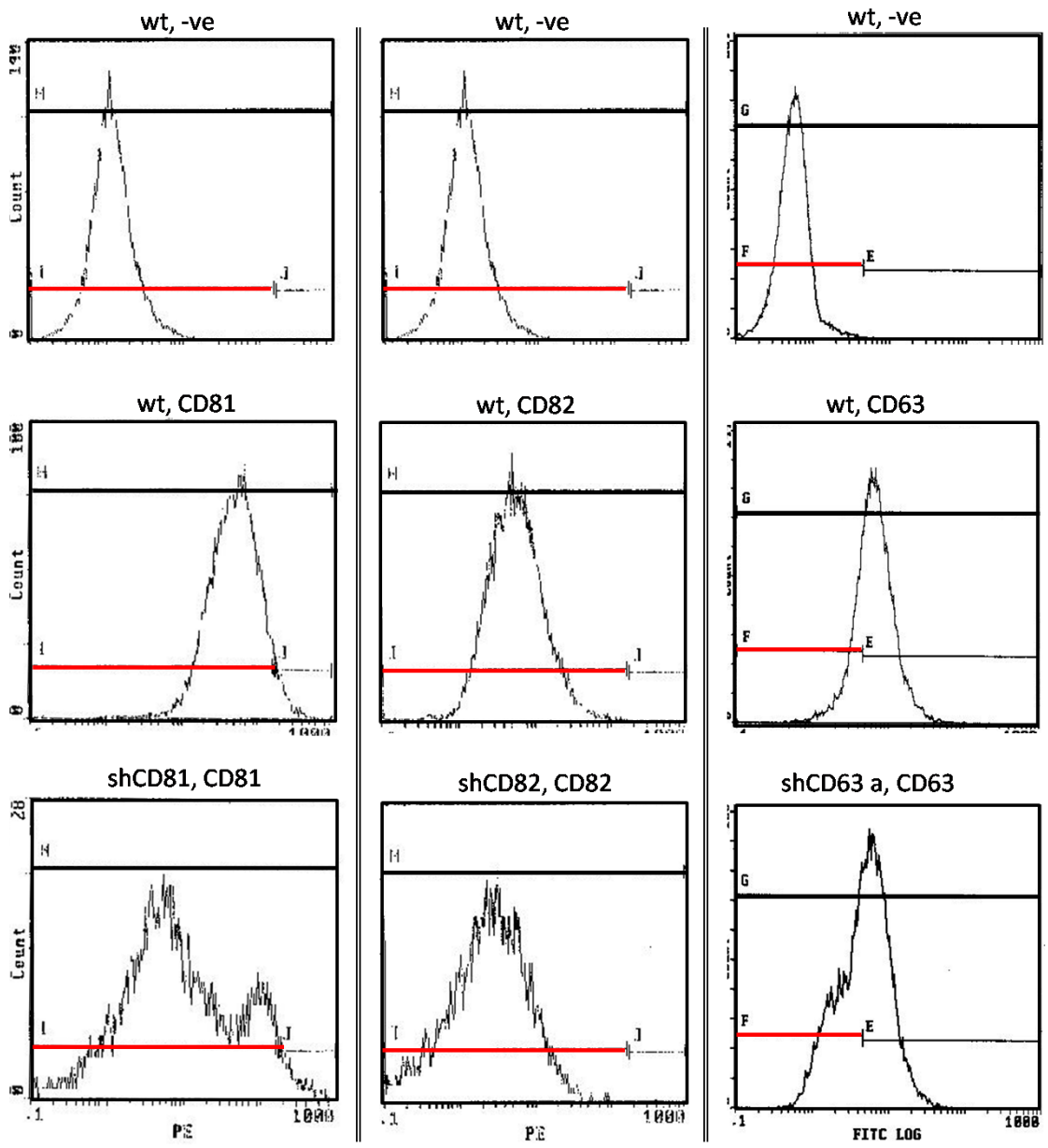


Figure 3.4: Assessment of shRNA knockdown efficiency cloned into pSuperior in HeLa cells

HeLa cells were transfected with pSuperior containing shRNA constructs targeting sequences within the proteins CD81, CD82 and CD63 (see 2.1.6.1). A) Western blotting of whole cell lysates shows a substantial knock down of the targeted proteins CD81 and CD82. For CD63 no Western blotting was conducted. B) Knockdown was confirmed by flow cytometry using antibodies against CD81, CD82 or CD63, top row: unmanipulated (wild type: wt) cells incubated with an irrelevant primary antibody; middle row: unmanipulated cell stained with antibodies specific for CD81 (left panel), CD82 (middle panel) and CD63 (right panel); bottom row: shRNA transduced cells stained as described above. None of these knock downs did affect the expression of other relevant members of the tetraspanins family including CD81, CD82, CD63, CD151 (data not shown).

3.1.4.3 Lentivirus production and LCL infection

Following their identification in HeLa cells, the functional shRNA sequences were subcloned from pSuperior into the lentivirus vector pLV-THM. These lentiviral vectors were then transfected into the packaging cell line HEK293T along with the packaging construct psPAX2 and the envelope plasmid pMD2G-VSVG to generate lentiviruses. Initial experiments using GFP, which is co-expressed by the lentivirus, to monitor infection efficiency showed that HeLa cells were readily transduced with these viruses whereas the efficiency of infection of LCLs was very low (data not shown). Testing different HEK293 packaging cell lines, different media and different commercial and non-commercial transduction reagents increased the efficiency of LCL transduction, although levels were still low. Concentrating the lentivirus by centrifugation prior to LCL infection gave the largest increase in infection efficiency, allowing up to 30% of LCL to be infected, and was therefore used routinely. Since transduced cells also express GFP, fluorescence-activated cell sorting (FACS) was then used (Institute of Biomedical Research, IBR, Birmingham) to select infected cell lines. Following several rounds of infection and cell sorting two different types of LCLs were produced, one with low levels of CD63 (Fig: 3.5) and a second transduced with the same virus lacking an shRNA insert to serve as a control. In the case of CD81 and CD82, despite multiple rounds of infection combined with three separate rounds of cell sorting, LCLs with reduced levels of these proteins could not be established (Fig: 3.5B, C) Note that in the transfection with shRNA targeting CD82 caused even a slight increase in the total cell lysates before sorting. The fact that the lentivirus infection was 100% raised the possibility that the high levels of CD81 and

CD82 expressed by LCLs made them resistant to knockdown by these shRNA molecules that had been shown to work in HeLa cells.

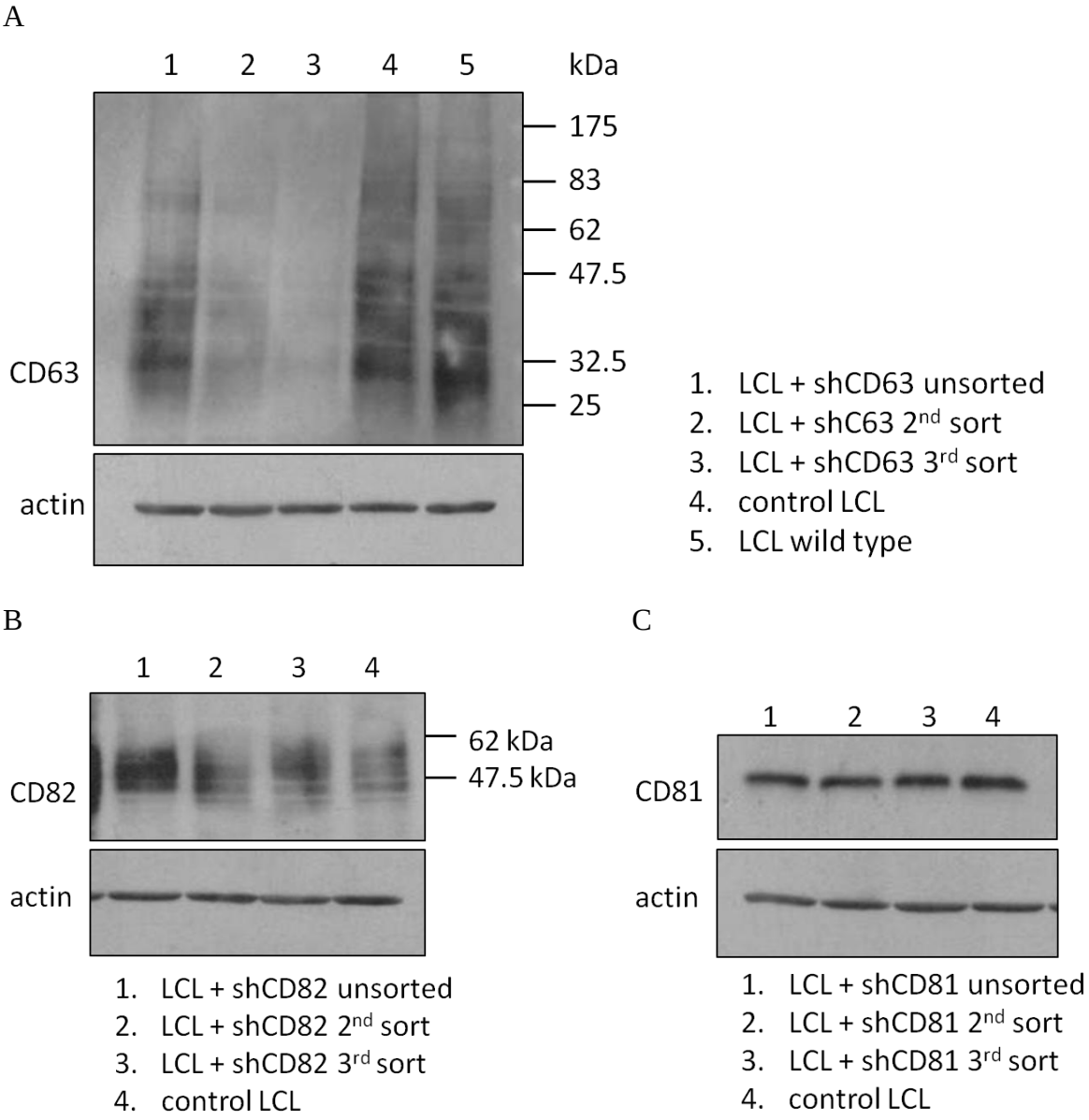


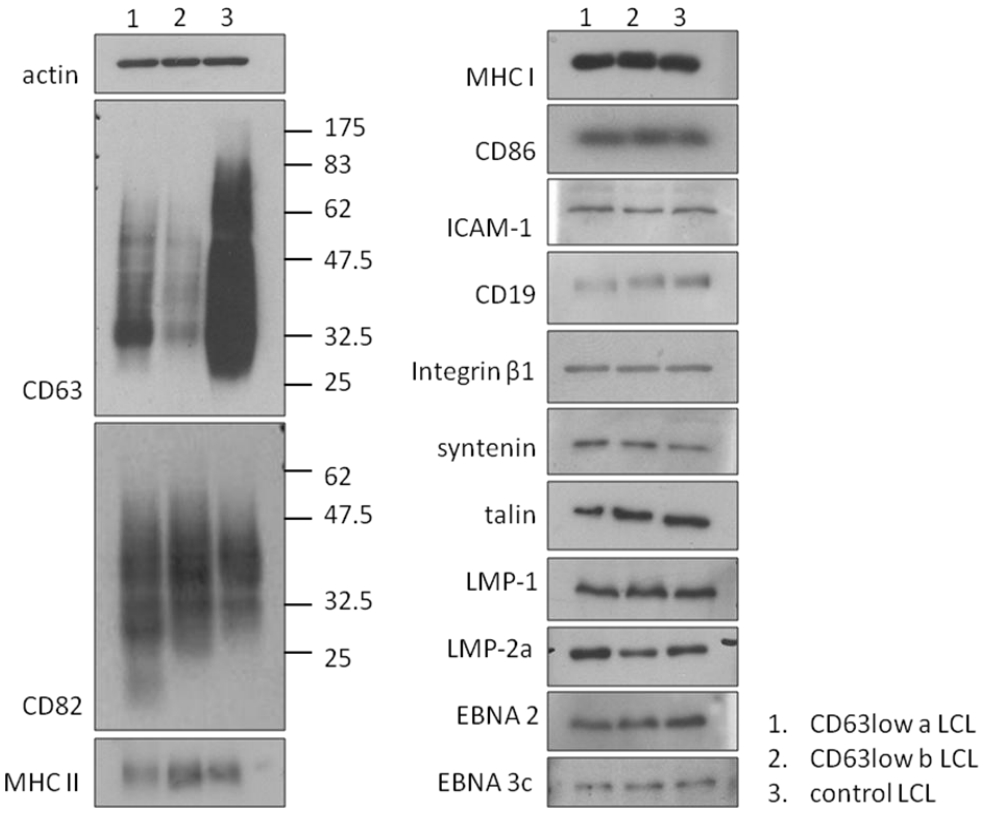
Figure 3.5: Western blot for knock down of tetraspanins in LCL
LCL PER241 was infected with lentivirus concentrated from HEK293T supernatant. A) LCL infection with shRNA constructs targeting CD63 combined with subsequent cell sorting for low expressing populations did lead to a stable knock down of this protein. Additional flow cytometry data support this observation (data not shown). B+C) Transducing the same LCL with lentivirus expressing shRNA targeting CD81 or CD82 did not result in stable knockdown of these proteins even after three separate rounds of cell sorting. For all experiments "control LCL" represents the LCL PER241 infected with empty pLV-THM whereas "LCL wt" is untreated LCL.

3.1.4.4 Knock down of CD63 in LCLs

As it proved impossible to knock down CD81 and CD82 in LCL despite intensive efforts, these two tetraspanins were not investigated any further. I therefore focused on the tetraspanin, CD63, which could be knocked down in LCLs. In order to rule out the possibility that any results achieved with the CD63 shRNA were due to off-target effects, a different shRNA sequence able also to knock down CD63, was sought. Five other shRNA sequences, each targeting different sequences within CD63, were cloned into pSuperior and tested for their efficacy. The shRNA causing greatest knockdown of CD63 in HeLa cells was then sub-cloned into pLV-THM. Lentivirus was prepared and used to infect PER241 LCL and a new line was generated as described above with CD63 now knocked down in the same cell background by a different shRNA. Western blotting and flow cytometry using the original CD63low LCL (CD63low-a) and the newly generated line (CD63low-b) revealed that the CD63 expression level was even further decreased in CD63low-b (Fig. 3.6A, B). Regarding other proteins, Western blotting showed only minor differences in total protein expression of MHC II, CD82, CD19 and LMP2 between the control and CD63low cell lines. In contrast, flow cytometry detected a number of changes in the cell surface levels of these proteins. CD63low-a cells exhibited lower cell surface levels of CD82 and CD151. Cell surface levels of ICAM-1 in both CD63low-a and CD63low-b cell lines increased by 13% and 32% respectively. These discrepancies between total and cell-surface levels could be due to differences in recognition by the antibody of epitopes in proteins in their cell surface context. For example, an altered distribution of ICAM caused by CD63 knockdown could potentially increase the availability of the antibody's epitope. Another explanation for these differences could be natural

variation between identical cell lines grown in parallel cultures. I observed fluctuations in the expression of certain proteins including MHC II and CD19 between control and CD63low cell lines over the months of monitoring. However, the overall picture over time was that the expression of all tested membrane proteins in control and CD63low LCLs oscillated around the same average value.

A



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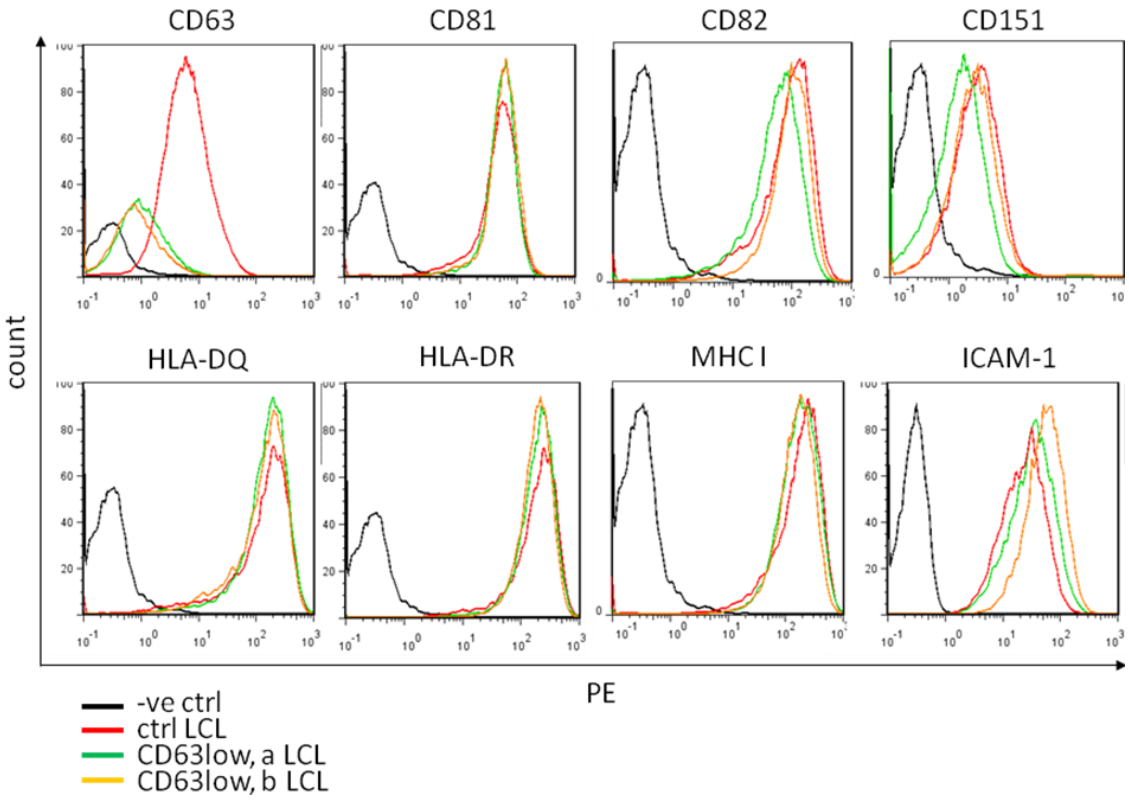


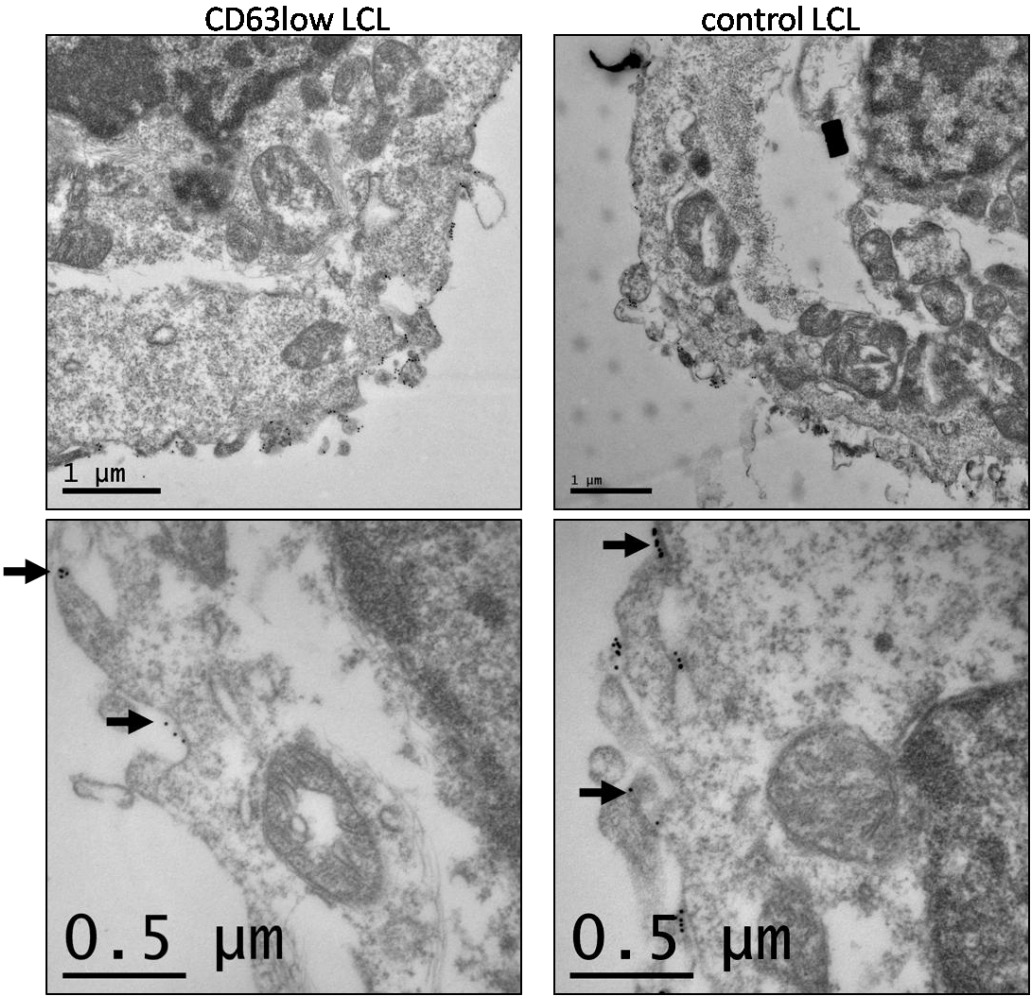
Figure 3.6: CD63 knockdown does not alter the level of other relevant cellular and viral proteins
A) Total levels of relevant proteins were analysed for the two LCLs in which CD63 expression is stably decreased, and the control LCL stably transfected with a lentivirus not expressing shRNA. The expression of the membrane proteins MHC class II, MHC I, ICAM-1 and CD86 as well as the viral nuclear proteins EBNA2 and EBNA3c was unaltered upon CD63 knockdown. B) Surface expression of proteins including CD81, two different HLA II molecules (DQ and DR) and MHC I were not altered by either CD63-specific shRNA. Alterations were observed for CD82, CD151 and ICAM-1. Negative control (-ve ctrl) see Fig. 3.1.

3.1.5 Analysis of MHC class II distribution in LCLs by transmission electron microscopy (TEM)

To analyse the density and distribution pattern of MHC class II on LCLs after knocking down CD63, cells, immunogold labelled using an antibody specific for HLA-DR, were quantitatively analysed by electron microscopy (Fig. 3.7A). In total approximately 550 gold particles, representing labelled MHC II molecules, were

counted for each cell line. No apparent differences could be seen in the number of surface-bound gold particles between the CD63 knockdown (CD63low-a) LCL and the control LCL (Fig. 3.7B). The distribution of surface MHC II was observed to mainly occur in patches. The average number of MHC II molecules present in these patches (Fig. 3.7C) and also the number of patches in ratio to their size was indistinguishable between the control and the CD63low LCL (Fig. 3.7D).

A



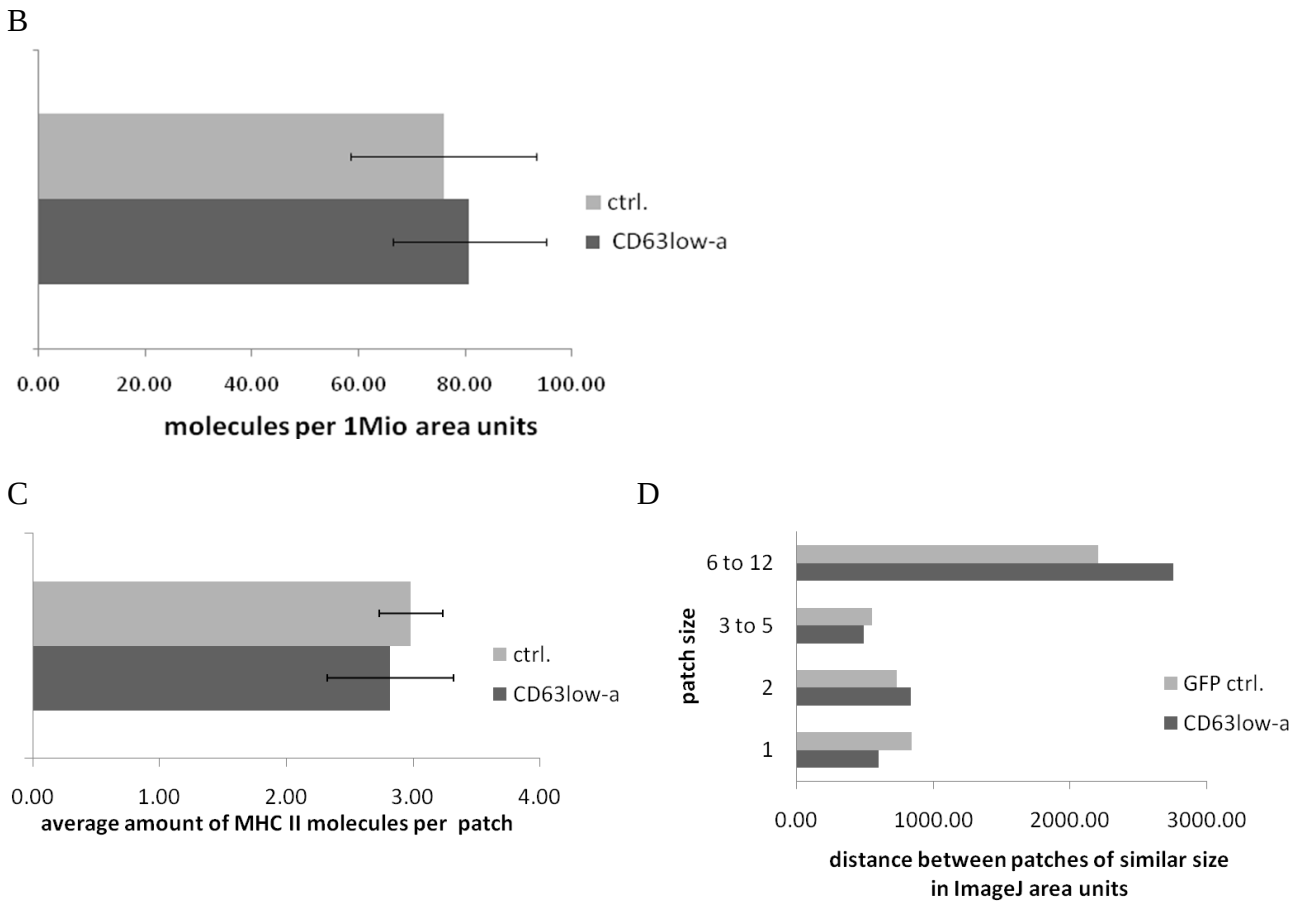


Figure 3.7: Analysis of quantity and distribution of surface MHC II on LCLs
A) CD63low-a and control LCLs, were negatively stained using uranyl acetate and immunogold labelled using an antibody specifically recognising MHC class II-DR molecules (L243) (arrows). A negative control was conducted and resulted in cells without labelling (data not shown). B) Detailed quantification using the image processing software ImageJ reveals an unaltered quantity as well as surface distribution of MHC II after knocking down CD63 in LCL. The number of molecules was assessed in relation to the cell's perimeter which was given by ImageJ in arbitrary area units. The error bars represent the mean +/- standard deviation of MHCs per perimeter unit counted on each analysed cell. For each cell line, between 4 and 5 cells were analysed for quantification purposes. C) MHC II molecules are organised into patches on the LCL's outer membrane. The average amount of MHC II molecules per patch is similar for CD63low and control LCL. The error bars represent the mean +/- standard deviation of the average MHCs amount per patch per cell. D) Different sizes of MHC II clusters could be observed on each cell. The calculated mean distance between patches of similar size are similar for CD63low and control LCL.

Summary

This chapter has focussed on the development of an experimental system to allow the influence of tetraspanins on MHC II antigen processing and presentation to be

explored. Despite intensive efforts, it proved impossible to knockdown CD81 and CD82. This is likely due to the naturally high levels of these tetraspanins present in LCLs. Only for CD63 could an adequate depletion of proteins be achieved. This tetraspanin and its influence on antigen processing and presentation to CD4+ T cells would be the subject of the next part of the project. Importantly, the total protein expression of relevant proteins such as MHC II, ICAM-1, CD86 or the EBV nuclear proteins EBNA3c and EBNA2 was not affected by knocking down CD63 in LCLs. Surface expression did not vary either and analysing the surface distribution of MHC II on CD63low and control LCL did not reveal any change upon CD63 depletion. Thus, any changes in presentation to CD4+ T cells would represent changes due to CD63 rather than other molecules involved in antigen presentation.

3.2 Exosomes from B-LCLs, purification and characterisation

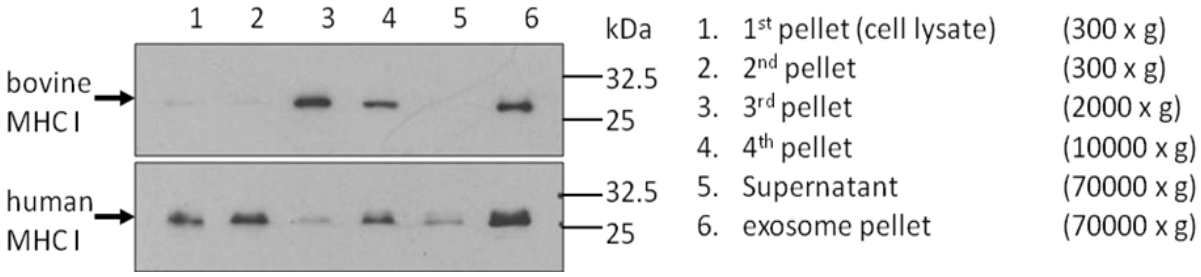
3.2.1 Purification of LCL-derived exosomes

3.2.1.1 Optimising exosome purification: i) depletion of serum contaminants

An important part of the research project was to study the contribution made by exosomes in the recognition of LCLs by CD4+ T cells. Therefore, it was important to optimise methods for purification of exosomes secreted into the culture medium by LCLs. Exosome purification is typically achieved by differential centrifugation. To optimise this method, the progress of purification was tracked by samples taken from

the pellets produced after each centrifugation step. These samples were studied by Western blotting using antibodies to human MHC II, a known exosome component. Apart from cellular debris, the foetal bovine serum (FBS) used as a supplement to the medium LCLs are cultivated in, is also a potential source of contaminants. Serum naturally contains bovine microvesicles, which due to their morphological characteristics can co-sediment with human exosomes. Firstly, the degree of contamination by bovine material was investigated by Western blotting using an antibody specific for bovine MHC class I. Bovine material was present in fractions 3 and 4 of the differential centrifugation procedure (Fig. 3.8A; top panel, lane 1, 4) as well as in the final exosomal pellet (lane 6). The strength of bovine MHC I band in those exosomal fractions was only marginally smaller compared to human MHC I derived from LCLs (Fig. 3.8A; bottom panel, lane 6). In order to avoid any potential influence of bovine material in the planned T cell assays, all exosomal preparations were made from LCLs grown in bovine-microvesicle depleted medium. Such medium was prepared after high speed centrifugation (100 000 x g, overnight) of FBS diluted to 20% with RPMI. The supernatant was decanted and the pellet of bovine cell material, including bovine vesicles, was discarded. When using this depleted medium, Western blotting using anti-bovine and anti-human MHC I antibodies shows a substantial change in ratio of bovine to human MHC I in exosomal lysates (Fig. 3.8B).

A



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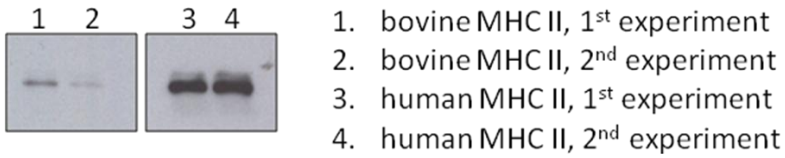

Figure 3.8: Depletion of bovine cellular material from exosomal samples
A) The purification of exosomes was monitored for contamination with bovine cellular material using an anti-bovine MHC I antibody (ILA88) and an anti-human MHC I antibody (W6/32). Bovine MHC I could be detected in fractions 3 and four (top panel, lane 3, 4) and the final exosome pellet (top panel, lane 6) whereas human MHC I was seen in all fractions (bottom panel). B) LCL-derived exosomes from two separate experiments (1 and 2), using bovine-microvesicle depleted medium for culturing LCLs, were analysed for remaining contamination by bovine exosomes. Using anti-bovine and anti-human MHC I antibody for Western blotting showed a markedly reduced ratio of bovine (lane 1, 2) to human MHC I (lane 3, 4).

3.2.1.2 Optimising exosome purification: ii) separation from cellular debris

The quality and purity of exosomal preparations were regularly checked by looking for the presence of calnexin, a transmembrane chaperone which predominantly resides in the endoplasmic reticulum and known not to be present in exosomes. Calnexin is commonly used as a control to monitor purity of exosomal fractions (Asea et al. 2008;Gastpar et al. 2005;Gomes et al. 2007;Mears et al. 2004). It was found that the cell material contamination in exosomal fractions was substantially diminished after the initial spin with decreased g-force was added to the protocol to carefully pellet/remove live cells from the medium (Fig. 3.9A). Exosomes were used for functional studies only if the amount of calnexin in the exosomal samples was less

than 10% of that present in the cellular lysate (in samples equalised by the amount of protein material). Note the substantial reduction in the ratio of calnexin and MHC II in lane 1 (whole cell lysate) and lane 6 (exosomes). Moreover, for electron microscopy experiments, microvesicles and potential contaminating membrane fragments were separated by their specific density by flotation on a sucrose gradient. This gradual separation revealed a broad overlap in density of MHC II and calnexin containing material of unknown constitution (Fig. 3.9B, lane 3 to 8). Exosomes characteristically appear in densities ranging from 1.10 to 1.19 g/ml. So the MHC II seen in fractions of higher density derives from unknown cellular compartments. Later, especially for analyses by electron microscopy an even higher purity was achieved by floatation on sucrose cushion. This cushion was prepared to a density of 1.2 g/ml which allows exosomes of this and lower density to remain in the cushion where it can be harvested while debris of higher density penetrates the cushion and forms a pellet.

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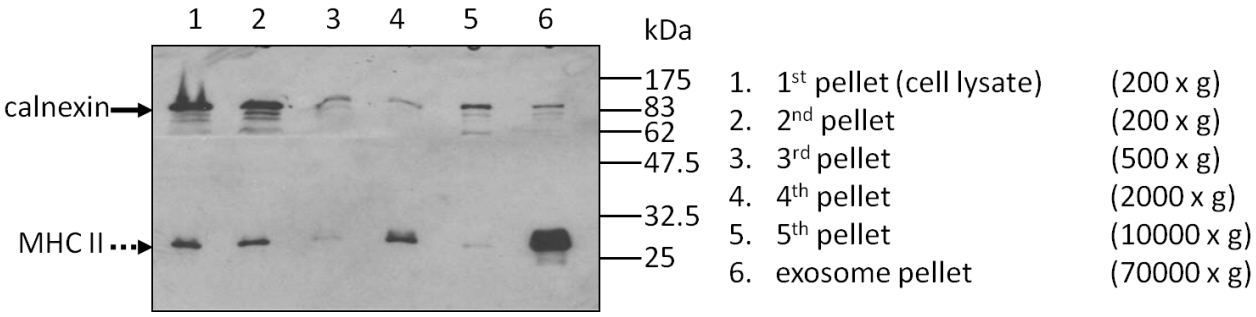

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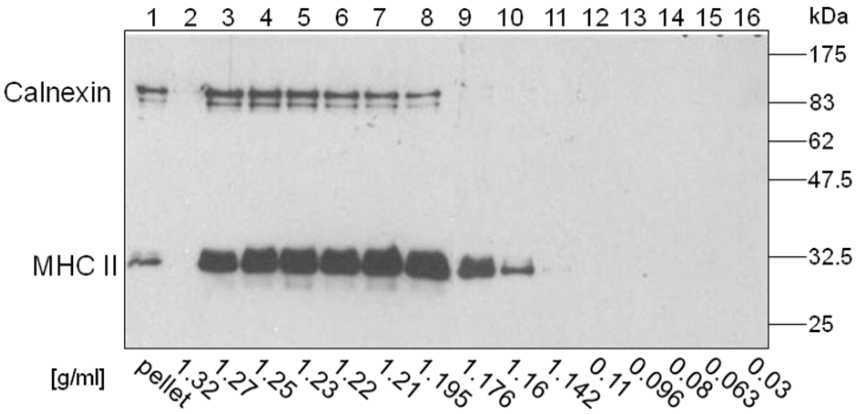


Figure 3.9: Depletion of cellular debris from exosomal samples
A) Exosomes were purified from cell supernatant by differential centrifugation in 5 separate steps. Lane 1:1st spin, Lane 2: 2nd spin to separate cells, Lane 3: sedimentation of remaining dead cells, Lane 4: sedimentation of cell debris, Lane 5: soluble proteins in supernatant after pelleting exosomes, Lane 6: exosomes (arrow: calnexin (90kDa), dashed arrow: human MHC II (28 kDa)). B) Exosomes were further analysed by sucrose gradient centrifugation. Cellular material originating from the MIIC is visualised using anti-human MHC II antibody (30kDa), contaminating debris using anti-human calnexin antibody (95 kDa). Characteristically floating on a sucrose gradient with a density range from 1.10 to 1.19 g/ml, exosomal fractions (lane 7 – 11) overlap with fractions containing cytoplasmatic material, represented by calnexin (lane 7 and 8).

3.2.1.3 Characterisation of exosomes by Western blotting

After optimising the protocol for purifying exosomes by differential centrifugation (Fig. 3.10), the distribution of proteins during the separate purification steps was analysed by Western blotting using antibodies against CD82, CD63, CD53, heat shock protein (HSP)90, CD19, MHC II, MHC I, actin and tubulin (Fig. 3.10), all of them known to be present in exosomes (Mears et al. 2004). Since these proteins are

present in cells, in the cell membrane, in membrane fragments derived from apoptotic or dead cells and in the membrane and lumen of exosomes, the proteins were found in all fraction including the cell lysate (lane 1) and the purified exosomes (lane 7), but not in the non sedimented fraction (lane 6) as would be expected for these proteins located in the membrane or lumen of exosomes.

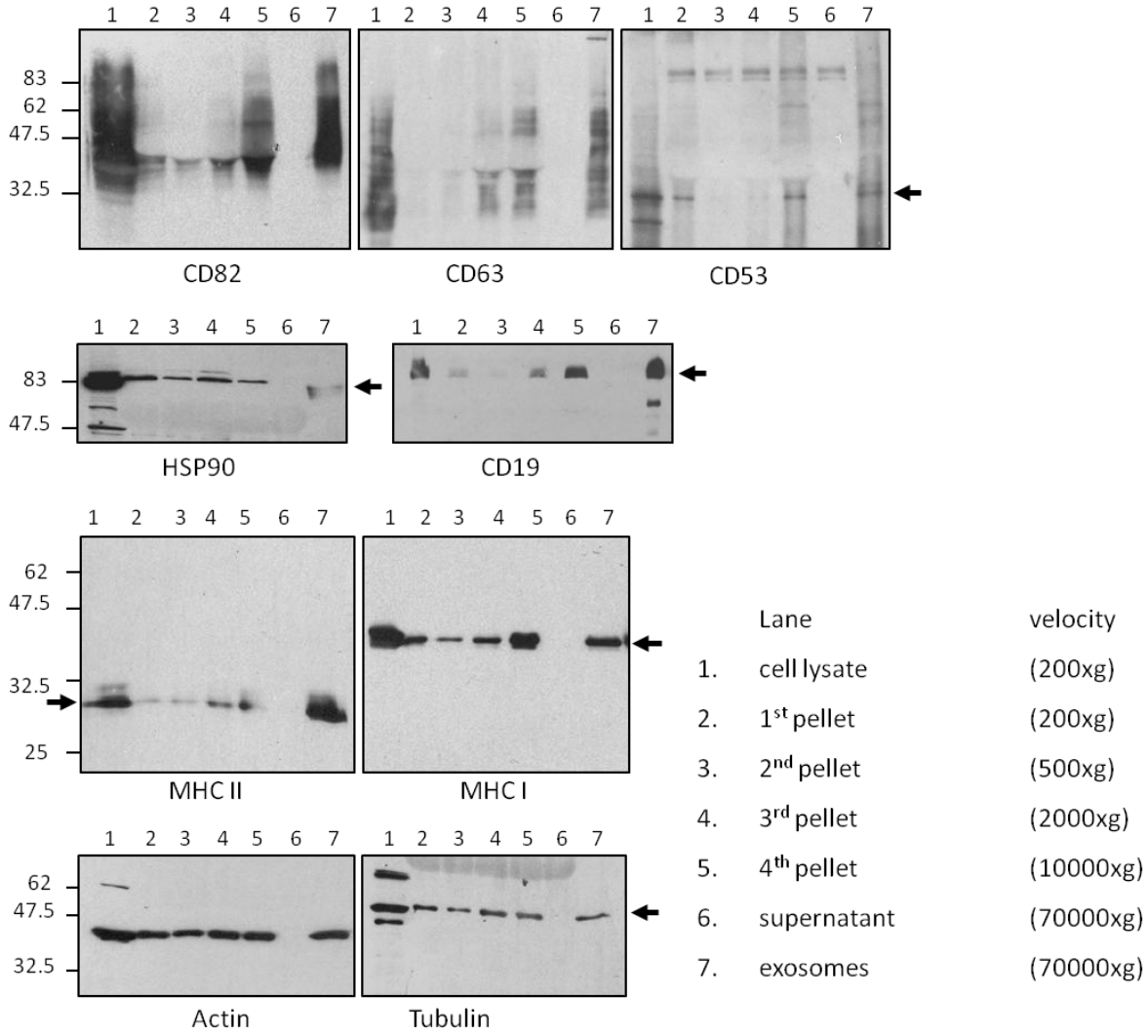


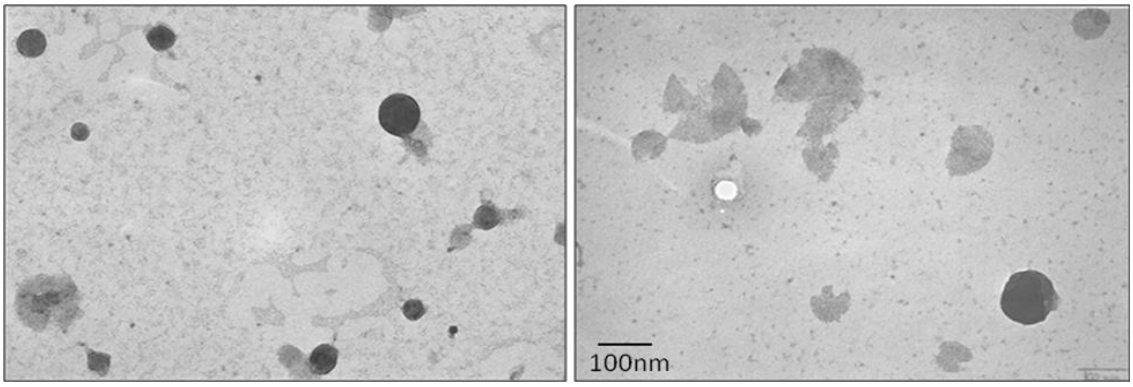
Figure 3.10: Analysis of the purification of LCL-derived exosomes
Protein samples from each of the seven different stages of a single purification were probed by Western blotting using antibodies to CD82, CD63 (glycosylated form, MW: 83-30kDa), CD53, HSP90, CD19, MHC class II (MW: 32kDa, arrow), MHC class I, actin and tubulin. These proteins were not present in the unsedimented supernatant in fraction 6 but clearly seen in the cell lysate in fraction 1 and in the exosomal fraction (#7).

3.2.2 Morphological analysis of LCL-derived exosomes

3.2.2.1 Transmission electron microscopy

The morphology of LCL-derived exosomes was studied by electron microscopy. For this purpose exosomes purified by differential centrifugation were centrifuged again and harvested from the sucrose cushion (density 1.2g/ml). The first exosome samples examined by electron microscopy revealed that high proportions of vesicles had been damaged (Fig. 3.11A). Several months of investigation into the methodology of exosome purification and preparation of samples suitable for electron microscopy were undertaken. Among others, new centrifugation tubes were purchased, different procedures for cleaning these tubes after use were tested and each step of the protocol for preparing the exosomes for electron microscopy was assessed. Eventually, the major reason for the exosome damage turned out to be due to generation of air bubbles during resuspension of exosomal pellets by pipetting with a P200 Gilson fitted with a yellow 200µl pipette tip. The shear forces were sufficient to almost completely destroy the fragile exosomes. Conducting this resuspension very gently, avoiding the generation of air bubbles and hence reducing the shear force, made it possible to resuspend the exosome pellet without causing damage producing vesicles morphologically consistent with exosomes. Immunogold labelling using antibodies binding tetraspanin CD81, CD82 or CD63 and MHC II, showed presence of the proteins on exosomes and their co-localisation with MHC II molecules (Fig.3.11B). While LCLs in culture can release EBV virions, only a small number of the cells enter lytic cycle replication, no virus particles were ever observed in the EM work.

A



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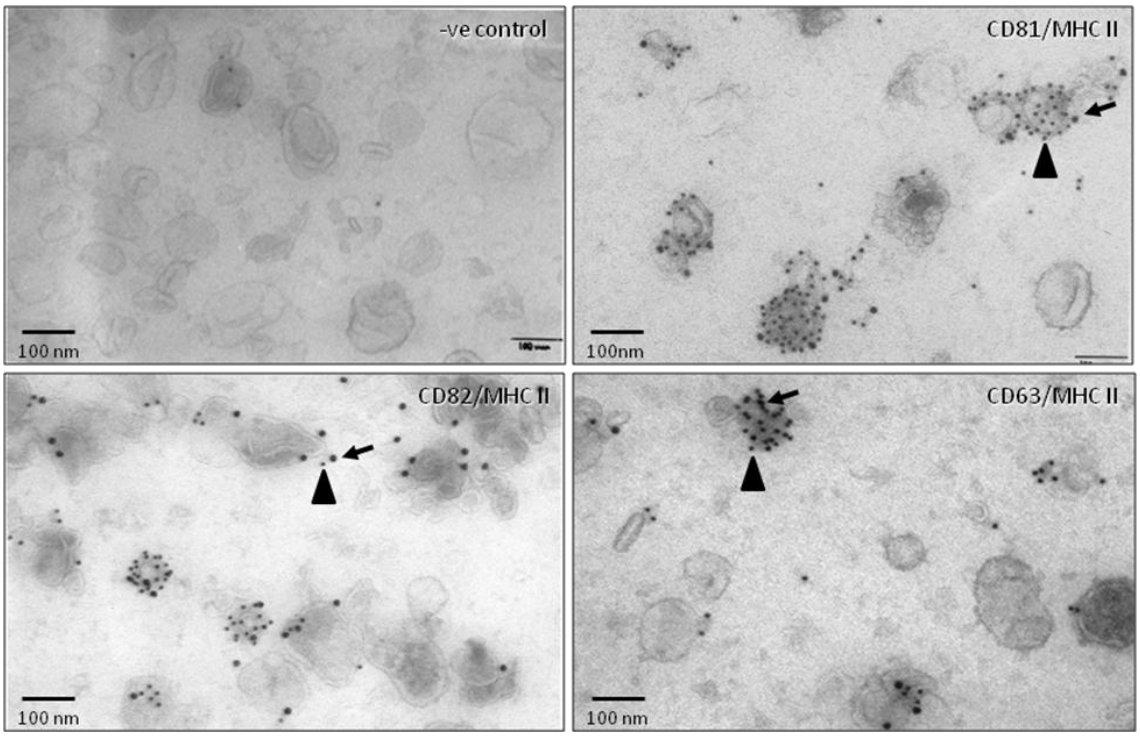

Figure 3.11: Vesicles visualised by transmission electron microscopy
A) The morphology of uranyl acetate stained exosomes was analysed via TEM. First attempts at purification resulted in almost complete destruction of exosomes. B) After further optimisation of the purification method, electron microscopy confirmed vesicles of size and morphology consistent with exosomes (upper left). Immunogold co-labelling of tetraspanins CD81 (upper right), CD82 (lower left) and CD63 (lower right) together with MHC class II molecules confirmed their co-localisation on exosomes. Here: tetraspanins - 10nm gold particles (arrowhead), MHC class II - 15nm gold particles (arrow).

3.2.3 Functional analysis of exosomes

3.2.3.1 Exosomes interact with CD4+ T cell clones

Since exosomes contain MHC II, co-stimulatory proteins and adhesion proteins and are able to directly interact with T cells (Thery et al. 2002a) the ability of LCL-derived exosomes to bind T cells was studied. Here, the fact that LCLs transduced by the control or CD63 shRNA expressing lentivirus also express GFP was taken advantage of. Since exosomes capture proteins of the cell's cytoplasm they should contain some GFP and so be detectable by flow cytometry. Exosomes purified from PER241, transduced with the control lentivirus, were co-incubated with a CD4+ T cell clone at 4°C or 37°C for one hour. The T cells were then washed and analysed by flow cytometry. There was an increase in fluorescence upon incubation with exosomes at 4°C, a larger increase was observed when the co-incubation was performed at 37°C (Fig 3.12A). To increase the fluorescent signal, the LCLs described above were first pre-labelled with CFSE, a fluorescent dye that binds intracellular proteins, prior to the two day culture to allow exosomes to be secreted. Different concentrations of CFSE were tested for LCL-labelling. The aim was to maximise fluorescence while minimising toxicity, since dying cells can release microvesicles that are not true exosomes. Labelling the LCL with 40µM CFSE decreased cell viability to 55%, whereas 20µM yielded strongly fluorescent LCLs and exosomes with an acceptable LCL-viability of 70% after the 48 hour culture used to accumulate exosomes in the cell supernatant (Fig. 3.12C). A remarkable increase of T cell fluorescence was achieved when incubating T cells with exosomes labelled with 20µM or 40µM CFSE at 37°C (Fig. 3.12B, right panel). At 4°C however, CFSE

labelling of LCLs using 5 or 20µM was apparently not sufficient to cause any enhancement of T cell fluorescence after co-incubating T cells with labelled exosomes. A shift of T cell fluorescence was only seen when incubated with exosomes labelled with 40µM CFSE (Fig. 3.12B). However, due to the high rate of cell death caused by this concentration of CFSE, the result has to be interpreted cautiously.

Generally, the higher increase in fluorescence intensity of T cells when incubated with labelled exosomes, either GFP or CFSE labelled, at 37ºC suggests that this temperature allows the T cells to take up exosomes and further allows additional exosomes to bind at their place. At 4ºC however, these exosomes are possibly not taken up and only bind on the T cell's surface.

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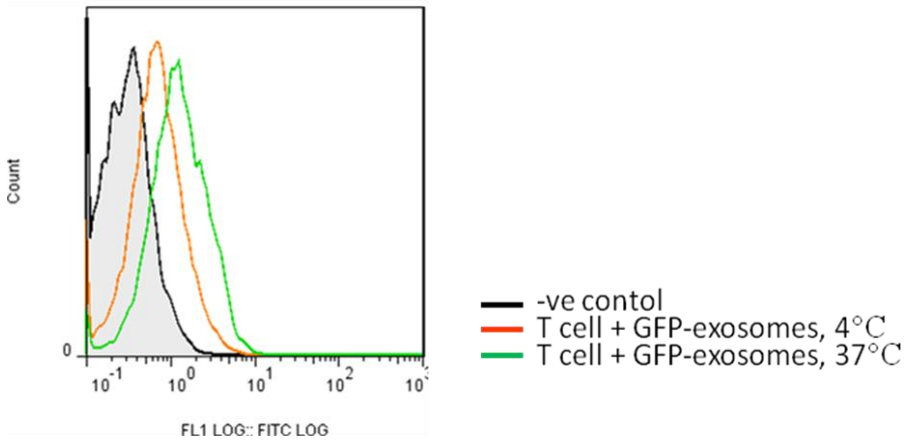

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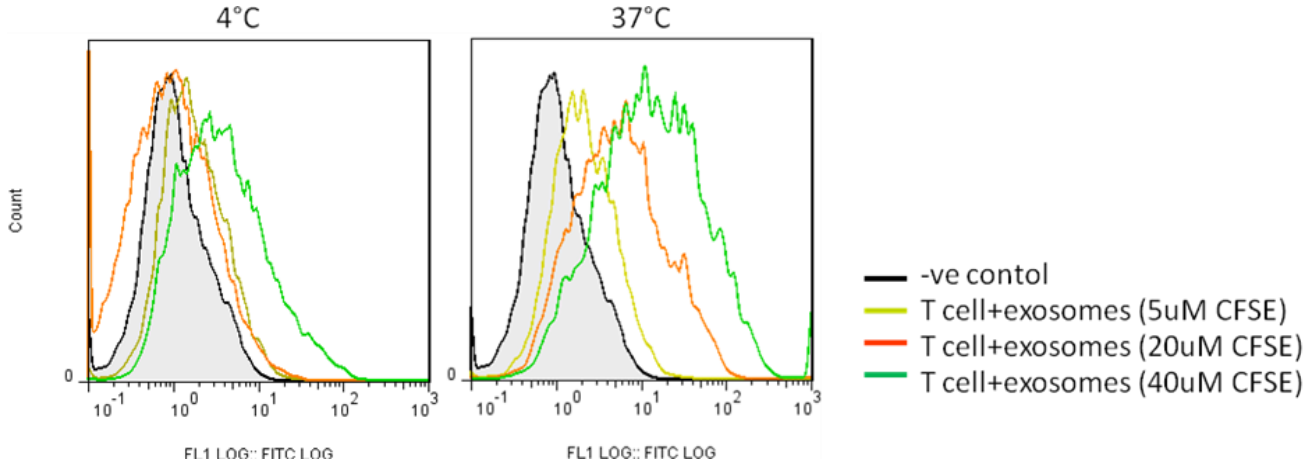

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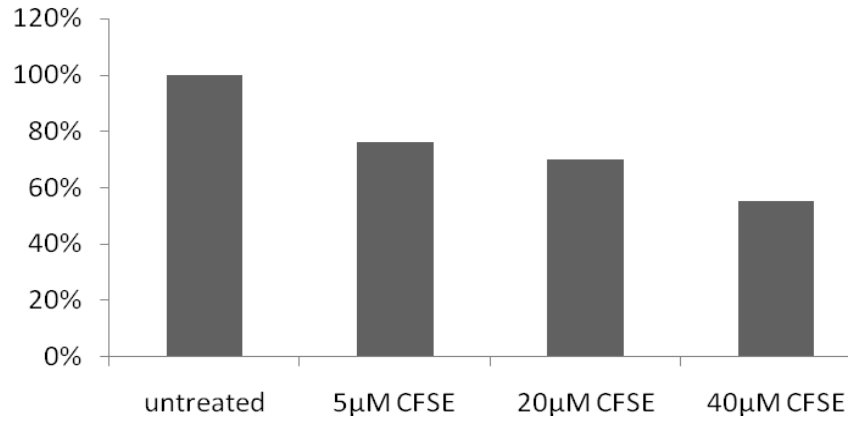

Figure 3.12: Analysis of binding of exosomes to T cells by flow cytometry
A) The CD4+ T cell clone c38 was incubated with HLA-matched GFP-containing exosomes purified from LCLs. After washing off remaining exosomes analysis by flow cytometry revealed increased fluorescence in the T cells, confirming exosomal binding to T cells. The negative control (-ve control) is T cells incubated with non-fluorescent exosomes. B) Staining LCL prior to isolating exosomes using 5 or 20μM CFSE showed a similar effect and only a slight shift using 40μM when incubating these exosomes with HLA-matched T cells at 4°C. Incubation at 37°C leads to even higher degree of fluorescence in this T cell clone. The negative control (-ve control) was provided by T cells incubated with unmanipulated exosomes. C) LCL's viability was determined before and after the 48 hour incubation with CFSE using Trypan blue. The viabilities were normalised in relation to 100% viability for untreated cells.

3.2.3.2 Exosomes are capable of stimulating CD4+ T cells

After demonstrating that exosomes from PER241 could bind to the CD4+ T cell clone, the ability of the exosomes to activate the T cells was studied. Exosomes purified from HLA matched or mismatched LCLs were added directly to CD4+ T cells. Following overnight incubation, levels of IFNγ released by the T cells was measured by ELISA. Exosomes were able to stimulate CD4+ T cell clones specific for the EBNA2 epitope "PRS" (c93, DR52b restricted) or the EBNA 3C epitope "SDD" (c38, DQ5 restricted), but only when derived from a suitable HLA matched LCL (Fig. 3.14). The same quantity of exosomes purified from an HLA-mismatched LCL lacking these alleles (PER213, Table 2.4) was completely unable to activate the

T cell clone. This result demonstrates that exosomes are able to directly activate CD4+ T cell clones in a HLA restricted manner.

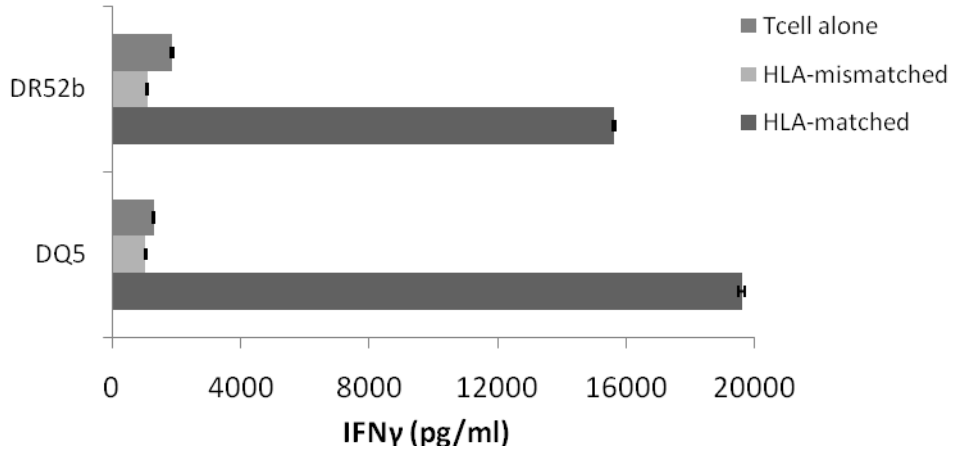

Figure 3.13: Direct stimulation of CD4+ T cell clones by purified LCL-derived exosomes
CD4+ T cell clones (5000 T cells/well) specific for the DR52b restricted epitope PRS (EBNA2) or the DQ5 restricted epitope SDD (EBNA 3C) were incubated with equal quantities of exosomes purified from LCLs either HLA matched or HLA mismatched for both alleles. IFNγ released by activated CD4+ T cells was analysed by ELISA. Shown is one representative result from at least 5 experiments. Error bars show the mean +/- standard deviation of triplicate ELISA-wells.

3.2.3.3 Stimulation of CD4+ T cells by LCL-derived exosomes is MHC II dependent

To confirm that the ability of LCL-derived exosomes to stimulate the CD4+ T cell clones was indeed MHC II restricted, T cells were exposed to exosomes in the absence or presence of blocking monoclonal antibodies specific for HLA-DQ or HLA-DR. Sensitisation of the HLA-DQ5 restricted SDD epitope was blocked by the HLA DQ but not the HLA-DR specific antibody (Fig. 3.14A). Likewise, sensitisation of the HLA-DR52b restricted PRS epitope was reduced by the HLA-DR specific antibody while the HLA-DQ antibody had only a minor effect (Fig. 3.14B). Note that as exosomes bear both DR and DQ molecules on the surface, this small reduction

seen following incubation with the "incorrect" antibody could be caused by non-specific steric hindrance.

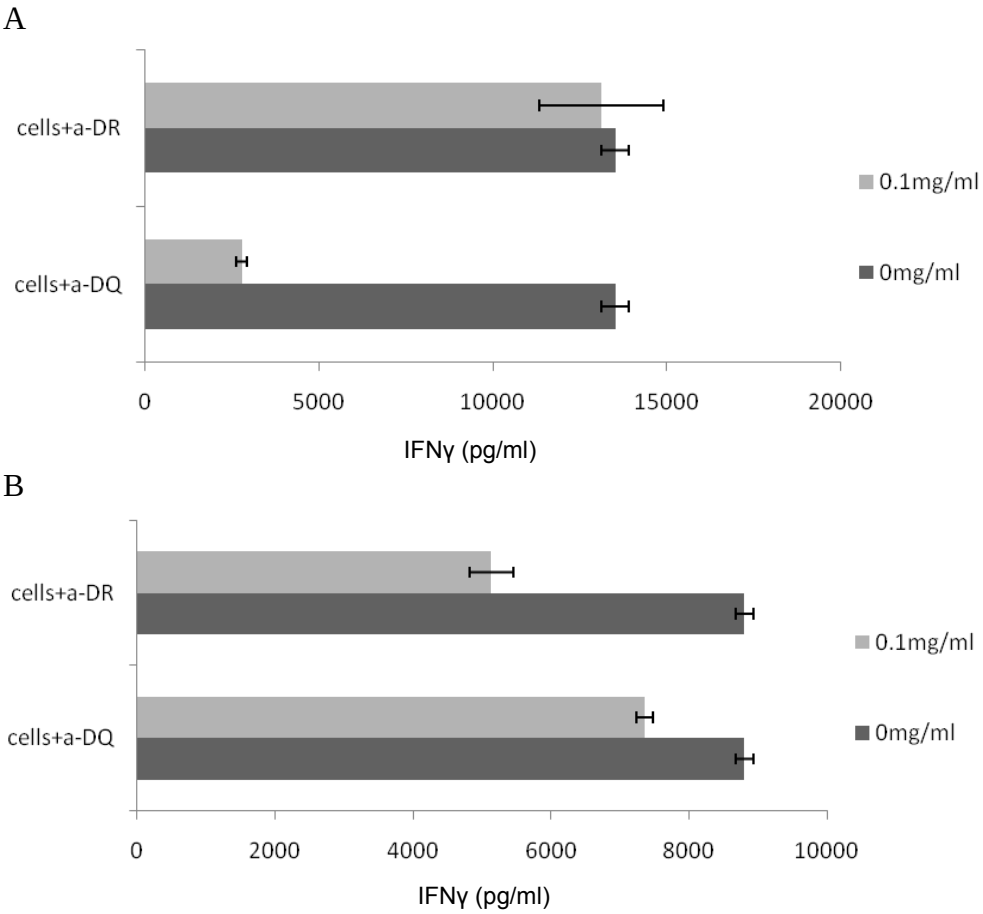

Figure 3.14: Analysis of MHC II-restriction of CD4+ T cell clone activation
Antibodies specifically binding to HLA-DQ or -DR were added to exosomes at a concentration of 0.1 mg/ml prior to addition of T cells (5000 T cells/well) to determine the role of exosomal MHC class II-peptide complexes in CD4+ T cell stimulation. A) The activation of DQ5-specific T cell clones was reduced by adding α-DQ antibody but almost unaffected by the addition of α-DR52b. B) Similarly, the recognition of the DR52b-specific T cell clones was markedly reduced upon adding α-DR antibody. However, here the addition of α-DQ also caused a small decrease in HLA-DR specific T cell activation. This could be due to steric hindrance rather than real inhibition of the HLA-DR molecule. Shown is one representative result from two experiments. Error bars show the mean +/- standard deviation of triplicate ELISA-wells.

3.2.4 Analysis of exosomes derived from CD63low LCL

3.2.4.1 Characterisation of CD63low LCL-derived exosomes

Exosomes purified from the two CD63low LCLs, a and b, were analysed for their protein content and compared to exosomes derived from control LCL transduced with the control lentivirus that contains no shRNA sequence. CD63 was markedly reduced in exosomes derived from CD63low LCLs compared to the control LCL. All other membrane proteins tested, including MHC I, MHC II, ICAM-1 and LMP-1 were not affected by the knock down of CD63 in exosomes (Fig. 3.15). Interestingly, while, as expected, LMP-1 was present in exosomes, Western blotting also revealed the presence of the EBV nuclear protein EBNA2 in exosomes. Other EBV proteins including EBNA3c and LMP2a as well as the adhesion molecule β1-integrin subunit could not be detected in any of the exosomes (data not shown).

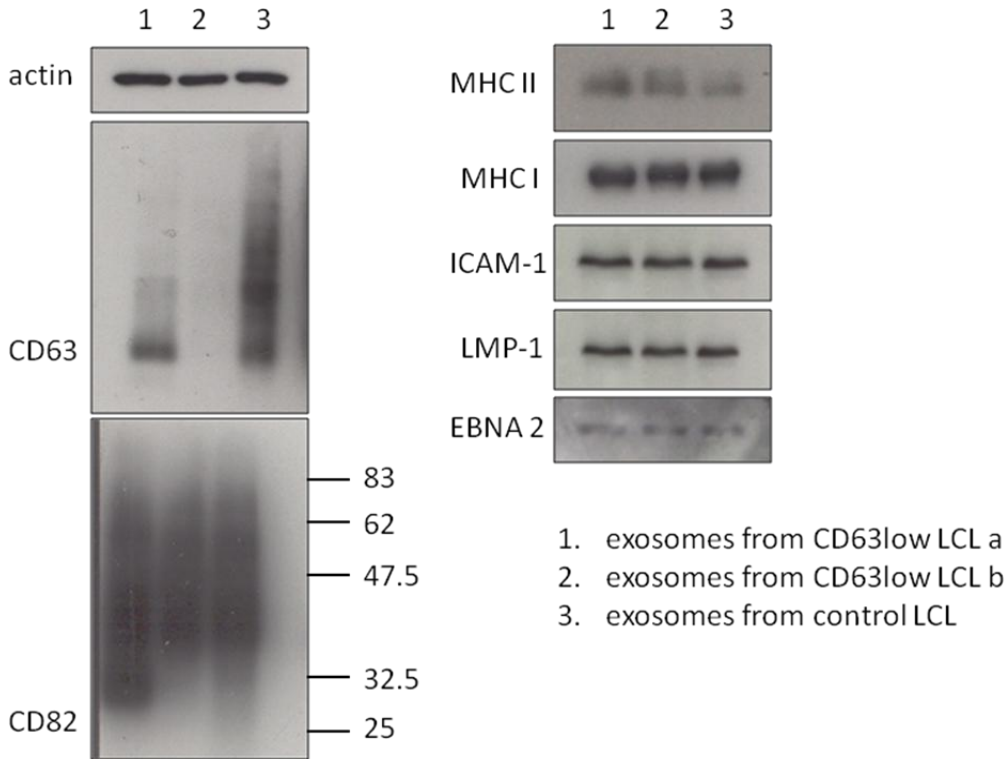

Figure 3.15: Characterisation of CD63low exosomes
Western blotting of exosomal lysates using antibodies recognising CD63, CD82, MHC II, MHC I, ICAM-1, LMP-1 and EBNA2 demonstrated that CD63 depletion (lane 1 and 2) does not influence the amount of these proteins compared to the amount found in exosomes purified from control LCL (lane 3).

3.2.4.2 Quantification of exosomal MHC II by electron microscopy

To investigate the effect of CD63 knockdown on the density and distribution of MHC class II on individual exosomes, immunoelectron microscopy analysis was performed (Fig. 3.16A). The analysis involved counting up to 1500 gold particles on exosomes per cell line. As expected low CD63 labelling was observed on exosomes purified from CD63low LCLs. Double labelling experiments (CD63-MHCII) were carried out using an anti-HLA DR primary antibody along with a CD63-specific primary antibody. The lower amount of CD63 on exosomes purified from CD63low LCLs was confirmed by this technique. However, interestingly, in contrast to previous Western blotting results, the level of MHC II labelling on CD63low exosomes was about 30%

higher (Fig. 3.16B). Interpreting this result, it has to be taken into account that the quantification by TEM was conducted using exosomes purified from a single batch of cells per LCL. This difference in MHC II level may reflect a transient variation in MHC II expression as it was observed for this particular LCL over the years of cultivation (data not shown). Alternatively, the discrepancy with the WB data may be due to the differences in techniques. Immunolabelling of the exosomal surface measures what is available for the antibody to bind and would not recognise target epitopes of some of the MHC II molecules on the surface of the control exosomes if these are occluded by adjacent proteins. On the other hand, depletion of CD63 may somehow increase the avidity of MHC II to this antibody.

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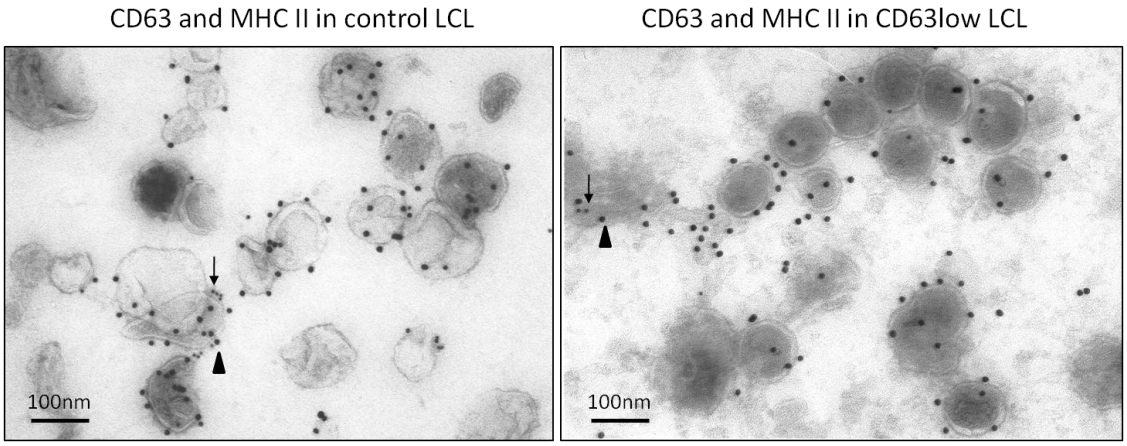

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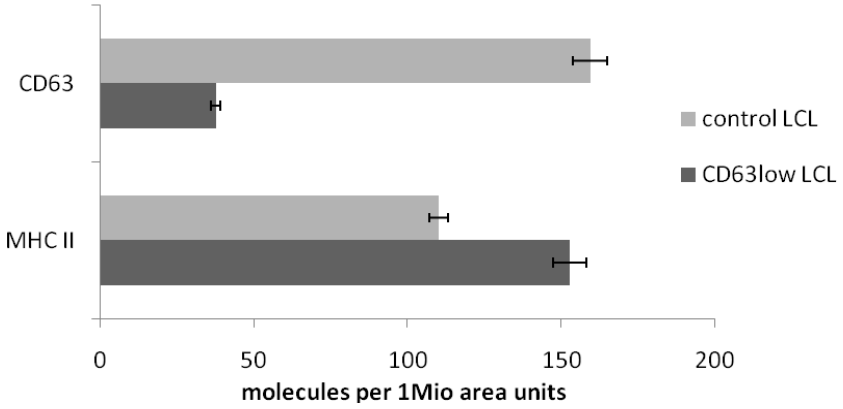

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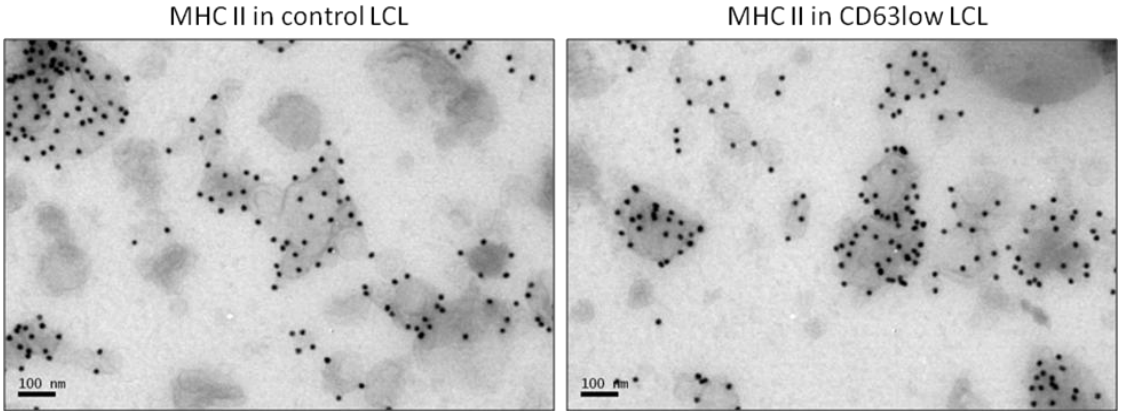


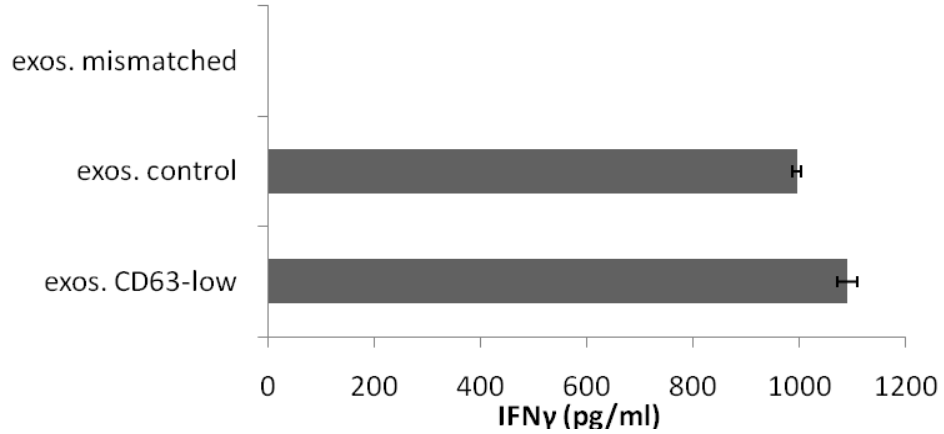
Figure 3.16: CD63 and MHC II content of control and CD63low
A) The quantity of MHC II and CD63 molecules was determined by immunogold labelled exosomes analysed using the image processing software ImageJ. Antibodies specifically recognising CD63 and MHC class II (HLA DR) where used for immunogold labelling. B) Lower level of CD63 was confirmed on the CD63low-a LCL. The level of HLA DR on the exosome surface was about 30% lower on exosomes purified from the control LCL compared to the CD63low LCL. The number of molecules was assessed in relation to the exosome's perimeter which was given by ImageJ in arbitrary area units. Isotype controls were used to check for unspecific staining. C) HLA DR single labelling of exosomes purified from a

different batch of the same cells as used above did not confirm this difference. However, conclusive quantification was not done in this analysis.

3.2.4.2 Analysis of direct CD4+ T cell activation by CD63low exosomes

To examine whether lower levels of CD63 on exosomes has any effect on exosomal antigen presentation, CD63low exosomes were tested for their ability to activate CD4+ T cell clones. Exosomes purified from the CD63low and the control LCL induced comparable levels of IFNγ production from both CD4 T cell clones, c38 (SDD) (Fig. 3.17A) and c93 (PRS) (Fig. 3.17B).

A



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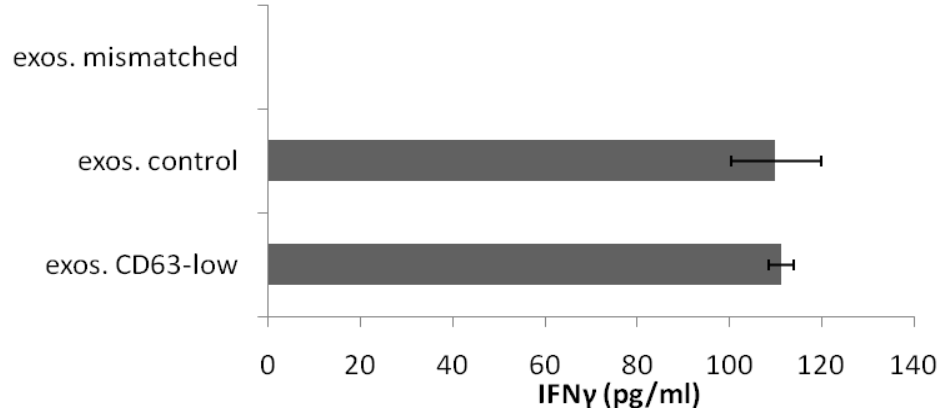


Figure 3.17: CD4+ T cell stimulation induced by exosomes purified from control or CD63low LCLs

Exosomes purified from control or CD63low LCLs caused a comparable degree of T cell activation after co-incubation with CD4+ T cell clones c38 (A) or c93 (B). Exosomes purified from an HLA-mismatched LCL did not stimulate any detectable IFNγ secretion by T cells. Note that a lower number of T cells (500 cells/well) were used in this experiment accounting for the lower levels of IFNγ seen in this experiment compared to earlier experiments. Shown are the data from a representative experiment out of three. The error bars represent the mean +/- standard deviation of triplicate ELISA wells.

To exclude the possibility of oversaturation of the system by exosomal material, titration experiments were carried out. Exosomes were quantified by Bradford protein assay and titrated for subsequent T cell assays. 5000 cells of each CD4+ T cell clone, c38 and c93, were incubated overnight with 10, 30 and 55µg of exosomes purified from control or CD63low-a LCL in a total volume of 200µl. Comparable activation of c38 and c93 by control and CD63low exosomes was observed when 10, 30 or 55µg of

exosomes were added to the T-cell clones (Fig. 3.18). Interstingly, the T cell clone c38 was stimulated substantially better by the control exosomes at 10µg/reaction (Fig. 3.18A). However, given the low reading observed at this concentration of exosomes, this difference is likely to be a result of an experimental error due to pipetting errors during setting up of the assay. Taken together, these results suggest that depletion of CD63 did not affect the ability of LCL-derived exosomes to stimulate T cells.

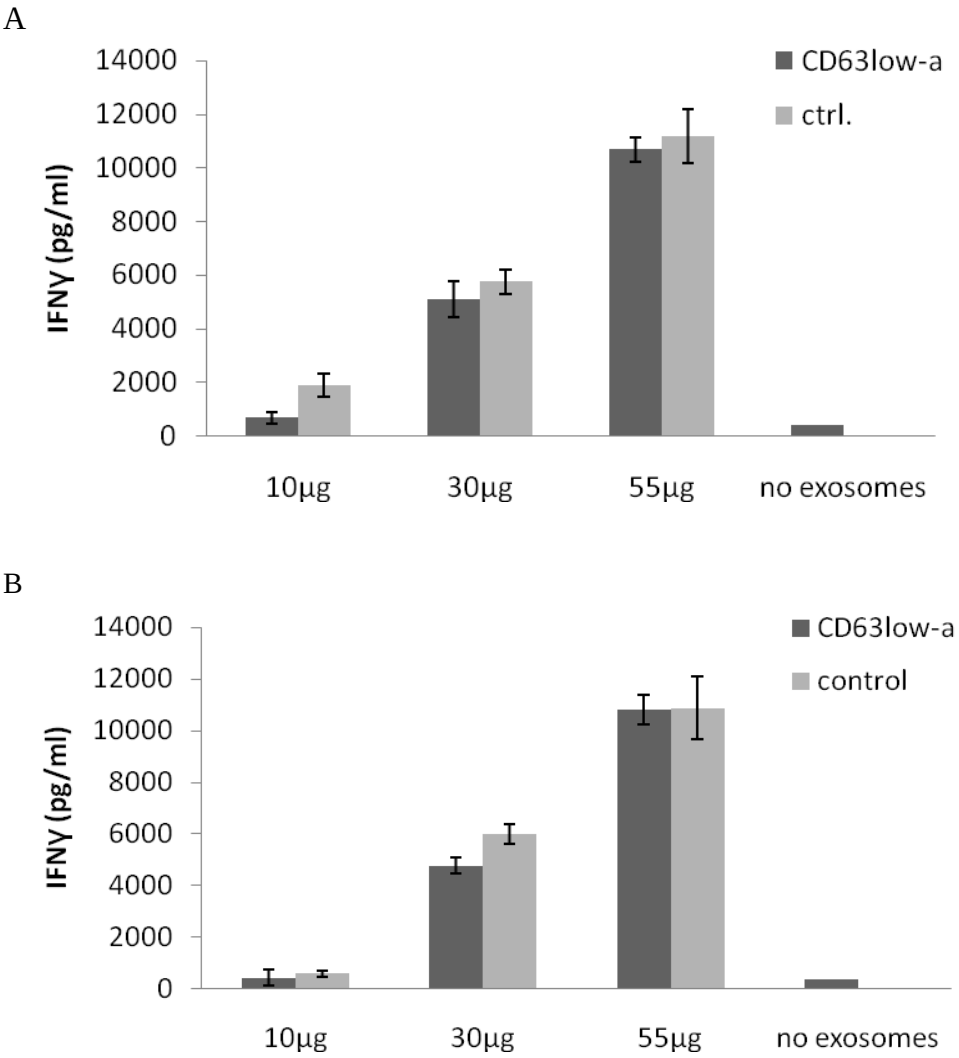


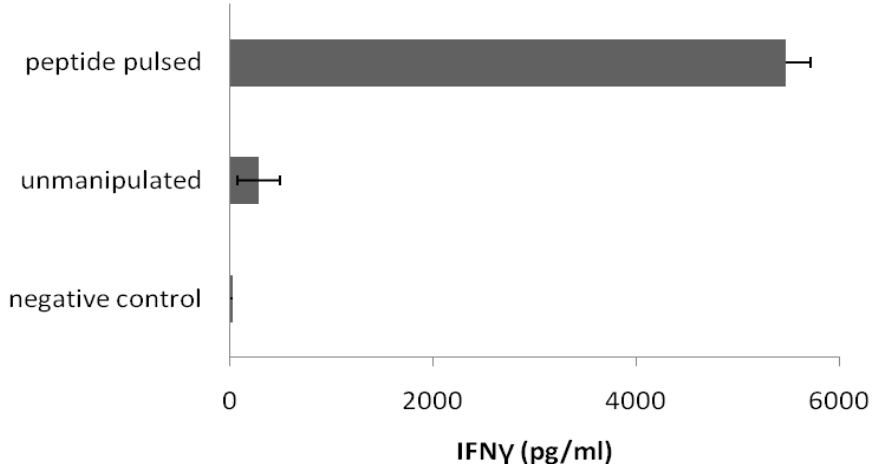
Figure 3.18: Analysis of direct exosomal CD4+ T cell activation by exosome-titration
Exosomes purified from control and CD63low LCLs were quantified by Bradford protein assay. The amounts of 10, 30 and 55µg were given to CD4+ T cell clone c38 (A) or c93 (5000 cell/well, respectively) (B). Exosomes purified from control LCL caused a higher stimulation of c38 than exosomes purified from CD63low LCL when 10µg of exosomes were added to T cells. Otherwise, T cell stimulation by exosomes from control and CD63low LCL was comparable for all tested concentrations.

3.2.4.3 Analysis of the capability of CD63low exosomes to sensitise a EBV-antigen negative LCL

To investigate whether exosomes purified from control and CD63low-a LCLs are able to sensitise other B cells for CD4+ T cell activation we used PER282 as a mediator LCL. PER282 is transformed with the type II Ag876 strain of EBV. This LCL is HLA-matched for the CD4+ T cell clone c93 but, due to sequence variations in the endogenous antigen, does not stimulate this CD4+ T cell clone by itself. This means that the amount of IFNγ induced by this LCL should be at the background level. However, being HLA-matched, this LCL can still present epitope peptides derived from conventional LCLs or corresponding exosomes to T cells.

As expected, unmanipulated Ag876 LCL did not stimulate c93 but caused a substantial IFNγ secretion when pulsed with the antigenic peptide (PRS) prior to co-incubation with T cells (Fig. 3.19A). Next, exosomes purified from control and CD63low-a LCLs were added to the Ag876 LCL (unmanipulated or peptide pulsed) prior to co-incubation with T cells. The Ag876 LCL supplemented with exosomes derived from either CD63low-a or control LCL stimulated c93 T cells to the same degree (Fig. 3.19B). The results of this experiment confirmed the equal stimulatory power of these two different classes of exosomes. Note, that exosomes were not washed off but co-incubated together with the T cells and the Ag876 cells.

A



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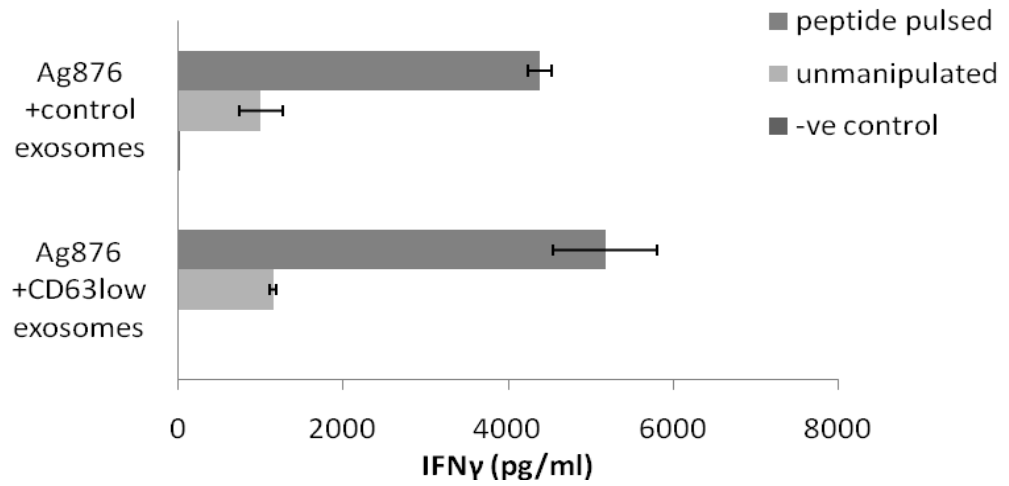


Figure 3.19: Capability of CD63low exosomes to sensitise EBV-antigen negative LCL
The LCL PER282 does not stimulate CD4+ T cells by itself but causes IFNγ release by the CD4+ T cell clone c93 when pre-pulsed with exogenous antigen-peptides (A). Exosomes purified from CD63low and control LCL were mixed with PER282 cells (unmanipulated or antigenic-peptide pulsed) prior to co-incubation with CD4+ T cells (clone 93, 2500 cells/well). Both types of exosomes sensitised LCL282 to a similar degree which is seen by a comparable level of IFNγ release upon CD4+ T cells encounter. Exosomes were washed off the LCL before incubation with CD4+ T cells. (B). Negative controls were T cells incubated without any stimuli.

Summary

Here I have shown that LCLs secrete exosomes and have optimised the purification of LCL-derived exosomes by differential centrifugation. I found that it was first necessary to remove bovine microvesicles from the FBS and also use a low speed spin

to remove cells in order to minimise contamination with protein residents of other intracellular compartments (e.g. calnexin). After isolating exosomes of sufficient purity, I showed that exosomes purified from CD63low LCLs have a reduced level of CD63 compared to exosomes purified from a control LCL. MHC I and adhesion molecules remained constant over time. Seemingly contradictory results were obtained in MHC II quantification experiments when Western blotting and immune-electron microscopy were used. This difference however, may be due to the effect of temporal fluctuations in the protein expression of the LCLs used in this study. Alternatively, the discrepancy with the WB data may be due to the differences in techniques. Immunolabelling of the exosomal surface measures what is available for the antibody to bind and would not recognise target epitopes of some of the MHC II molecules on the surface of the control exosomes if these are occluded by adjacent proteins. On the other hand, depletion of CD63 may somehow increase the avidity of MHC II to this antibody. LCL-derived exosomes were capable of directly stimulating CD4+ T cell clones in a HLA-restricted manner but depletion of CD63 in LCL-derived exosomes did not affect the activation of CD4+ T cell clones by exosomes directly or added to a epitope-negative LCL prior to incubating with T cells.

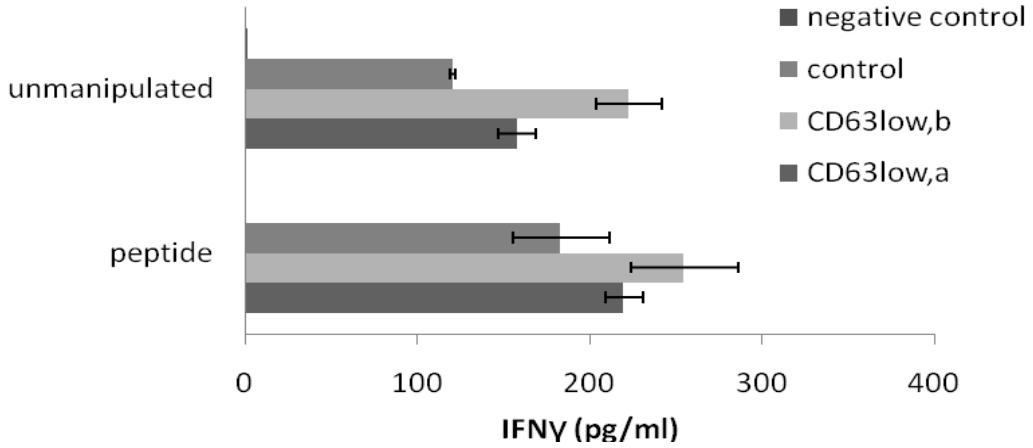
3.3 Influence of CD63 on T cell recognition of EBV positive LCLs

3.3.1 The role of CD63 in cellular T cell stimulation

To investigate the role of CD63 in antigen presentation by cells, both of the CD63 depleted LCLs and the control LCL were co-incubated with CD4+ T cell clones c38

or c93. In contrast to the results obtained using exosomes, both CD63low LCLs stimulated markedly higher levels of IFNγ release by the two different T cell clones. These results suggested that depletion of CD63 increased levels of antigen specific CD4+ T cell recognition compared to the control LCL (Fig. 3.20). The enhanced T cell stimulation by the CD63 low LCL was also seen when the LCLs were loaded with synthetic epitope peptides.

A



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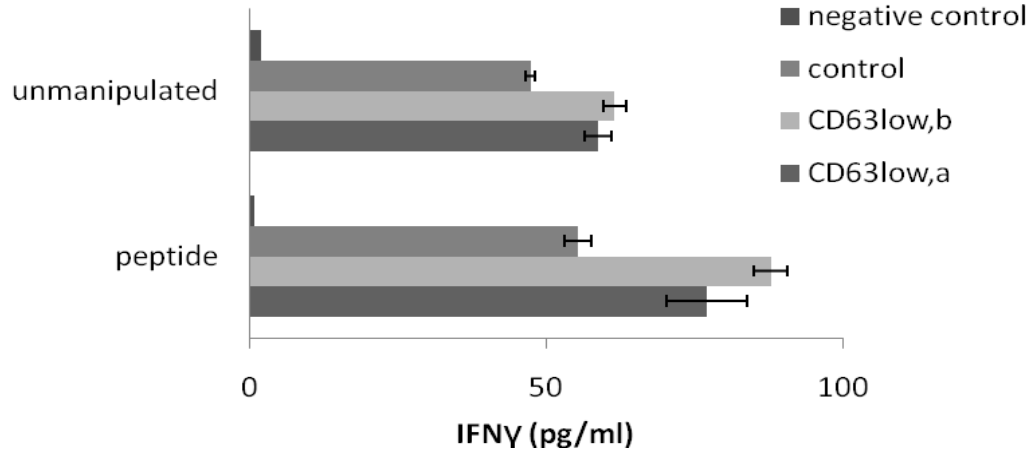


Figure 3.20: CD63 knock down potentiates cellular CD4+ T cell activation by LCLs
DQ5- (A) as well as DR52b- (B) specific CD4+ T cell clones exhibit an increased activation after co-incubation with CD63 low LCLs (CD63low-a and -b) compared to the control LCL. This is the case for peptide pre-loaded LCLs (peptide, 10^{-5}M) as well as for unmanipulated cells. The graph shows a representative experiment out of 13 performed. The error bars represent the mean +/- standard deviation of the ELISA triplicate wells. Each incubation was conducted using 200 T cell/well. Negative controls were T cells incubated without any stimuli.

3.3.2 Titration of exogenous antigenic peptides

As an enhancement of CD4+ T cell stimulation was seen for both, unmanipulated and peptide loaded (10^{-5}M) CD63low LCLs, the next step was to titrate the peptides in order to identify the concentration for optimal T cell stimulation. In the range of 0 to 1 x 10^{-5} M of peptide that was added to the LCLs prior to then being washed and mixed with CD4+ T cells, a concentration of 1 x 10^{-7} M was sufficient to cause a maximum IFNγ-release. Indeed, higher concentrations of peptide caused the T cell stimulation to actually decrease to a similar degree for all three LCLs (Fig. 3.21). A similar effect of high dose peptide inhibition was reported before for CD8+ T cells (Alexander-Miller et al. 1996) and has also been described for CD4+ T cells (Haigh et al. 2008;Long et al. 2005b). The fact that T cell recognition of exogenous peptide was increased upon CD63 knockdown was an unexpected observation since it suggests that the phenotype we have observed is more general than just an effect on the EBV epitopes themselves.

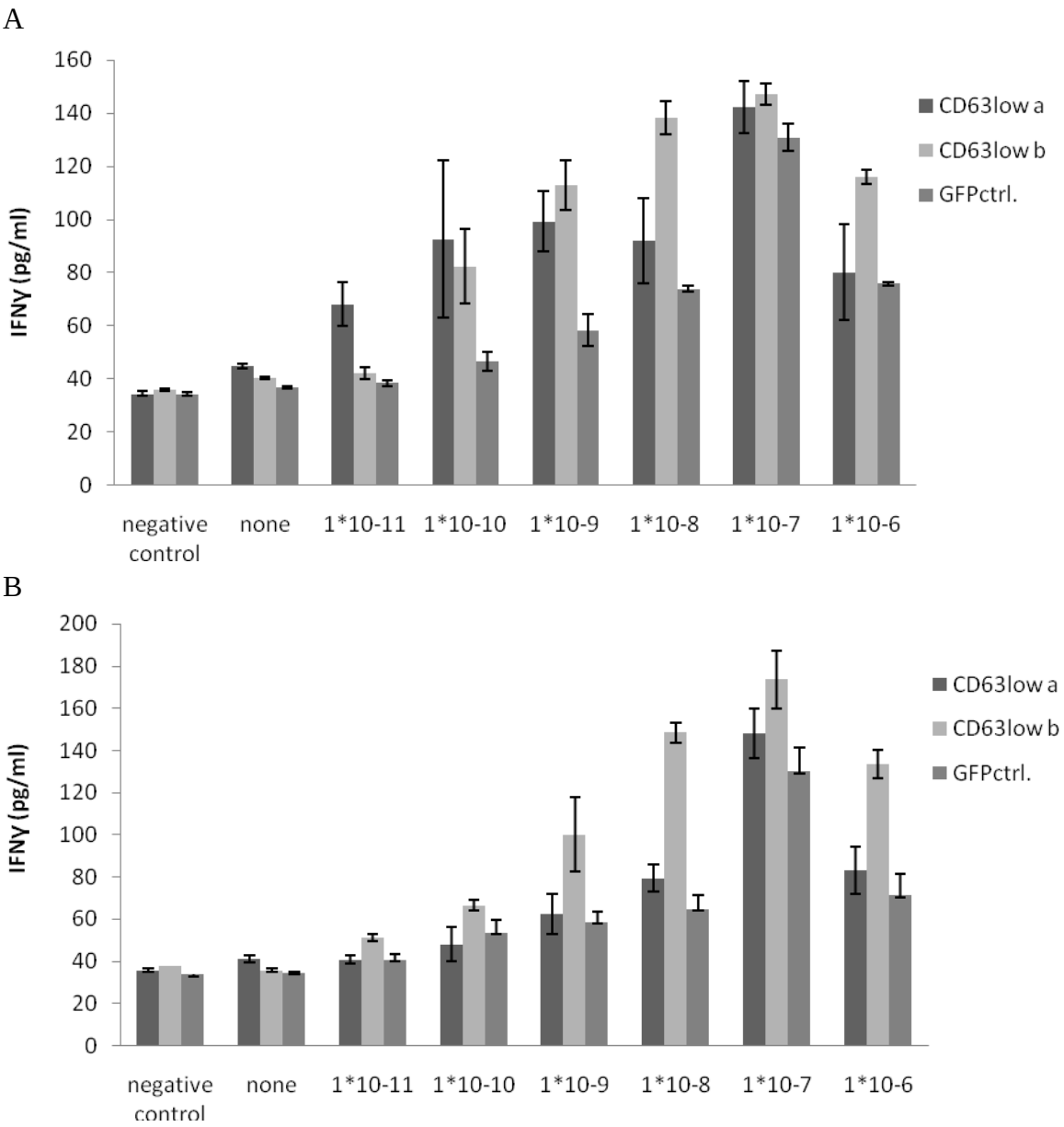


Figure 3.21: Titration of LCL-loading of exogenous antigenic peptides
The LCLs, CD63low-a, CD63low-b and control, were pulsed with the antigenic peptides "SDD" and "PRS" prior to co-incubating with CD4+ T cell clones overnight (250 cells/well). For both T cell clones, c38 (A) and c93 (B), a peptide molarity of 1 $x10^{-7}$ proved to be maximal stimulatory in addition to the innate LCL-derived T cell stimulation.

3.3.3 CD8+ T cell stimulation

In order to examine whether knocking down CD63 effects MHC class I driven antigen presentation, T cell assays were conducted as before but now using a CD8+ T cell clone (c6), This clone is specific for an epitope (GLCTLVAML) from the EBV

lytic protein BMLF1 which is known to be expressed by LCLs (Yang et al. 2000). C6 is HLA-A0201 restricted and therefore HLA matched for the control and CD63low LCLs established from PER241. In contrast to the results observed using CD4+ T cells, CD63low LCLs stimulated CD8+ T cell clone to the same degree as the control LCL (regardless of whether the LCLs were preloaded with synthetic epitope peptide or left unmanipulated) (Fig. 3.22).

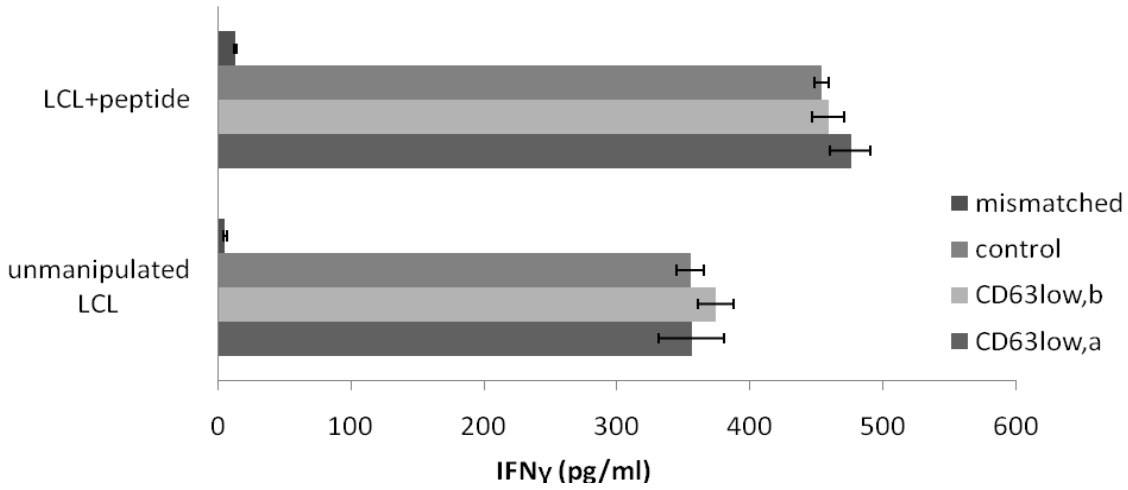


Figure 3.22: Influence of CD63 knock down on CD8+ T cell activation
CD63low-a, -b and the control LCL were tested for CD8+ T cell recognition; 500 T cells were used per ELISA well and 50 000 lymphoblastoid cells. CD8+ T cells did not show any difference in activation caused the by CD63low or control LCL. This applies to LCLs either non-treated or peptide loaded with the particular antigenic peptide (GLC) recognised by this T cell clone. The HLA-mismatched LCL PER213 was not recognised. Shown is one representative experiment out of two. The error bars show the mean +/- standard deviation of ELISA triplicates.

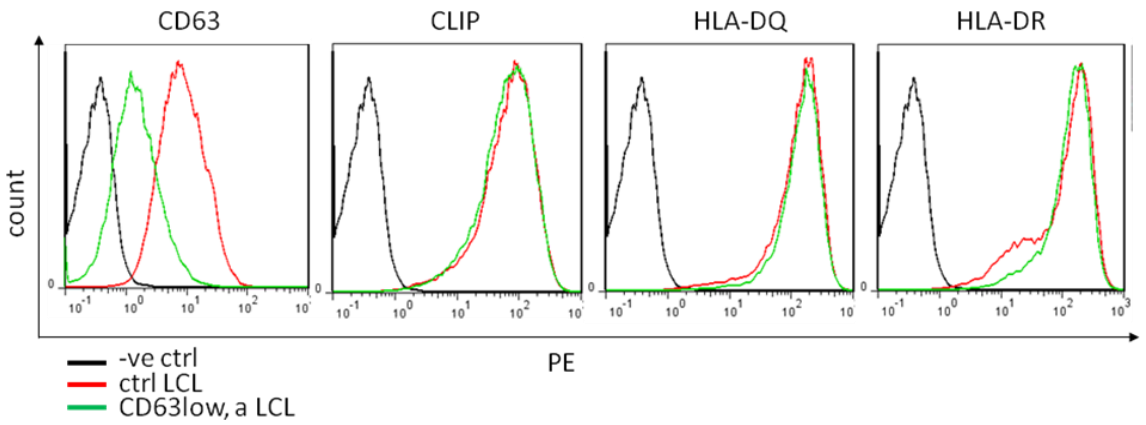
3.4 Mechanism how CD63 depletion enhances CD4+ T cell recognition

3.4.1 Expression level of CLIP following CD63 depletion

Since MHC class II expression, both total and surface, was unaltered in CD63 depleted LCLs (Fig. 3.6), the enhanced CD4 T cell recognition observed was clearly not due to a quantitative change in MHC II levels. Rather, it may have reflected a qualitative change in MHC II. As a first step, the level of CLIP expression on the cell surface of the LCLs was examined. CLIP binds MHC II with low affinity. Therefore, the observation that peptide loaded LCLs are recognised better upon CD63 depletion could conceivably be due to the presence of more CLIP-containing MHC II molecules on the surface of CD63low LCLs and CLIP being more readily displaced by the higher affinity epitope peptides. Flow cytometry showed that staining with α-CLIP antibody was comparable for control and CD63low-a LCLs (Fig. 3.23A). As an additional control, the human T2 cell line was used. These cells lack almost all of the MHC regions from chromosome 6p (Trowsdale et al. 1990). T2 stably transfected with HLA-DR11, or HLA-DR11 and HLA-DM, kindly provided by Dr. J. Blum (Indiana University), were stained for CLIP and analysed by flow cytometry. As expected, T2-DR11 and T2-DR11-DM express HLA-DR11 but no HLA-DQ (Fig. 3.23B). In terms of CLIP, T2-DR11 exhibits a noticeable expression of CLIP, whereas T2-DR11-DM, overexpressing HLA-DM (which chaperones the exchange of CLIP with antigenic peptides in the MIIC) does not. This control therefore confirmed the specificity of the anti CLIP antibody. Note that the CLIP expression of LCLs is substantially higher than of the T2 control cell line. Similarly, high levels of CLIP

were observed on LCLs established from other healthy lab donors (S. Sabbah, personal communication). The lower expression of CLIP on T2 cells is in correspondence with their lower expression of total HLA (here HLA-DR).

A



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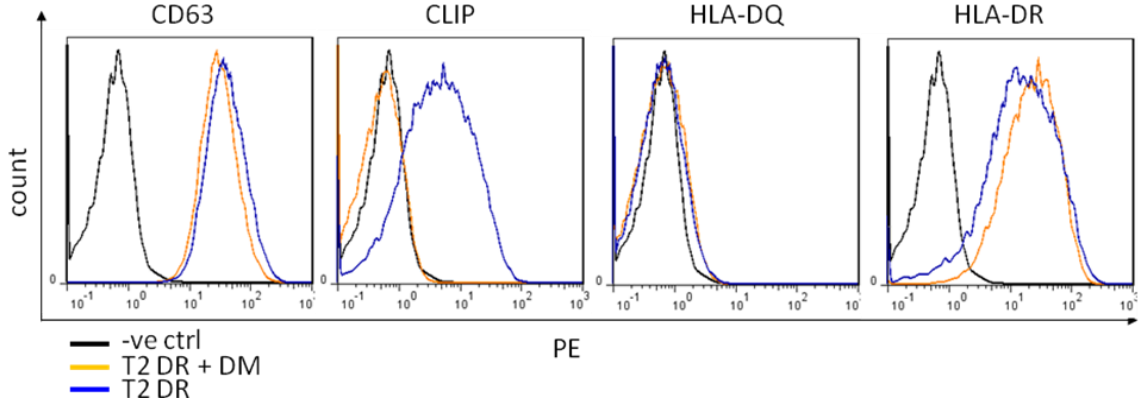


Figure 3.23: CLIP expression in CD63low and control LCL
A) Flow cytometry was conducted using α-CD63 (left), α-CLIP (middle left), α-HLA-DQ (middle right) and α-HLA-DR (right). Surface expression of CLIP is equal in control LCL and CD63low-a LCL. Both HLA molecules, DQ and DR, are equally expressed as already shown in 3.1.4.4. B) The two T2 cell lines, T2-DR11 and T2-DR11-DM express equal amounts of CD63 and HLA-DR, HLA-DQ is not present in these cell lines. As expected, CLIP was only detectable for the T2-DR, not for the T2-DR-DM cell line expressing the chaperone protein HLA-DM that mediates the exchange of CLIP for antigenic peptides in MIICs.

3.4.2 MHC class II stability in CD63-low LCL

While levels of cell surface CLIP were equivalent in control and CD63low cells, it was possible that MHC class II molecules in the latter are less stable and so better able to bind exogenously provided synthetic epitope peptides. This prompted a second experiment to examine stability of MHC II molecules in the different LCLs. Lysing cells in Triton X 100 (1%) for 30min at room temperature results in the disassembly of unstable MHC class II dimers into monomers that can be identified by non-reducing SDS-PAGE. This experiment therefore allows the stability of MHC II to be determined by analysing the ratio of monomers to remaining dimers. The relative amounts of MHC class II dimers from control and CD63low LCLs was assessed by immunoblotting using an antibody specific for HLA class II (DA6-14) which can bind both dimers and monomers alike. CD63low-a, CD63low-b LCL and the control cell line did not show any substantial difference in the ratio of HLA mono- and dimers (Fig 3.24), which is in agreement with the previous result examining CLIP surface expression level (see Fig. 3.23A). The same experiment was also conducted after pulsing LCLs with antigenic peptides before lysis. As for the non-pulsed LCLs, the ratio of HLA monomers and dimers was equivalent for all LCLs tested (Fig. 3.24B).

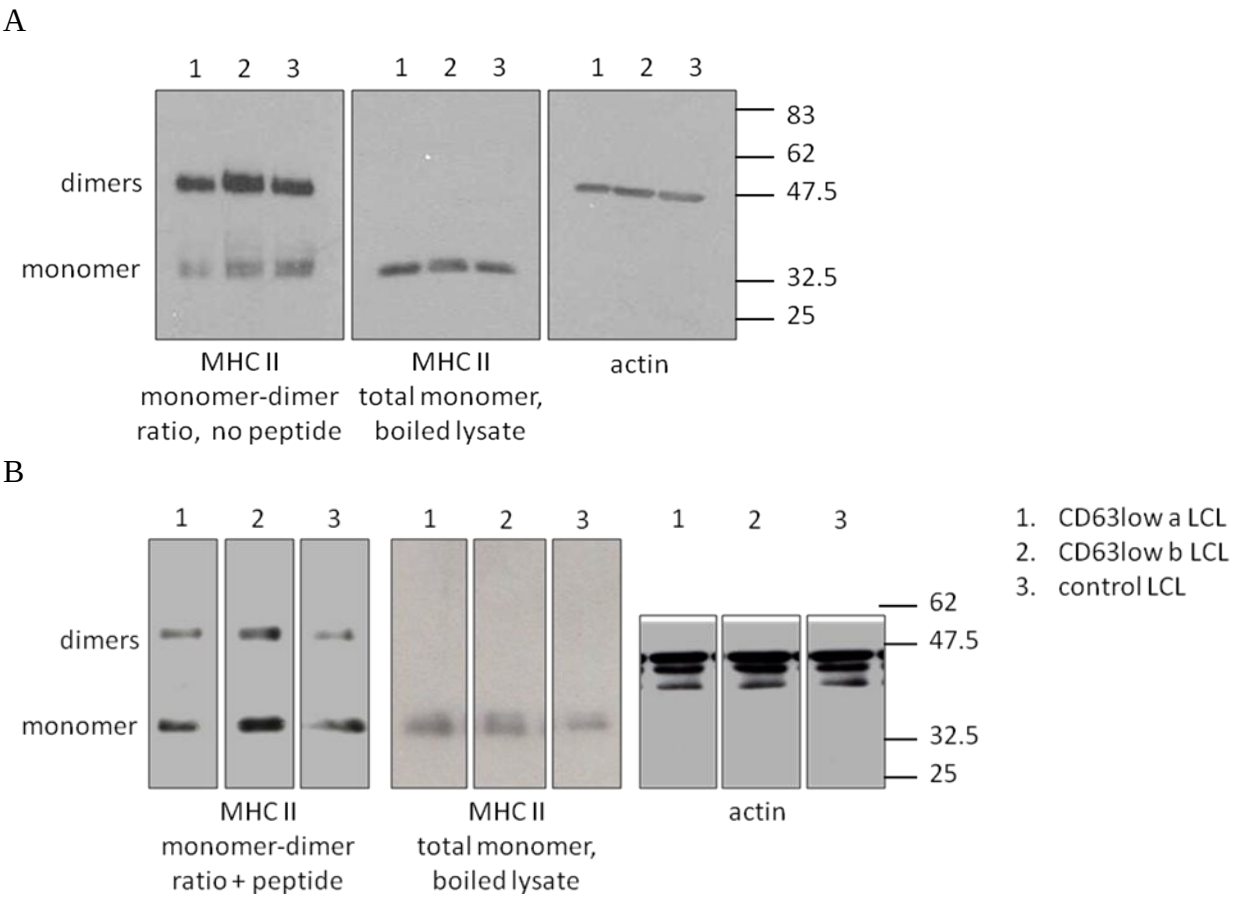


Figure 3.24: Analysing HLA-stability in CD63low and control LCL
A) LCLs were lysed for 30min on ice in Triton X-100 prior to incubating the lysates for 30min at room temperature. Analysis by non-reducing SDS-PAGE and Western blotting revealed comparable dimer/monomer ratios of MHC class II in the control and CD63-depleted LCLs. An aliquot of the lysates was reduced and boiled to detect the total amount of MHC II monomers; blotting for actin serves as a loading control. Shown are blots from one representative experiment out of four. B) Peptide pulsing of LCLs with antigenic peptide prior to lysis did not cause any change in the ratios.

3.4.3 Total binding capacity of MHC II molecules in control and CD63low cells

To test for potential differences in peptide binding properties, CD63low-a and CD63low-b as well as the control LCL were incubated with biotinylated PRS or SDD peptide sequences. Staining with PE-Avidin allowed quantification of bound peptides by flow cytometry (Fig 3.25C). In order to demonstrate that the process of biotinylation of the peptides did not sterically hinder the binding to MHC II

molecules, a standard T cell assay using the LCL PER241 and the CD4+ T cell clone c38 and c93 was conducted. The biotinylated and non-labelled SDD peptides were equally able to stimulate T cells (Fig. 3.25A). Biotinylated PRS showed a somewhat reduced ability to stimulate T cells (Fig. 3.25B). Nevertheless enough peptide bound to the cells to allow differences in binding to control or CD63low cells to be studied in subsequent experiments.

Flow cytometry experiments showed that LCLs loaded with biotinylated peptides display low overall levels of fluorescence. Nonetheless, the enhancement of fluorescence after peptide loading was consistent and equal for the control and both CD63low LCLs after pulsing with biotinylated SDD (Fig. 3.25C), suggesting that depletion of CD63 did not affect peptide binding. Similar results were obtained when biotinylated PRS peptide was used (data not shown). Therefore, the enhanced T cell recognition that is seen for peptide pulsed CD63low LCLs is not due to just more synthetic epitope peptide binding to the CD63low LCLs but is likely to involve another mechanism(s).

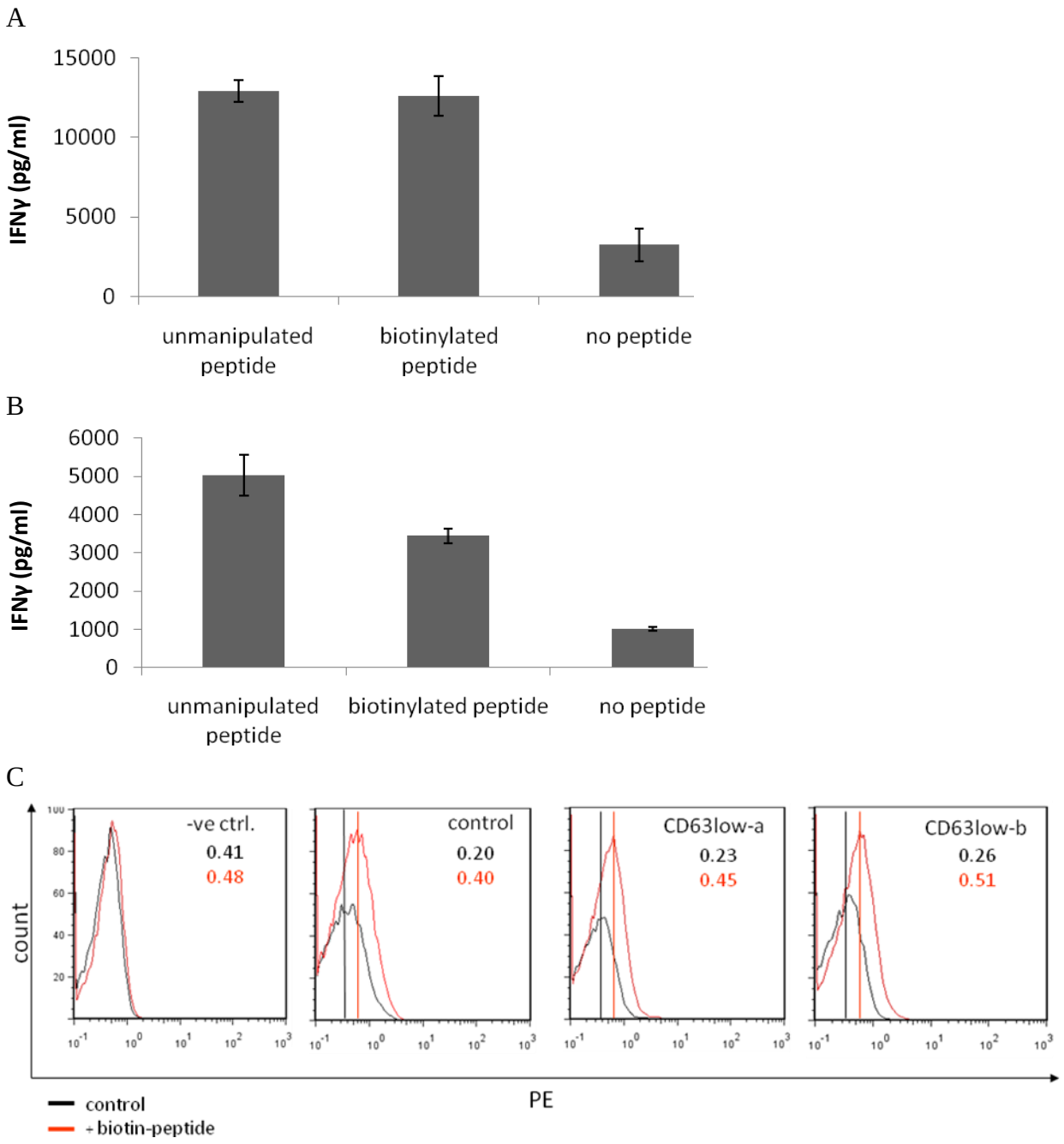


Figure 3.25: Analysis of peptide binding properties
A) Synthetic epitope peptides, unmodified or biotinylated, were tested for their ability to bind MHC II molecules. Peptides were loaded onto the LCL PER241 and analysed for their ability to increase T cell stimulation. The T cell stimulation was equal when loading LCL with non-labelled or biotinylated SDD. B) The same experiment was conducted using PRS. Here, the biotinylation caused a app. 30% reduction in T cell activation compared to unmanipulated PRS. Error bars show the mean +/- standard deviation of ELISA triplicates. C) Peptide binding assays were conducted pulsing control LCL, CD63low-a and CD63low-b LCLs with biotinylated peptides and subsequently staining the peptide-labelled LCLs using PE-Avidin. LCL incubated with PE-Avidin but not pulsed with any peptides served as a negative control (-ve control). No difference in the amount of peptides bound to the surface was observed. The black and red numbers represent the mean fluorescence intensity of control cells without peptides and cells loaded with biotin-peptide complexes, respectively.

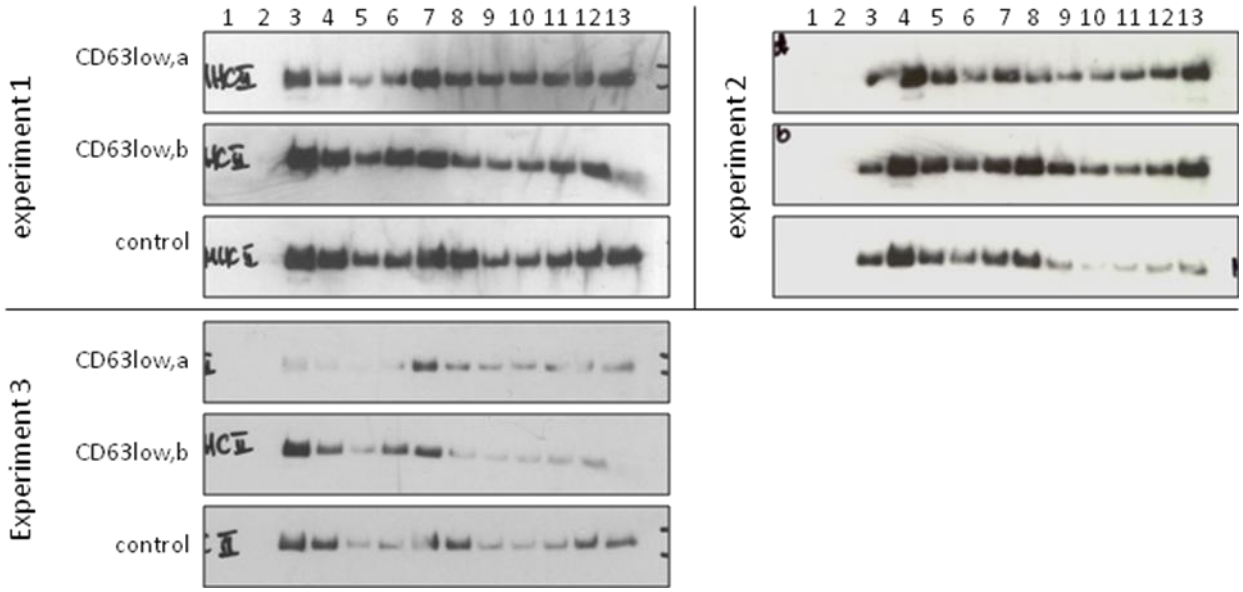
3.4.4 Compartmentalisation of MHC class II molecules

To test whether CD63 depletion altered the compartmentalisation and distribution of MHC class II molecules on the cell surface, lysates of CD63low-a, CD63low-b and control LCLs were separated by rate zonal centrifugation on a sucrose gradient and detected by Western blotting after protein separation in SDS-PAGE. Three separate experiments were conducted, homogenising the cells without detergent. Consistent differences could not be detected in these experiments (Fig 3.26A, experiment 1-3). MHC II was not detected in the fractions 1 and 2 whereas fraction 3 and 4 showed strong MHC II presence throughout the experiments. The low staining of these lanes in CD63low-a lysates in experiment 3 can be rather attributed to poor antibody binding to this particular membrane than a true difference in protein compartmentalisation. For the fractions 5 to 8, contradictive results were obtained. Whereas an almost even distribution in experiment 1 and 2 was observed, experiment 3 revealed a weaker staining of MHC II in these fractions. Also the result for fractions 9 to 12 were not consistent as we saw a comprehensive presence of MHC II in these fractions for experiment 1, whereas MHC II seems to be less abundant in the same fractions when conducted experiment 2 and 3. Lane 13 exhibited a strong signal for MHC II throughout all 4 experiments. Staining for MHC class I and CD82 did not show any alteration in distribution in any of the four experiments. Shown are representative results from experiment 1 for the control and the CD63low-a LCL. MHC I was not or only scarcely present in lane 1 and 2 but abundantly found in fractions 3 to 8. Fraction 9 to 12 exhibited a lower amount of MHC I with an increase seen in fraction 13. CD82 was not present in the fractions 1 and 2 but abundantly found in the fractions 2 to 9 showing peaks in fraction 4 and 8. A weaker signal was

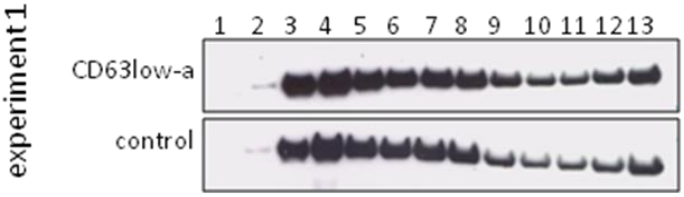
observed for CD82 in the fractions 10 to 13. Following fractionation experiments under lysis conditions using detergents focussed investigating the protein distribution of MHC II. The distribution of MHC II when the LCLs were lysed with Triton X-100 (here: CD63low-a, control LCL, experiment 4) was almost indistinguishable (Fig. 3.27B). An even distribution was seen for the CD63low LCL throughout the fractions 8 to 12 and a lower signal in fraction 13. In control LCL this pattern was repeated with the exception of a light shift towards fractions of lower density. A faint signal was seen in fraction 7 whereas hardly any MHC II was detectable in fraction 13. Lysing cells in Brij98 prior to performing the sucrose centrifugation gave a similar result (Fig. 3.26C, experiment 5). Here, the most noticeable difference could be seen between the two CD63 knockdowns whereas the MHC II molecules of the control LCL were distributed in a pattern similar to those of the CD63low-a LCL (Fig. 3.26C). No protein was detected in fraction 1, in fraction 2 MHC II was present in CD63low-a and control LCL but not in CD63low-b lysates. Heavy fractions combine fraction 2 and 3 in all three cell lines. Light fractions follow from fraction 5 to fraction 7 and medium fractions are remaining from fraction 8 to fraction 13. Additionally, the distribution of CD19 was analysed for all three LCLs. No apparent change under each of those three lysing conditions could be detected (data not shown).

A

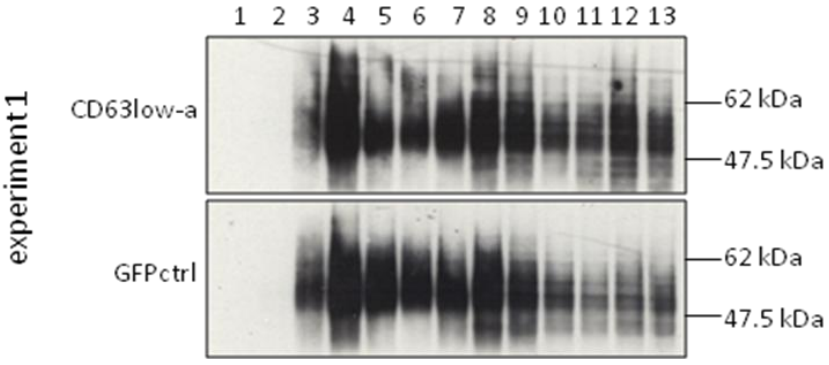
no detergent, MHC II



no detergent, MHC I

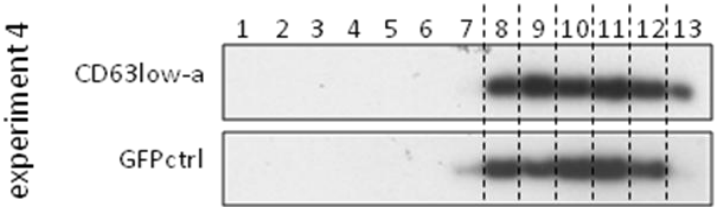


no detergent, CD82



B

Triton X-100, MHC II



C

Brij 98, MHC II

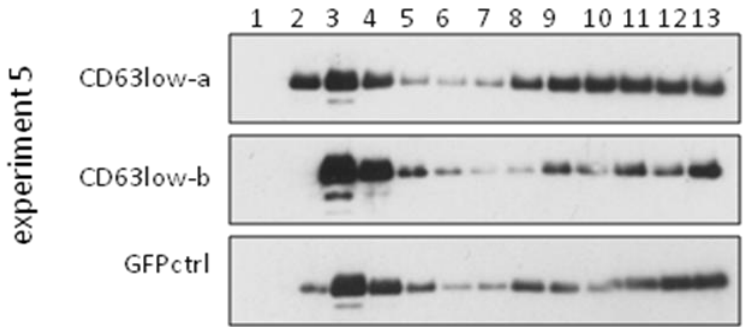


Figure 3.26: Distribution of selected membrane proteins
A) Three fractionation experiments were conducted after the control or CD63low LCLs were homogenised without detergent. The compartmentalisation of relevant proteins was then analysed by Western blotting. While MHC II was identified in different fractions in one experiment when control and CD63low LCLs were analysed, this was not observed in four other repeat experiments. Analysing the same cell lysates for compartmentalisation of MHC I and CD82 did not show any difference between CD63low and control LCL either (shown is one representative blot). B) MHC II does also appear in the same fractions when lysing CD63low-a and control LCL in Triton X-100 (1%). C) A difference in MHC II distribution between CD63low-a and -b can be seen in the one experiment in which cells were lysed using Brij 98. However, comparing the pattern for the control LCL to the pattern of both CD63low LCLs does not suggest a CD63 knockdown-driven alteration in MHC II compartmentalisation.

3.4.5 Analysis of protein compartmentalisation by chemical cross-linking

In addition to rate zonal density gradient centrifugation, protein compartmentalisation can be analysed by protein cross linking using DSP (Dithiobis (succinimidyl propionate). Chemical crosslinking is used to determine proximal partners of a given molecule/protein. DSP is a homobifunctional, thiol-cleavable and membrane-permeable protein crosslinker. It contains an amine-reactive *N*-hydroxysuccinimide (NHS) ester which reacts with lysine residues in protein side chains and the N-terminus of each polypeptide. DSP cross-linked proteins can be cleaved in the presence of reducing agents. LCLs were treated with DSP prior to lysis in TritonX-100 (1%). No difference in the patterns of crosslinked complexes was observed when

CD63low and control cells were treated with DSP (Fig: 3.27) Since staining for MHC II after cross linking resulted in equal bands for both LCLs, the interaction of MHC II with proximal partners seems not to be affected by the knockdown of CD63. However, it was noted that there was a slight difference in the mobility of MHC II molecules when non-crosslinked lysates separated by SDS-PAGE on a non-reducing gel. Such a difference in size could reflect a difference in post-translational modification, for example the degree of glycosylation. However, this size difference was not detected in repeated experiments suggesting it represents a subtle transient difference between the two cultures rather than a mechanism responsible for differential T cell recognition.

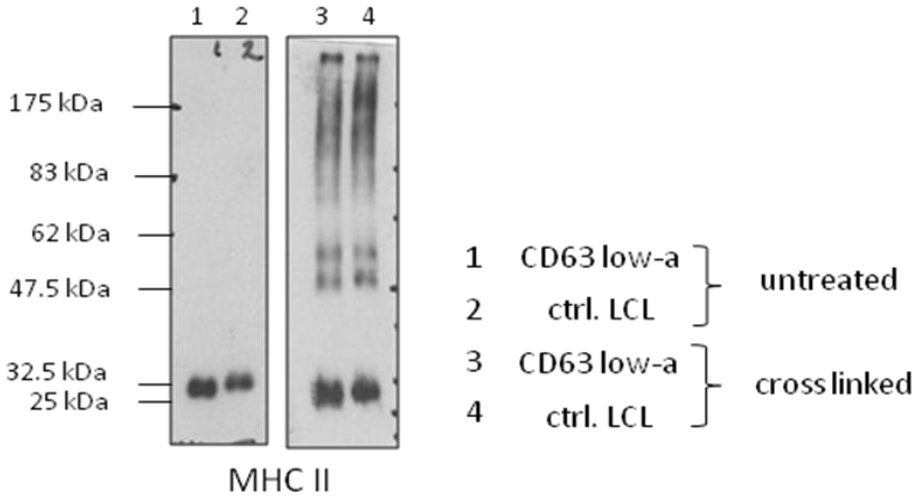


Figure 3.27: MHC II multimer pattern after protein cross-linking
MHC class II molecules in LCLs, either untreated (lane 1 and 2) or treated with the cross-linking agent DSP (lane 3 and 4) were analysed by SDS-PAGE on a non-reducing gel using the α-MHC class II antibody DA6-14. An equal pattern of MHC II for both LCLs was seen after protein cross-linking (lanes 3 and 4) which suggests equal protein interaction partners for MHC class II in both LCLs. In contrast, analysing lysates from cells not treated with DSP revealed a small difference in the molecular weight for MHC II from control and CD63low LCLs (lanes 1 and 2). This difference was not seen in 3 subsequent experiments.

3.4.6 Immunological synapse (IS) formation between LCLs and CD4+ T cells

Since no biochemical difference in MHC II molecules in control and CD63low LCLs were observed, the next step was to explore whether there was any difference in the interaction between MHC II on the surface of the LCLs with the TCR on the CD4+ T cell clone. When these cells interact, MHC II complexes are clustered within the immunological synapse. An immunological synapse (or immune synapse) is the interface between an APC and a lymphocyte which coordinates a number of signalling and adhesion molecules towards the contact area. This well structured assembly of molecules, also called supra-molecular adhesion complex (SMAC), facilitates an effective T cell activation (Cemerski and Shaw 2006;Kupfer and Kupfer 2003).

Control and CD63low LCLs were co-incubated with antigen specific CD4+ T cell clones and then fixed, allowing the cell conjugates to be studied by immunofluorescence microscopy. LCLs were additionally stained with the cytoplasmic, membrane permeant green fluourescent dye BCEFC (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) allowing LCLs to be differentiated from T cells when analysing conjugates. Staining was performed using monoclonal antibodies specific for MHC class II, the adhesion protein ICAM-1, which has been shown to be localised in the synapse (Almeida and Davis 2006;Batista and Saito 2010;Goldstein et al. 2000;Jo et al. 2010) or the EBV membrane protein LMP-1, which has also been suggested to be recruited to the synapse (Dr. M. Pegtel, personal communication). A number of different permeabilisation protocols were tried including TritonX-100 (Fig. 3.28), Brij98 and CHAPS (data not shown). The experiments were also performed without permeabilisation. For each condition 10 to

12 images were taken and analysed by confocal microscopy. Images were taken from cell conjugates which demonstrate a clear intercellular interaction and a pronounced straining of the relevant protein. This enabled us to compare the exact distribution of the stained proteins between control or CD63low LCLs conjugated with T cells. MHC II was present on the outer membrane and internal vesicles which is seen by a mainly cortical staining. A clear localisation towards the intercellular contact area can be observed. ICAM1 appears to exhibit a more dispersed and even distribution with presence on the surface as well as in the cytoplasm. The localisation of LMP-1 however resembles MHC II's with a cortical distribution and presence on inner vesicles. However, a marked difference in synapse formation or protein distribution of these three proteins could not be detected between control and CD63low LCLs.

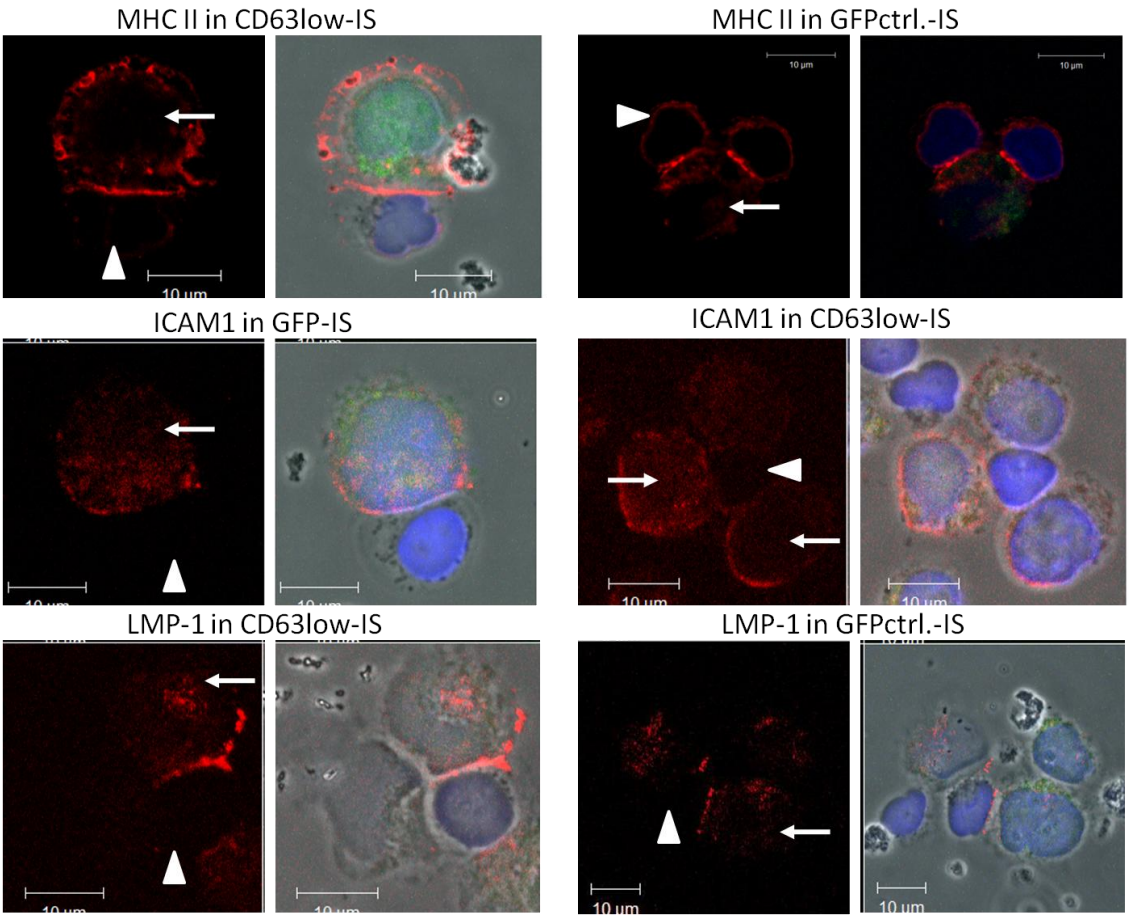


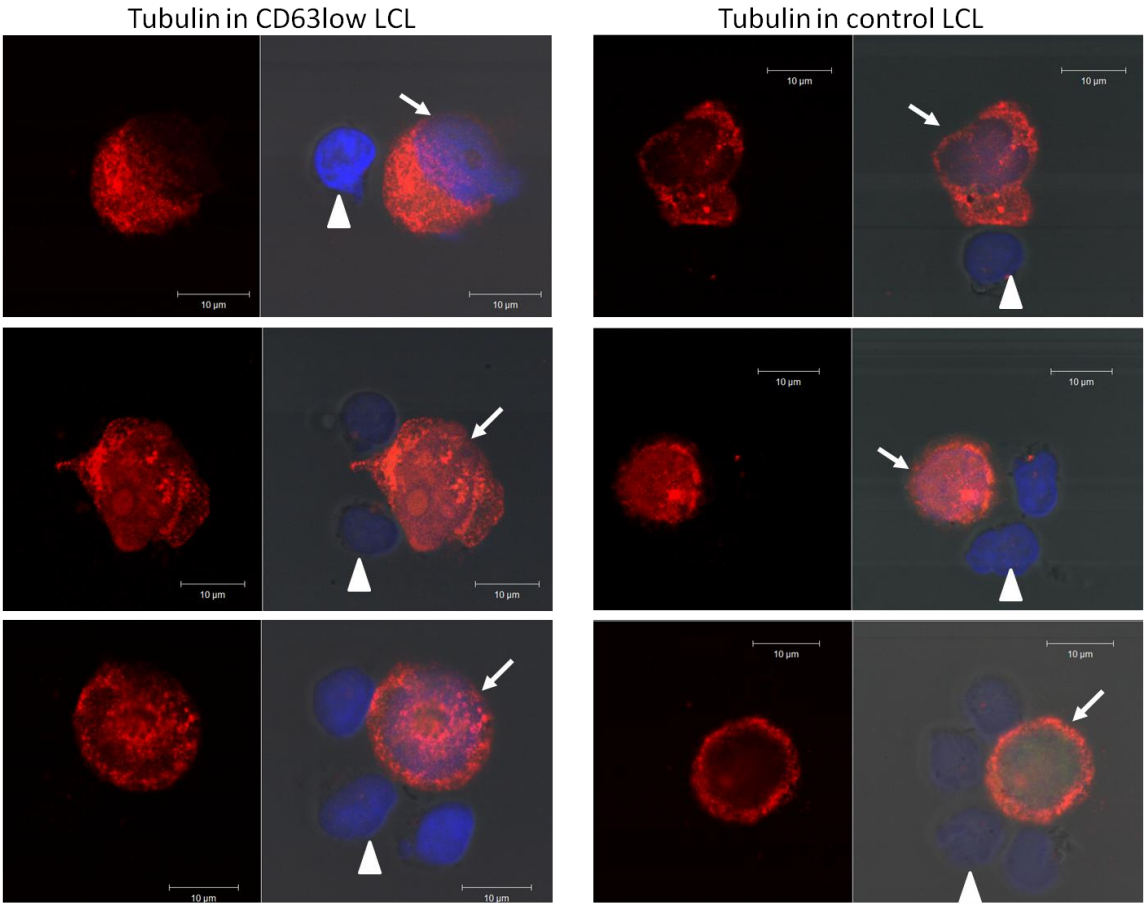
Figure 3.28: Analysis of the LCL-T cell IS after permeabilisation with Triton X-100
Triton X-100 treated control or CD63low-a LCLs were labelled with BCEFC (2_,7_-Bis(2-carboxyethyl)-5-carboxyfluorescein; green). As this weak green staining is difficult to see in the printed images these cells are identified with an arrow. LCLs were conjugated with antigen specific CD4+ T cell clones (arrowhead) and immunostained for ICAM-1, LMP-1 or MHC class II (all red stained). Cellular DNA was stained with Hoechst 33258 (blue). No apparent difference in protein recruitment to the IS could be seen in CD63low cells for any of the analysed proteins. This also applies to staining using no detergent or using Brij 98 or CHAPS to permeabilise cells (data not shown).

3.4.7 Microfilaments and microtubules in LCL-CD4+ T cell conjugates

While membrane proteins such as MHC II or ICAM-1 play a crucial role in the direct intercellular contact between APC and T cell, cytoskeletal proteins are also involved in the formation and maintenance of immunological synapses (Billadeau and Burkhardt 2006;Gomez et al. 2005;Rodriguez-Fernandez et al. 2010). Here, we investigated the distribution and organisation of the cytoskeletal proteins tubulin and

actin. CD63low-a and control LCLs were co-incubated with the CD4+ T cell clone c38 for 30min at 37ºC to allow the cells to form conjugates. The conjugates were resuspended in PBS and cytospun onto slides prior to fixation with paraformaldehyde. After permeabilisation, cells were stained with an anti-tubulin antibody (see 3.4.6) or with phalloidin which binds actin filaments. For both LCLs a pronounced localisation of tubulin in the microtubule organisation centre (MTOC) facing towards the contact area between the LCL and the T cell and even at the immune synapse itself could be observed. The overall tubulin organisation in CD63low and control LCLs appeared indistinguishable (Fig. 3.29A). Actin also shows polarisation towards the immune synapse for both the CD63low-T cell and the control LCL-T cell conjugates. Likewise tubulin, polarisation of actin fibres shows no difference in the CD63low LCL conjugated to the T cell clone compared to the control LCL (Fig 3.29B). Using confocal microscopy imaging to produce optical slices through the cell cytoplasm allowed more readily the analysis of actin polarisation and confirmed previous data (data not shown). In total three separate staining experiments were conducted and 10-12 images taken from each experiment.

A



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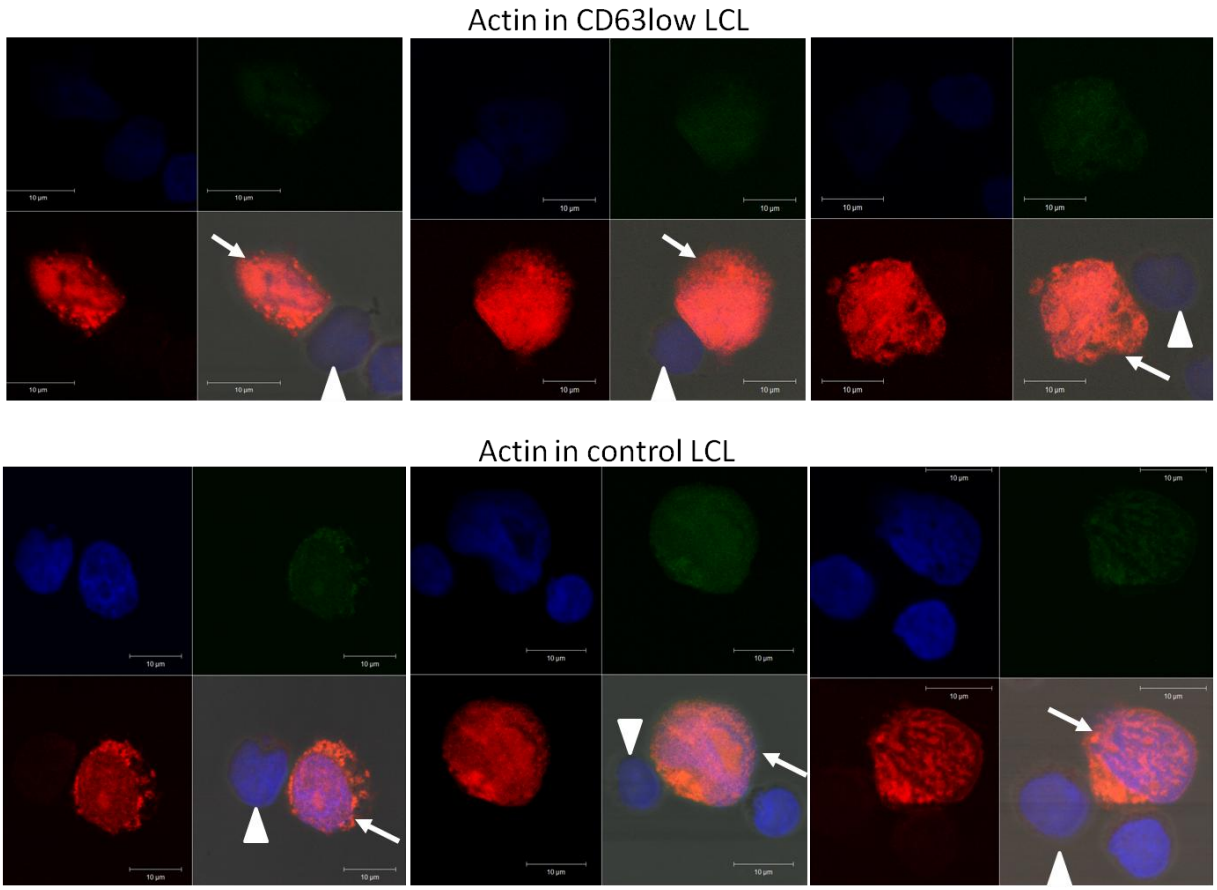

Figure 3.29: Analysis of cytoskeletal protein in the CD63low LCL conjugated to the CD4+ T cell clone c39
Images of three representative cells from the CD63low and the control LCLs are shown. For each cell an image was taken in the blue (top left, shown in B), green (top right, shown in B) and red (bottom-left) channel as well as in transmitted light (not shown). A merged image is shown on the bottom right of each panel. Staining was conducted as for Fig. 3.27. A) CD63low-a and control LCL (arrow bar) were co-incubated with the CD4+ T cell clone c38 (arrowhead) to allow the formation of cell conjugates. Subsequent staining of these conjugates using antibodies binding tubulin did not show any difference in the distribution of tubulin in either the CD63low or the control LCL whilst conjugated to a T cell. B) Similarly, actin distribution seems to be somewhat located towards the IS but does not show pronounced differences between control and CD63low LCL.

3.4.8 Effect of CD63 depletion on co-stimulation activity of LCLs

Another possibility is that the enhanced CD4+ T cell activation observed for CD63low cells is due to a difference in co-stimulation of the T cell by the LCL; that is, operating independently of the synapse. To test this, another CD4+ T cell clone

(c4) was used which is specific for an EBV EBNA1 epitope restricted through HLA-DR1. As the control and CD63low LCL, derived from the same parental line, lack HLA-DR1, they cannot activate these T cells through MHC II-TCR interaction. Any differences observed using the LCLs would therefore be due only to differences in co-stimulatory activity. In the same experiment it is still necessary to activate the clone's TCR in MHC II independent way to allow co-stimulation to be studied. In order to activate the T cell clone's TCR, different concentrations of α-CD3 antibody were added to the T cells in the presence or absence of what are now HLA-mismatched LCLs (either control or CD63low). In the absence of α-CD3, no T cell activity was observed as expected (Fig. 3.30). In the presence of α-CD3, the T cell clone was activated, producing IFNγ. However, no differences were observed in levels of IFNγ in the absence or presence of the LCLs. Even though different amounts of α-CD3 antibody were tested, it has to be taken into consideration that T cell clones require little in the way of co-stimulation to be activated and subtle differences in co-stimulation may not be detected in this experiment. A difference in co-stimulation between the control and CD63low cells can therefore not be fully excluded by this experiment.

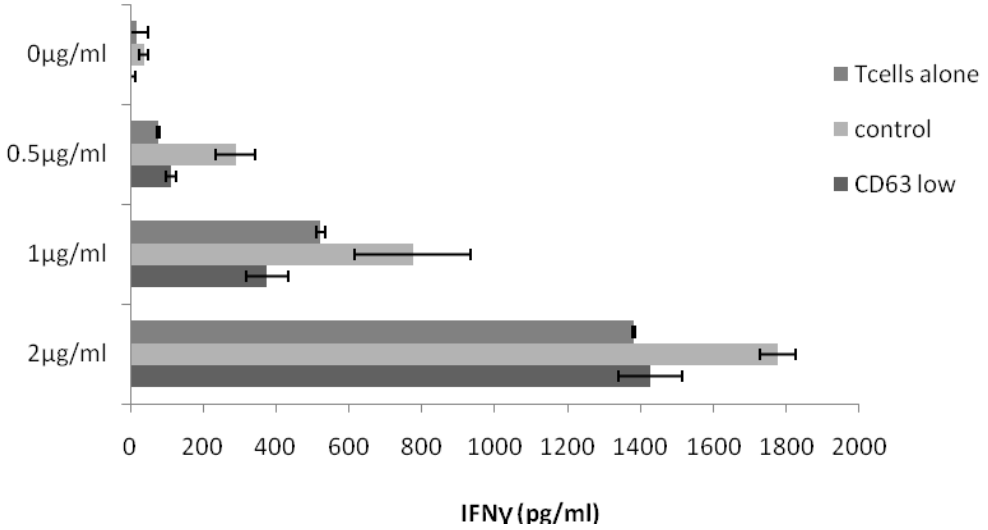

Figure 3.30: Co-stimulation assay using CD4+ T cell clones
LCL co-stimulatory activity was assessed by coating Maxisorp 96-well plates (Nunc) with α-CD3 antibody to activate T cells and adding CD63low-a and control LCLs that are HLA-mismatched to the particular T cell clone used for this experiment. The antibody concentrations used for coating were 0, 0.5, 1 or 2µg/ml. No difference in co-stimulatory activity was detected between CD63low and control LCLs. The graph shows a representative experiment out of two, the error bars indicate the mean +/- standard deviation of ELISA triplicate wells.

3.4.9 Knock down of tetraspanins in HeLa CIITA cells

CD63 has been suggested to interact with the EBV LMP-1 protein, localising LMP-1 to the immunological synapse (Dr. M. Pegtel, personal communication). LMP-1 has been reported to have an immunosuppressive effect (Middeldorp and Pegtel 2008b). A model to explain the enhanced T cell recognition exhibited by CD63low cells could be that depletion of CD63 causes less LMP-1 to be present at the synapse, which in turn results in less LMP-1-mediated suppression of the T cells. While I had observed no difference in LMP-1 distribution following CD63 depletion in LCLs (Fig. 3.29) it was important to test this hypothesis by asking if CD63 depletion could enhance the CD4+ T cell recognition of a cell lacking LMP-1.

HeLa cells express MHC class II when transfected with the vector pXPG which express the MHC II transactivator CIITA. This cell line is commonly used for studying MHC II, for example MHC II trafficking, in a background of non-professional APCs (Walseng et al. 2008). Since HeLa cells are HLA-DQ5 positive they could be used, along with the CD4+ T cell clone c38 (DQ5-restricted) already used on the LCLs, to investigate the influence of tetraspanins on antigen presentation in the background of a cell lacking EBV.

HeLa CIITA cells were transduced with lentivirus encoding the two shRNA constructs used to target CD63 in LCLs (section 3.1.4.2, 3.1.4.4). As expected a large reduction in the expression level of CD63 was achieved with both shRNAs. Cells transduced with CD63low-a shRNA maintained low levels of CD63 (Fig. 3.31A, B). Cells transduced with the CD63low-b shRNA, however, did not maintain knockdown and were not used in subsequent experiments. HeLa CIITA cells transduced with a lentivirus not containing any shRNA construct served as a negative control cell line. The expression of other members of the tetraspanin family was tested by flow cytometry for these knocked down cells. The depletion of CD63 did not cause any difference in the level of other tetraspanins (data not shown). This experiment was particularly important as the expression of MHC II was not altered by the CD63 shRNA lentivirus.

In addition to CD63, we also targeted CD81 and CD151 with shRNAs cloned into lentiviruses. We used the same CD81 lentivirus that had previously been shown to function in HeLa cells (Fig. 3.4) but that had failed to knock down their respective gene targets in LCLs (Fig. 3.5). The CD151 shRNA lentivirus was kindly provided by Dr. F. Berditchevski and has been shown to work in other cell lines (Baldwin et al. 2008). While CD81 and CD151 levels were reduced, in contrast to the CD63 shRNA

lentivirus, we observed a somewhat altered expression of MHC II for these two constructs. MHC II levels were increased slightly in the cell line transduced with the CD81 shRNA lentivirus. In cells transduced with the CD151 lentivirus two populations of cells, one with low the other one with higher MHC II, were observed (Fig. 3.31C). It is possible; therefore, that CD81 and CD151 affect MHC II, a possible avenue of research for future work.

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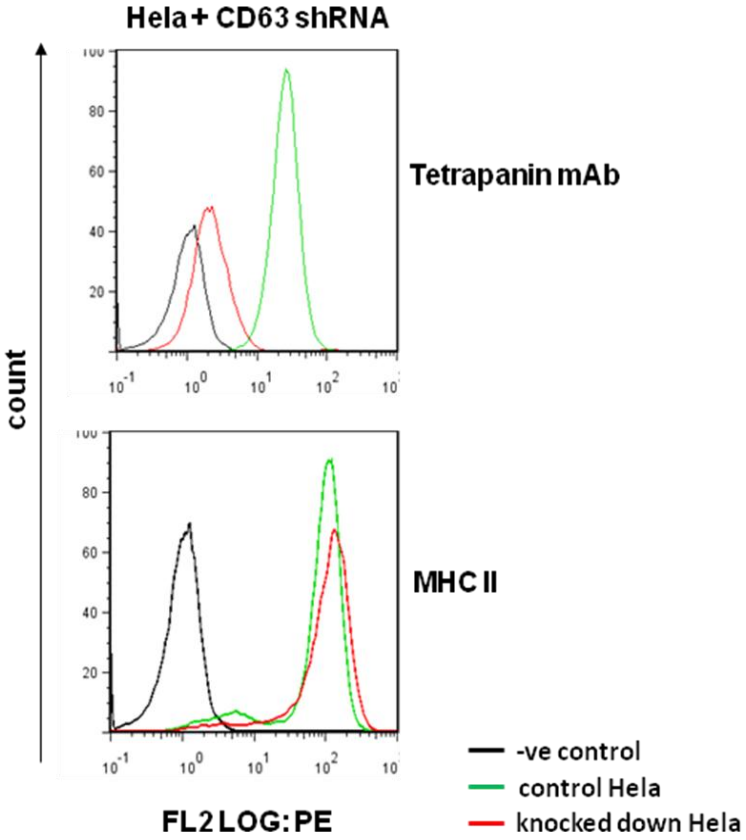

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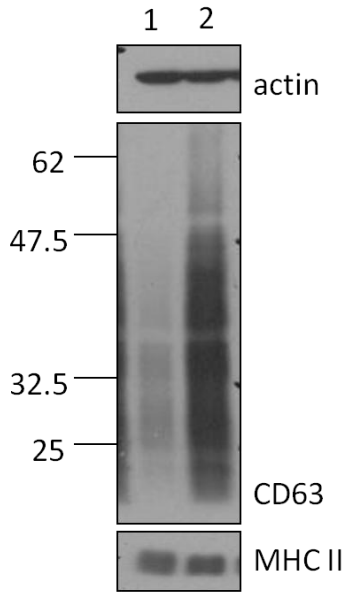

1. Hela CIITA CD63low,a
2. Hela CIITA control

C

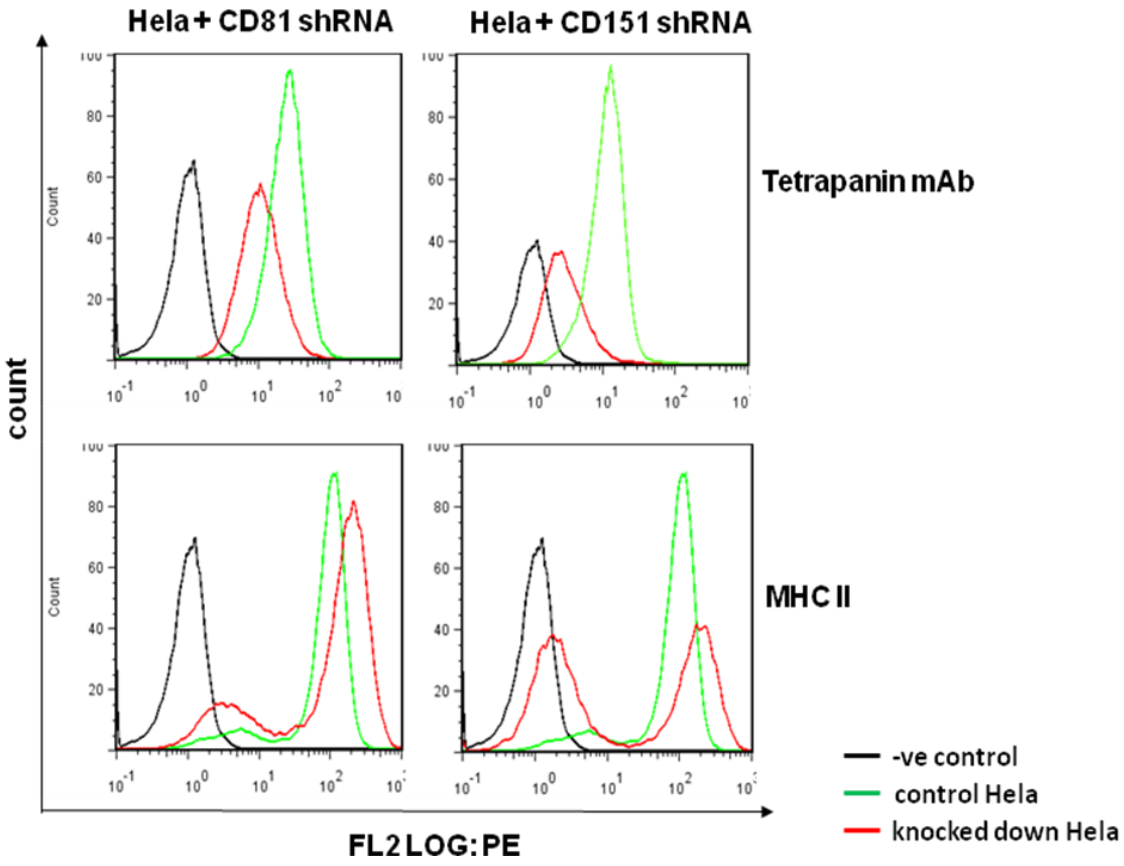

Figure 3.31: Knock down of CD63, CD81, CD151 in HeLa CIITA
A) Expression of tetraspanins CD63 was reduced in HeLa CIITA cells transduced by lentiviruses targeting the respective protein. MHC II levels were unaltered in the CD63low shRNA transduced cells. B) The knock down of CD63 and unchanged expression of MHC II expression upon CD63 depletion was confirmed by Western blotting. Actin serves as a loading control. C) Expression of tetraspanins CD81 or CD151 were also reduced using respective shRNA constructs cloned into lentivirus. MHC II was altered in these two lines.

3.4.10 CD4+ T cell activation by HeLa CIITA cells

Following generation of the CD63-, CD81- and CD151-low HeLa CIITA lines (Fig. 3.31), these cells together with the control HeLa CIITA cell line were tested in T cell assays using CD4+ T cell clone c38.

As expected, due to their lack of EBV antigen expression, HLA-DQ5 expressing HeLa CIITA cells by themselves were not recognised by CD4+ T cell clone c38 However, tetraspanin knockdown cells and the control line did stimulate the clone

when exogenously loaded with the antigenic EBV peptide “SDD” (Fig. 3.32A). The key result is shown in Fig 3.32B. Importantly, in accordance with the results seen for LCLs, the CD63low HeLa cell line was much more efficient at stimulating the CD4+ T cell clone than the control cell line. Note that the two lines were pulsed in parallel with equal quantities of epitope peptide before their use as targets in the assay and the same result was seen in six independent experiments. This important result, showing that enhanced CD4+ T cell recognition occurs in the complete absence of LMP-1, strongly suggests an LMP-1 independent mechanism to be responsible for the phenotype observed in LCLs. Furthermore, HeLa cells, unlike LCLs, do not express the co-stimulatory molecule CD86 nor the adhesion molecule ICAM-1 (data not shown) suggesting these molecules are also not involved in the enhanced stimulation of CD4+ T cells. Interestingly, both CD81 and CD151 knockdowns also increased CD4+ T cell stimulation (Fig. 3.32C), albeit not to the same degree as the CD63 knockdown. Since MHC II levels were altered in both the CD81 and CD151 knockdown lines, some caution must be exercised in interpreting these data. After a few weeks of cell-culture upon virally infecting HeLa CIITA cells, we noticed the divergence into two populations in terms of MHC II expression. For the CD81 knockdown we see the majority of the transfected cells expressing a somewhat higher level of MHC II compared to the control-HeLa cell line, whereas a small population exhibits a substantially reduced expression level. In the case of shRNA-CD151 transfection, we obtained two populations of similar size showing comparable MHC II expression levels as seen for CD81 low HeLa cells. However, these results indicate that tetraspanins other than CD63 could also affect CD4+ T cell activation.

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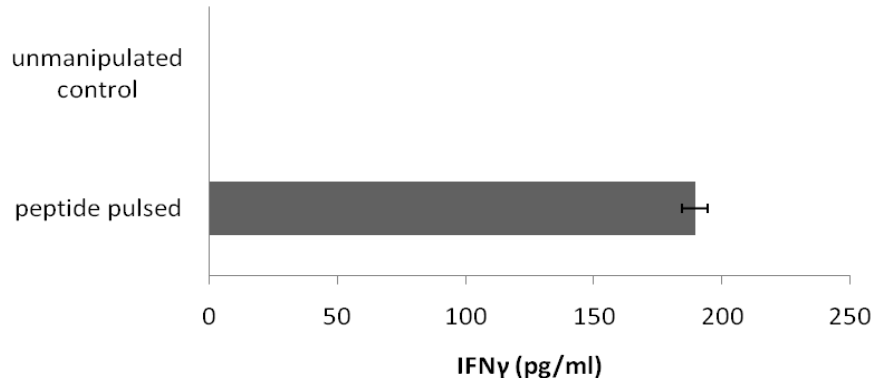

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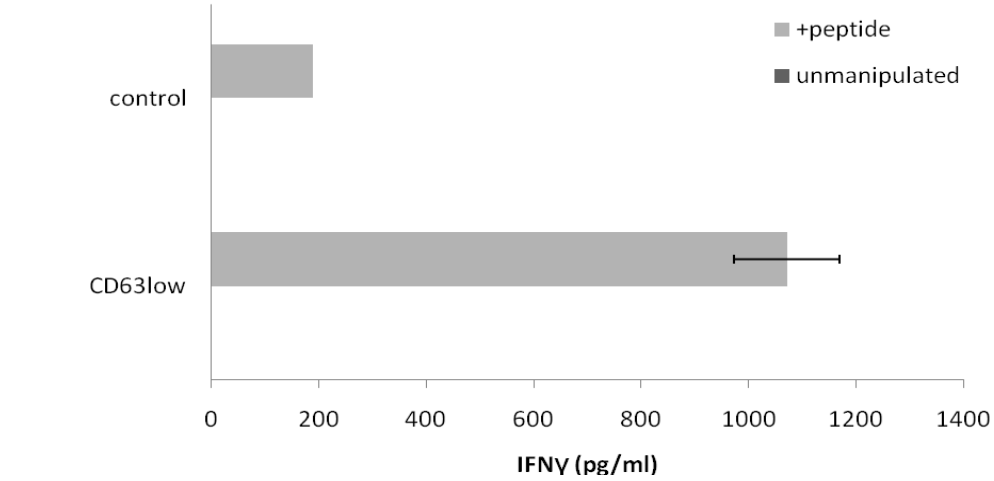

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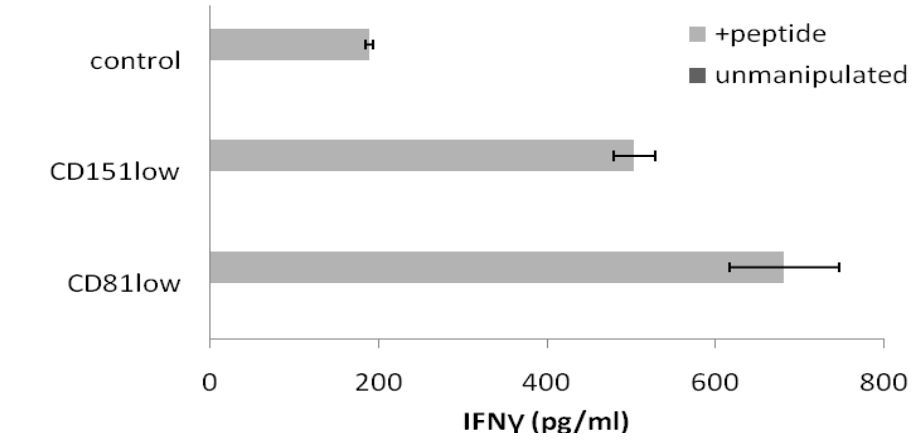

Figure 3.32: CD4+ T cell activation by HeLa CIITA cells
A) HeLa CIITA cells were analysed via IFNγ ELISA for their ability to stimulate the CD4+ T cell clone c38. When exogenously loaded with antigenic peptide ($1x10^{-7}$M), HeLa CIITA cell were recognised by the T cell clone whereas no T cell stimulation was observed for unloaded HeLa cells. B) The control and CD63-deficient HeLa CIITA-cells were peptide pulsed ($1x10^{-7}$ M) and washed before use as targets in the assay as described in A. A much higher level of T cell stimulation was observed with the CD63 knockdown HeLa cell line compared to the control. C) Depleting CD81 and CD151 also increased the level of T cell stimulation by HeLa CIITA cells. The shown data are from one representative experiment out of six. The error bars show the mean +/- standard deviation of triplicate ELISA wells.

3.4.11 Reconsideration of the effect of exosomes and their impact on CD4+ T cell stimulation

The above result clearly shows that CD63 depletion yields a robust increase in the ability of MHC II-positive cells to activate CD4+ T cells. Given that exosomes bear a very similar collection of membrane molecules, it was surprising that in earlier experiments there was no difference in CD4+ T cell activation when T cells were exposed to exosomes purified from control and CD63low LCLs. This lack of differential T cell activation exhibited by exosomes formed the basis of a final series of experiments seeking to discover the mechanism responsible for the observations made thus far.

3.4.11.1 Quantification of exosome secretion by control and CD63low LCLs

In all previous experiments with exosomes same quantities of exosomes (as determined by Bradford assay) were added to the T cells. For some of the experiments exosomes were prepared from the cells which were seeded exactly at the same density, and cells were recounted at the end of the 48 hours culture. The control and both CD63-low LCLs grew equivalently and similar cell numbers were recorded at the end of the culture period. Exosomes were purified in parallel from the three supernatants, resuspended in the same volume of PBS and quantitated by Bradford assay. The total amount of exosomes purified from a single experiment is shown in Fig. 3.33A. A larger amount of exosomes were prepared from the CD63-deficient LCLs. In total this experiment was repeated six times and the combined results, standardised per million cells (Fig. 3.33B), clearly shows that both the CD63low

LCLs secreted 50-70% more exosomes than the control. To confirm that the Bradford assay accurately reflected levels of exosomes, Western blotting was conducted running 10% of the exosome preparations made from the CD63low-a and control cell lines onto a reducing gel (Fig. 3.33C). Levels of both actin, which is present in the lumen of exosomes and MHC II, present on the surface of exosomes, were higher confirming the Bradford assay results. Western blotting was not performed on exosomes from CD63low-b cells.

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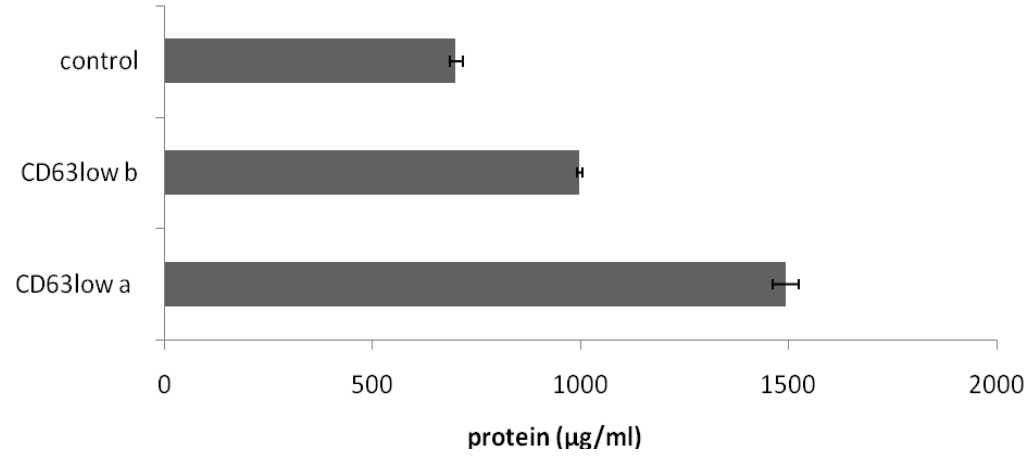

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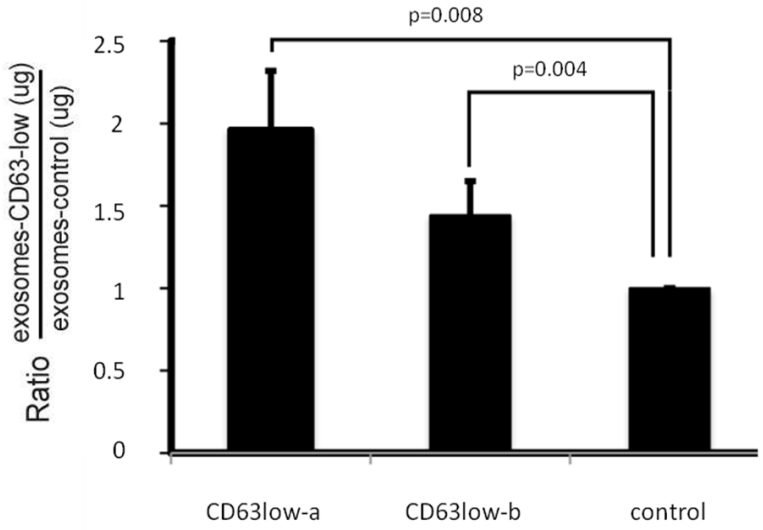

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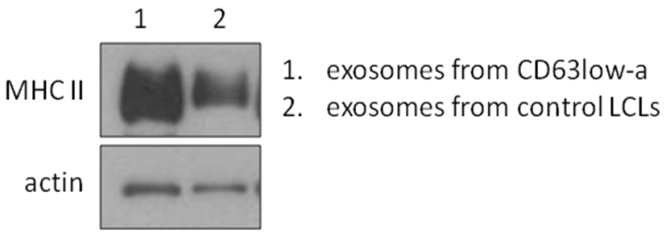

Figure 3.33: Quantification of exosome release by LCLs
A) Exosomes production was quantified by determining the protein concentration of the final exosome product. Both CD63low LCLs yielded more exosomes. The error bars represent the mean +/- standard deviation of the triplicate Bradford assay wells. B) The average amount of exosome secretion was determined by standardising the amount of exosomal protein produced by 1 x 10^6 cells (counted after the 48 hours culture) for three independent experiments. Approximately 50-80% more exosomes were secreted by CD63low LCLs compared to the control LCL. The error bars show the mean +/- standard deviation of the amount of exosomes per million cells counted at the end of the 48 hour culture period. $p<0.01$; ns=not significant, analysed by unpaired Student's t test. C) The increased exosome secretion by CD63low-a LCL was confirmed by Western blotting. For each lane the same proportion (~10%) of the exosomal sample were loaded onto the gel.

As exosomes are MHC II positive and themselves able to activate CD4+ T cells (see section 3.2.2.2), the observation that CD63low LCLs secrete larger quantities of exosomes suggested a number of possible mechanisms that could account for these cells' enhanced ability to activate CD4+ T cells. These mechanisms are explored below.

3.4.11.2 Exosome uptake by LCLs and CD4+ T cells

One hypothesis suggested by the previous observation is that the increased amount of exosomes, released by CD63low LCLs, indicates an increased level of exosome processing and trafficking accompanied by an increased re-uptake of autologous exosomes. In order to study the cellular uptake of exosomes, LCLs were stained with DIL (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) prior to incubating the cells for 48 hours and purifying exosomes from their supernatant. DIL is a hydrophobic and lipophilic membrane integrating dye which stains the outer cell membrane as well as the membrane of subsequently generated endosomes, MVBs and exosomes. Unstained LCLs, were incubated for 15min with a mix of DIL-labelled exosomes, derived from the CD63low-a and control LCL, and analysed by confocal microscopy (Fig. 3.34A). No apparent difference in exosome binding or uptake was seen comparing both LCLs. However, in this experiment LCLs were co-incubated not with a pure autologous exosome preparation but a mixed population of exosomes derived from both control and CD63low LCL. The binding properties of CD63low exosomes onto CD63low LCLs would need to be investigated in future work. Furthermore, to assure comparability of the results obtained for both cell lines, the amount of exosomes given to LCLs was quantified and equalised prior to co-

incubation. Note that this does not reflect the conditions seen under unmanipulated assays where CD63low LCLs were proven to release a higher quantity of exosomes into the medium than control LCLs.

In the same experimental set-up CD4+ T cells were tested for their ability to bind these DIL-labelled exosomes. Two CD4+ T cell clones, one HLA-DQ5-restricted (c38) and the other not recognising any of the HLA-molecules present on these exosomes (c4, DR1-restricted) were co-incubated for 15min with exosomes either derived from CD63low or from control LCLs. Since optical slices were not taken from these samples a strict distinction between surface-bound and internalised exosomes cannot be made. But both CD4+ T cell clones appear to interact with exosomes derived from CD63low or from control LCLs equivalently (Fig. 3.34B). Moreover, the CD4+ T cell clone c38 whose TCR is capable of recognising HLA-DQ5 molecules loaded with cognate peptide that are present on these exosomes did not appear to interact more strongly with exosomes than the other T cell clone which the exosomes are mismatched to. This result agrees with the observation that recruitment of exosomes by T cells is dependent on the interaction of LFA-1 and ICAM-1 rather than MHC and the TCR (Nolte-'t Hoen et al. 2009a;Segura et al. 2007).

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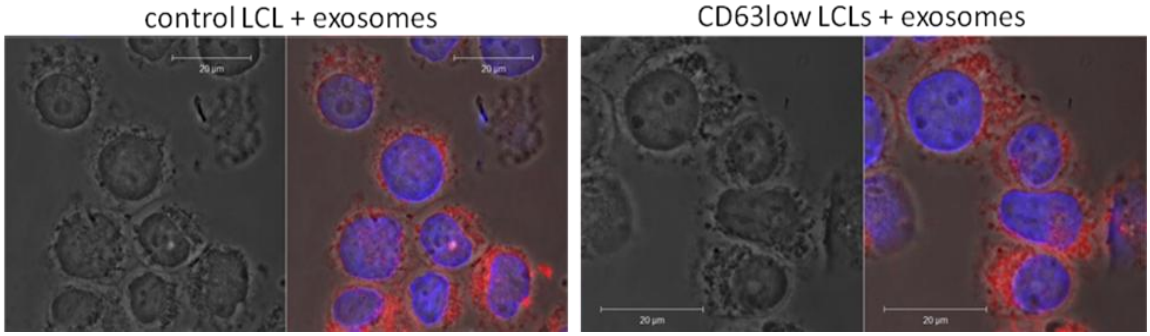

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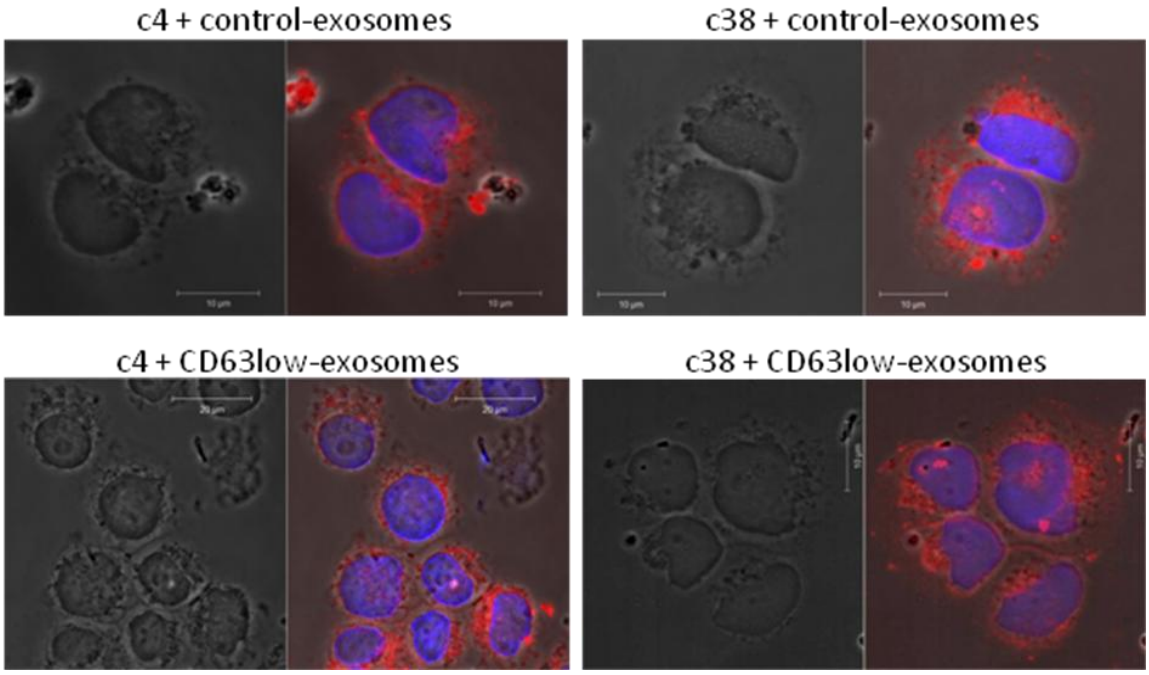

Figure 3.34: Interaction of exosomes with LCLs and CD4+ T cell clones
A) CD63low-a and control LCLs were co-incubated with a mixture of DIL-labelled exosomes (red fluorescent) purified from both CD63low-a and control LCL supernatants. Both LCLs appear to interact equivalently with these exosomes. The cellular DNA was stained using Hoechst 33342. B) CD4+ T cell clones were separately co-incubated with exosomes derived either from CD63low-a or control LCL. Exosomes derived from any of these LCLs interacted equivalently with the HLA-matched and the HLA-mismatched T cell clones.

3.4.11.3 Analysis of rebinding properties of exosomes to their mother cell

To address the possibility that increased exosome secretion could subsequently lead to a higher amount of autologous exosomes bound to the LCL's surface, flow cytometry experiments were undertaken using a slightly modified procedure. By avoiding cell

washing we allowed any weakly bound exosomes to remain bound to the LCL surface. Cells were fixed with PFA to immobilise exosomes onto the cell surface prior to staining with a specific antibody. Knowing that control and CD63low LCLs show an equal surface level of MHC II under conditions where surface bound exosomes are likely to be washed off the cell (Fig. 3.6), this modified procedure would reveal whether CD63low LCLs are binding back their own exosomes to a higher extent (Fig. 3.35A) since they were shown to secrete more exosomes compared to the control LCL (Fig. 3.33). No difference in the level of MHC II expression could be seen between CD63low and control LCLs (Fig. 3.35B) suggesting that the increased secretion of exosomes by CD63low LCLs does not lead to an increased amount of exosomes binding back to the LCLs.

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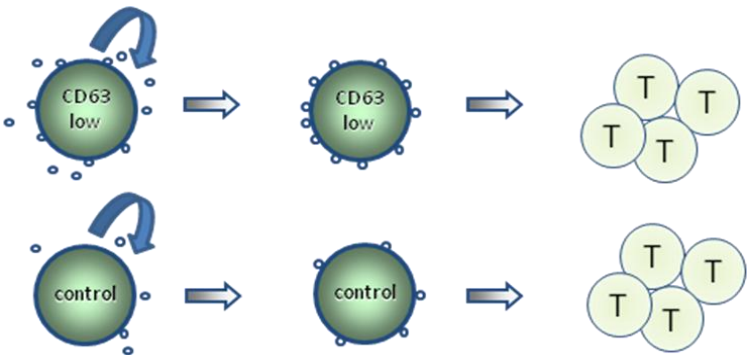

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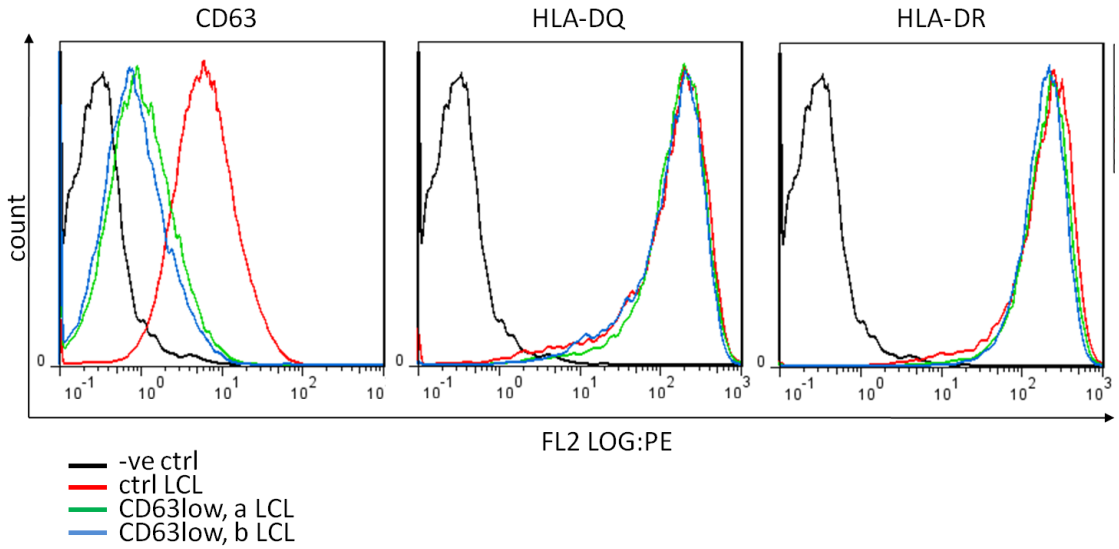

Figure 3.35: Analysis of exosomes binding to the surface of CD63low LCLs
A) The increased release of exosomes by CD63low LCLs could lead to a higher amount of autologous exosomes bound back to their mother cell's surface. B) Control and CD63low LCLs were gently washed only once and instantly fixed with PFA to ensure that any exosomes stuck to the LCL's surface remain bound. To measure any difference in cell surface exosomes, flow cytometry detecting total MHC II was performed. The expression of either HLA-DR or HLA-DQ was indistinguishable between both cell lines suggesting that there is no difference in the capacity of control and CD63low LCLs to bind their autologous exosomes, despite the larger production of exosomes seen for the latter.

3.4.11.4 Influence of increased exosome secretion upon CD4+ T cell activation

Once released into the extracellular medium, LCL-derived exosomes are capable of stimulating T cells (see 3.2.2.1). Moreover, CD63low exosomes have been shown to exert a similar CD4+ T cells stimulation as control exosomes (see 3.2.3.2). Therefore,

an increased amount of exosomes released from CD63low LCLs could be directly responsible for the increased T cell activation exhibited by these cells. To test this hypothesis, T cell assays were performed as described in section 3.3.1 by co-incubating both control and the CD63low-a LCL with the CD4+ T cell clones c38 and c93, respectively. This time however, previously purified exosomes derived from either the control or the CD63low-a LCL were added to the cell co-cultures. The rationale of this experiment was that if the enhanced recognition of CD63low LCLs was due to their increased release and binding of exosomes, an excess of exosomes would saturate the system causing the degree of CD4+ T cell stimulation by the control and CD63low cells to become equivalent.

The previously shown effect of increased antigen-specific T cell stimulation by the CD63low-a LCL compared to control LCL was first verified (Fig. 3.36A). Adding exosomes to LCLs before co-incubation with T cells did not equalise the CD63-related enhancement of stimulation. CD63low-a LCL, peptide loaded as well as the control, was still far better recognised by the CD4+ T cell clone c93 than the control LCL (Fig. 3.36B). Perhaps, the experimental design did not allow reaching saturation.

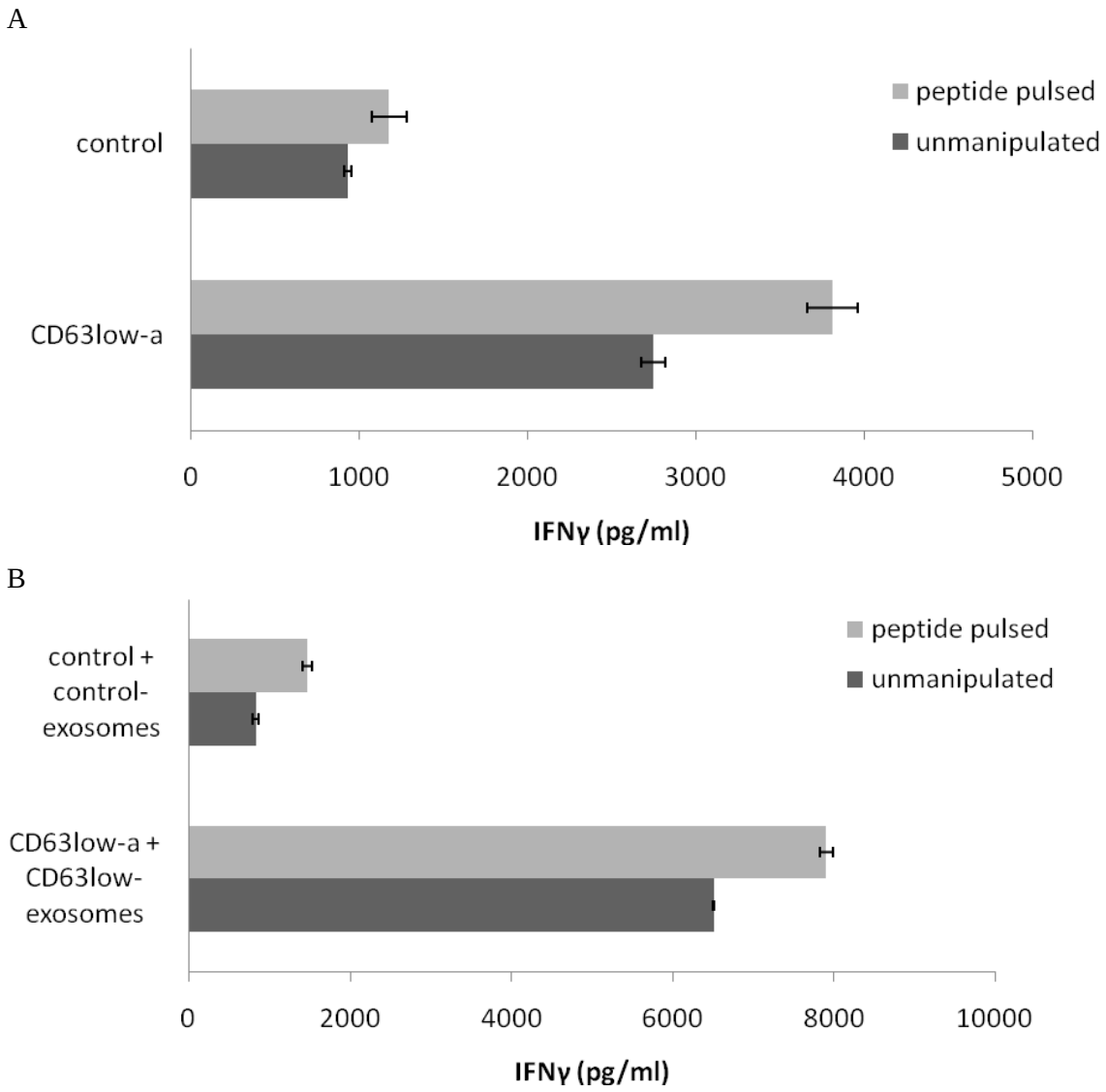

Figure 3.36: CD4+ T cell stimulation after incubation with LCL and exosomes
A) A T cell assay with CD63low-a LCL and control LCL does confirm an increased antigen specific CD4+ T cell stimulation by CD63low-a LCL compared to the control LCL. B) CD63low-a and control LCL were mixed with their own, previously purified, exosomes prior to being added to the CD4+ T cell clone c93. The addition of high amounts of exosomes did not change the previously observed intensity pattern for T cell activation. CD63low-a LCL was still recognised much better than the control LCL.

Summary

We showed that knocking down the expression of CD63 in a LCL increases antigen specific recognition by CD4+ but not CD8+ T cell clones. Exosomes derived from CD63low LCL also exhibit a pronounced reduction in CD63 but do not show the

enhancement of T cell stimulation as CD63low cells do. In search for the mechanisms, underlying the CD63-related stimulation enhancing phenomenon, we investigated the process of antigen presentation from different angles. The data show that 1) CD63 does not interfere with HLA-loading and peptide binding efficiency; 2) it does not influence the compartmentalisation and surface distribution; 3) CD63 does not have an apparent effect on the process of glycosylation of MHC II. Furthermore, CD63 depletion does not seem to impair the formation of the immunological synapse or the localisation of proteins involved in APC-T cell interaction, including MHC II and ICAM-1. I did also show that co-stimulation appears not to be involved. The only difference which could be determined was a clear increase of exosome secretion by CD63low LCL compared to the control LCL. However, CD63low and control LCLs did not exhibit any difference in the amount of exosomes stuck to their surface. Neither did the addition of exosomes to their parental LCL in T cell assays cause a levelling of the elevated CD4+ T cell stimulation by the CD63low-a LCL. When interpreting the latter experiment, suggesting that the higher number of exosomes secreted by CD63low LCLs is not responsible for this elevation, it has to be taken into account that this assay was only conducted once and would have to be repeated to allow solid conclusions.

4 Discussion

In order to study the role of the tetraspanin protein CD63 in the presentation of antigen to CD4+ T cells we firstly had to establish a model system. As APCs we used LCLs, B cells immortalised by EBV infection, which express EBV latent-cycle antigens. CD4+ T cell clones restricted through appropriate HLA molecules and specific for epitopes within those EBV-encoded antigens were used to detect changes in antigen presentation by the LCL. Antigen presentation by LCL-derived exosomes was also investigated.

4.1 Purification of LCL-derived exosomes

Depending on the purpose, different methods can be used for isolating exosomes. To purify exosomes suitable for clinical applications, techniques of filtering cell supernatant through a series of filters with gradually decreasing hollow size combined with separate ultracentrifugation steps are sometimes preferred (Lamparski et al. 2002). For analysing exosomes by flow cytometry however, vesicles need to be isolated by affinity extraction, for example using antibody coated magnetic beads (Clayton et al. 2001). For the purposes of this study we decided to use differential centrifugation (Raposo et al. 1996;Wubbolts et al. 2003b) that in certain cases, for instance for each preparation of exosomes analysed by electron microscopy, was supplemented by an additional centrifugation step using a sucrose cushion (Skriner et al. 2006).

Purity of exosome preparations is commonly determined by looking for the presence of calnexin, a marker of the ER that should be absent from purified exosomes (Asea et al. 2008;Ramachandra et al. 2010;Saunderson et al. 2008). We found that even after months of optimising the purification procedure the amount of calnexin in the exosome pellet, as measured by Western blotting, could only be decreased and was never completely abolished even using the additional sucrose cushion purification step. Personal communication with Dr Aled Clayton (University of Cardiff, UK) revealed that this phenomenon had previously seen by him and co-workers when purifying exosomes from LCLs. One possibility is that calnexin is not limited solely to the ER in some cell types. In support of this idea are observations showing direct membrane contacts between the perimeter membrane of MVBs and the ER occur to promote particular signalling processes (Eden et al. 2010). Indeed, in the case of Pompe disease, a disease which causes a dysfunction in the formation of organelles including lysosomes or melanosomes, ER proteins including calnexin were even found in lysosomes and late endosomes (Zhang et al. 2007). Eventually, we had to accept the fact that exosomes purified from LCLs inevitably contain a certain amount of ER-derived cell material. In this context it is conceivable that intracellular trafficking and sorting of proteins is altered in LCLs and a certain amount of ER proteins is translocated to endosomal compartments and eventually also to exosomes.

4.2 Recruitment of LCL-derived exosomes by CD4+ T cell clones

After establishing a suitable exosome purification protocol, we went on to investigate the ability of purified exosomes to interact directly with T cells. It is known that DC-derived exosomes can be recruited and bind to T cells, transfer molecules to T cells

and induce T cell activation (Andre et al. 2004a;Giri and Schorey 2008;Hao et al. 2006a;Luketic et al. 2007;Quah and O'Neill 2005;Raposo et al. 1996). In this context, it has been demonstrated that initial ICAM-1/LFA-1 driven exosome recruitment to T cells is crucial for the actual MHC-TCR interaction, the transfer of exosomal molecules and the subsequent T cell activation (Nolte-'t Hoen et al. 2009b;Segura et al. 2005). Furthermore, tetraspanins such as CD9, CD151 and Tspan8 as well as integrins on tumour-cell derived exosomes have all been proposed to be involved in exosome binding and uptake (Nazarenko et al. 2010b;Rana et al. 2011). In our study, exosomes purified from the LCL PER241 express ICAM-1 as well as several tetraspanins and are recruited to the CD4+ T cell clone c38. This was shown by co-incubating green-fluorescent LCL-derived exosomes with T cells prior to analysing the binding by flow cytometry.

Following their initial interaction exosomes can bind and be taken up by cells. Since exosomes express phophatidylserine (PS), it is suggested that they could be captured by phagocytes via direct or indirect recognition of PS (Al-Nedawi et al. 2009). More importantly for our study other PS-ligands like Tim1 and Tim4 are also expressed on activated lymphocytes and are shown to mediate exosome capture (Miyanishi et al. 2007). Whether the subsequent internalisation occurs via membrane fusion or endocytosis has been debated over the last decade (Morelli et al. 2004;Parolini et al. 2009). In support of endocytosis are two recent studies using live cell microscopy (Tian et al. 2010). Internalisation of exosomes was also demonstrated by visualising exosomes in phagosome-like compartments using electron microscopy (Feng et al. 2010). Membrane fusion and endocytosis is an energy dependent mechanism which is inhibited by low temperatures (Huth et al. 2006) whereas binding is energy

independent. We observed that co-incubation of exosomes and T cells at 37ºC resulted in a noticeably higher increase in T cell fluorescence than a 4ºC co-incubation. This suggests that the interaction between T cells at 4ºC is limited to surface binding whereas the co-incubation at 37ºC supposedly enables T cells to also take up exosomes and reprocess exosomal molecules leading to a higher intensity in fluorescence of the T cells. Labelling exosomes with a dye incorporating into the membrane and co-incubating these exosomes with T cells as well as with LCLs prior to analysis by confocal microscopy clearly showed an interaction between cells and exosomes in our experiments. Furthermore, our confocal images (i.e. localisation of exosomal membrane and proteins in intracellular vesicles) point to internalisation of added exosomes by T cell clones. This is consistent with a previous observation that exosomes were transported to endosomes and lysosomes after cellular uptake (Parolini et al. 2009).

4.3 Recognition of LCL-derived exosomes by CD4+ T cell clones

Stimulation of T cells requires the presentation of antigenic peptides loaded onto MHC I or MHC II molecules by APCs. Exosomes derived from APCs also express antigenic peptide-MHC complexes, which makes them potential mediators of immune responses. In the case of CD8+ T cells, exosomes derived from virtually any cell type bear MHC I molecules. However, since naive T cells require two signals for their initial activation, namely MHC-peptide complexes in addition to co-stimulation, tumour cell derived exosomes can only activate CD8+ T cells when HLA-matched DCs are present as mediator cells to supply the second signal (Andre et al. 2002;Wolfers et al. 2001). Exosomes from APCs such as DCs on the other hand, have

been shown to be capable of inducing CD8+ T cell's response either directly (Admyre et al. 2006;Luketic et al. 2007;Utsugi-Kobukai et al. 2003) or in the presence of allogeneic DCs demonstrating exosomes contain functional MHC I-peptide complexes (Chaput et al. 2004;Hsu et al. 2003). Importantly the T cell activation was more efficient using exosomes from mature DCs underlining the importance of co-stimulatory proteins in exosomal T cell activation (Admyre et al. 2006). In terms of MHC II, APC-derived exosomes bear functional MHC II molecules as they can be recognised by CD4+ T cells as allogenic antigens (Peche et al. 2003) and when antigen loaded directly stimulate cognate CD4+ T cell clones (Raposo et al. 1996), T cell lines (Admyre et al. 2007) or activated CD4+ T cells (Muntasell et al. 2007). Only the activation of naïve CD4+ T cells requires the uptake of exosomes by recipient DCs (Muntasell et al. 2007;Segura et al. 2005;Thery et al. 2002a). In accordance with this, exosomes secreted by LCL PER241 were capable of stimulating CD4+ T cell clones in an antigen-specific manner. As is the case with LCLs as target cells this exosome-mediated stimulation was due to the recognition of MHC II-peptide complexes by T cell receptors. We examined whether exosomes were able to properly present MHC II-peptide complexes to the TCRs or whether the T cell stimulation was due to the transfer of antigenic peptides or even whole antigens via exosomes to T cells that then processed and presented the epitope peptides to neighbouring T cells in the culture. Two lines of evidence suggested that it was the former scenario responsible for T cell activation. First, the rather short time of co-incubating T cells with exosomes (~18 hours) makes it unlikely that T cells can execute the whole process of exosome take-up and protein reprocessing. Second, and more importantly, by Western blotting we found that LCL derived exosomes contained EBNA2 but not EBNA3C protein. The presence of EBNA2, a nuclear

protein, in exosomes was surprising, nevertheless the fact that the EBNA3C-specific CD4 T cell clone was activated by exosomes in which EBNA3C protein could not be detected strongly suggests that T-cells were being directly stimulated by exosomes via pre-existing MHC-epitope peptide complexes.

4.4 Characterisation of LCL-exosomes upon CD63 knockdown

Tetraspanins are known to co-localise with MHC II molecules in MIICs and on exosomes of human B lymphocytes as shown by electron microscopy (Escola et al. 1998) and flow cytometry (Rialland et al. 2006). Immunogold labelling using antibodies against MHC II and the tetraspanins CD63, CD81 and CD82 followed by analysis by transmission electron microscopy confirmed the presence and co-localisation of these tetraspanins with MHC II on exosomes derived from LCLs used in this study. Considering that B-cell derived exosomes are capable of stimulating T cell responses (Admyre et al. 2007;Knight 2008;Raposo et al. 1996), the abundant expression of CD63 on our exosomes led us to examine the potential involvement of this tetraspanin in exosome-mediated T cell stimulation.

We showed that knocking down CD63 in an LCL also caused a substantial reduction in the content of CD63 in exosomes derived from those cells. Depletion of CD63 did not affect the cellular expression levels of other relevant proteins including MHC II, MHC I, ICAM-I and CD86 in cells. This is in line with the previous observation that CD63 knockout mice did not exhibit any readily detectable alteration in lymphocyte function including lymphocyte stimulation, migration or interactions (Schroder et al. 2009). A slightly different set of results were obtained when exosomes were analysed.

All marker proteins, analysed by Western blotting, including MHC I, ICAM-1, LMP-1, CD82, CD86 and EBNA2 were unaltered in expression upon knockdown of CD63. However, while also total levels of MHC II, as measured by Western blotting, were unaffected by CD63 depletion, immunogold labelling and electron microscopy suggested that CD63 low exosomes had lower levels of MHC II. A possible explanation for the conflicting results regarding exosomal MHC II levels may be the differences in techniques. During immunolabelling of the exosomal surface with the antibodies, target epitopes of some of the MHC II molecules on the surface of the control exosomes could have been occluded by adjacent proteins and therefore would not have been available for binding of the antibody. Alternatively, epitope occlusion can occur where a modification on a nearby residue hinders the ability of the antibody to bind to its specific sites (Cheung 2004). CD81 is one example in which a tetraspanin has been shown to facilitate the accurate processing of its partner protein in the Golgi. In the absence of this tetraspanin, CD19, which is normally associated to CD81, migrates differently after reaching the surface. This is due to an alteration in the final N-glycan structure, suggesting that lack of CD81 has an effect on posttranslational modification of CD19 in the Golgi (Levy & Shoham 2005b). For CD63 such a role in posttranslational modification of its interaction partners is not yet shown but depletion of CD63 may somehow increase the avidity of MHC II to the antibody used in this study. To finally confirm this by electron microscopy quantification, a preparation of exosomes for TEM could be done in combination of Western blotting verifying an equal level of MHC II in this particular batch of LCLs. Unfortunately, this could not be performed in the limited time frame of this project. Nonetheless, we assume that the MHC II level was not altered by CD63 depletion.

This would be in line with the observation of an equal T cell stimulation by LCL-derived exosomes following knockdown of CD63.

4.5 Characterisation of LCL PER241 upon CD63 knockdown

Several tetraspanins such as CD82 (Hammond et al. 1998), CD37 and CD53 (Angelisova et al. 1994), CD81 (Kijimoto-Ochiai et al. 2004;Schick and Levy 1993b), CD9 (Unternaehrer et al. 2007;Zilber et al. 2005) as well as CD63 (Engering et al. 2003;Engering and Pieters 2001) have been shown to be associated with MHC II molecules and play a role in MHC II sorting, clustering and organisation in lymphocytes. Kropshofer and colleagues reported that MHC II is partly incorporated into TERMs showing that a monoclonal antibody, CDw78 which binds a particular region of the MHC-II molecule, specifically recognises MHC-II associated with tetraspanins (Kropshofer et al. 2002b). However, in a subsequent study Poloso and colleagues showed that CDw78 does not define TERM-associated MHC-II, but instead recognises a peptide-bound MHC-II in a conformation acquired through trafficking to lysosomal Ag-processing compartments. Nonetheless, they confirmed the association of MHC II molecules with tetraspanins in DC as well as B cells (Poloso et al. 2006).

We showed that the depletion of CD63 in an LCL itself, like in exosomes purified from these cells, does not affect the total cell or surface expression of MHC II (or any of the other relevant membrane protein tested in this work). Remarkably, we observed that CD63 low LCLs were able to stimulate two different CD4+ T cell clones to a much higher degree than control LCLs. Furthermore, this increased stimulatory

capacity was sustained even in the presence of high concentration of the exogenously added epitope peptide. Repeating this set of experiments in a different cell background, using HeLa-CIITA cells in which HLA-DQ5 expression is induced via stable expression of the class II transactivator (Gobin et al. 1997) showed that after pulsing with a limiting concentration of synthetic epitope peptide (as the HeLa cells do not express EBV proteins) T cell recognition of the CD63low HeLa cells was much higher than the control. This clearly showed the effect of CD63 depletion is not dependent on EBV-encoded proteins and therefore represents a much wider phenomenon. These data obtained for CD63 are in line with a similar observation obtained from experiments using mouse DCs from CD37 or CD151 knockout mice. These CD37- and CD151-deficient DCs were also hyperstimulatory to T cells (Sheng et al. 2009). In that study, the knockout of CD37 or CD151 appears to cause hyperstimulation by affecting different processes of T cell activation. CD151 depletion appears to act through altering co-stimulation whereas CD37 was shown to interfere with MHC-peptide presentation itself. In a more recent study the tetraspanin Tssc6 also appears to be involved with CD37 and Tssc6 and CD37 may have cooperative roles in modulating cellular immunity (Gartlan et al. 2010). Both in vitro T cell proliferative responses and dendritic cell stimulation capacity are significantly exaggerated in $CD37^{-/-}$/$Tssc6^{-/-}$ mice compared with single knockout counterparts. An opposite regulatory function in MHC II-dependent antigen presentation was already shown for the tetraspanins CD9 in mouse DCs (Unternaehrer et al. 2007). In this report the superior capability of DCs compared to B blasts to stimulate T cells was proposed to be due to a distinctive organisation of MHC II on the DC's surface mediated by CD9.

Since CD63 is incorporated into tetraspanin microdomains, it is conceivable that CD63 causes a similar deregulation of these microdomains followed by a diminished antigen presentation as observed for CD37 and Tssc6. Alternatively, CD63 may have a regulatory function in co-stimulation or induced inhibition of TCR activation. Apart from the MHC peptide-TCR mediated stimulatory signal, T cells require a second signal to become fully activated. This signal, the co-stimulatory signal, is antigen nonspecific and is provided by the interaction between co-stimulatory molecules expressed on the membrane of APC. One of the best characterized co-stimulatory molecules expressed by T cells is CD28, which interacts with CD80 (B7.1) and CD86 (B7.2) on the membrane of APC (Lenschow et al. 1996). Another co-stimulatory receptor expressed by T cells is ICOS (Inducible Costimulator), which interacts with ICOS-L (Wang et al. 2000). T cell co-stimulation is necessary for T cell proliferation, differentiation and survival. Activation of T cells without co-stimulation may lead to T cell anergy, T cell deletion or the development of immune tolerance. Another way T cell stimulation is regulated is by the binding of programmed death ligand-1 or -2 (PD-L1/-L2) on APC to PD-1 on T cells. PD-L1 binds to its receptor, PD-1, found on activated T cells to modulate activation or inhibition. Interestingly, PD-L1 also has an appreciable affinity for the co-stimulatory molecule CD80, but not CD86 (Butte et al. 2008). The related molecule PD-L2 has no such affinity for CD80 or CD86, but shares PD-1 as a receptor. Engagement of PD-L1 with its receptor PD-1 on T cells delivers a signal that inhibits TCR-mediated activation of IL-2 production and T cell proliferation (Sheppard et al. 2004). There is no evidence of an interaction of tetraspanins and the above mentioned proteins CD80, CD86 or PD-L1/-L2 thus far. But the involvement of CD63 in sorting, trafficking and surface distribution of a number of proteins including MHC II (Pols & Klumperman 2008) makes also an

association of CD63 with CD80, CD86 and PD-L1/-L2 conceivable. To test these hypotheses a series of experiments investigating the qualitative properties of MHC II-antigen presentation as well as potential co-stimulatory effects of CD63 depletion were conducted.

4.5.1 Role of co-stimulatory molecules in stimulation of CD4+ T cell clones

Exosomes derived from APCs, including B cells, are known to express co-stimulatory proteins (Galli et al. 2005;Thery et al. 2002a;Zitvogel et al. 1998) that provide exosomes with the ability to deliver a co-stimulatory signal. In this regard, exosomes secreted by mature DCs are more efficient at inducing CD4+ T cell activation than those of immature DCs indicating the importance of co-stimulatory molecules (Montecalvo et al. 2008;Segura et al. 2005). However, it is important to mention that the CD4+ T cell clones used in our study hardly needed any co-stimulation to be activated. Our T cell clones exhibited similar levels of IFNγ release upon inducing TCR-cross-linking using an α-CD3 antibody (Valitutti et al. 1995;Weiss 1991) with or without allogeneic LCLs to provide a second co-stimulatory signal. This was the case even when levels of anti-CD3 were reduced thus our T cell clones did not show any dependence upon co-stimulation, as has been observed using T cell clones in other settings (Jenkins et al. 1990).

Apart from that, LCL-derived exosomes were also able to sensitise HLA-matched but antigen-lacking B cells, indicating exosomes as a potent vehicle for antigen cross-presentation. This phenomenon was previously shown especially for DCs in the context of presenting tumour derived antigens (Andre et al. 2002;Wolfers et al. 2001).

4.6 Mechanism how CD63 knockdown increases CD4+ T cell activation

4.6.1 CLIP-antigenic peptide exchange and surface peptide presentation

Since CD63 depletion did not affect levels of MHC II, yet increased the cell's ability to stimulate CD4+ T cells, we investigated a possible qualitative change in MHC II. The invariant chain molecule transports newly synthesised MHC II molecules into the MHC II compartment whereupon it is degraded leaving the low affinity CLIP peptide in the MHC II groove. The exchange of high affinity peptides for CLIP is then catalysed by HLA-DM molecules. The process of CLIP exchange and peptide loading in MIICs is not 100% efficient and therefore a certain amount of CLIP is naturally found on the surface of APCs (Odorizzi et al. 1994;Roche et al. 1993;Wang et al. 1997). HLA-DM is incorporated in tetraspanin-MHC II complexes (Levy & Shoham 2005b;van Spriel and Figdor 2010b) and CD63 and CD82 bear internalisation signals which may enable them to contribute in MHC II (conventional HLA and HLA-DM) recycling from plasma membrane to peptide loading compartments (Kropshofer et al. 2002b;Vogt et al. 2002). Hence, CD63 depletion could conceivably alter HLA-DM function. One possible scenario is that by altering HLA-DM function CD63 depletion increases the level of low-affinity MHC II-CLIP complexes which are readily replaced by exogenously added epitope peptides (Liu et al. 2010) that bind with higher affinity. This would account for the increased T cell stimulation observed when CD63 low LCLs are pulsed with exogenous epitope peptide.

Three separate approaches did not support this hypothesis. Firstly, flow cytometry using a CLIP-specific antibody detected similar surface expression levels of CLIP-bound MHC for both CD63low and control LCLs. Secondly, adding biotinylated antigenic peptides to LCLs and subsequently staining with Avidin-PE allowed the binding of exogenous peptide to be compared (Busch and Rothbard 1990). These experiments showed that amounts of exogenously bound peptides did not vary between CD63low and control LCLs. Thirdly, biochemical analysis of the amounts of unstable (CLIP bound) and stable (epitope peptide bound) MHC II molecules showed no difference between CD63low and control LCLs whether untreated or pre-loaded with high affinity epitope peptide. Taken together these results indicate unaltered CLIP-antigenic peptide processing after CD63 knockdown. This in turn is in agreement with the observation that knocking down CD81 and CD82 in HeLa CIITA cell had no effect on the expression of MHC II-CLIP expression (Poloso et al. 2006). Moreover, tetraspanins are shown not to bind Ii-associated MHC II in endosomal-lysosomal compartments (Hammond et al. 1998;Kropshofer et al. 2002b) and are only found on the cell surface (Kropshofer et al. 2002b;Rohn et al. 2004) indicating only a little if any participation of tetraspanins in peptide exchange processes.

4.6.2 Compartmentalisation of MHC II on the LCL's surface

As demonstrated for CD82, tetraspanins can regulate the distribution and compartmentalisation of associated proteins on the outer membrane (Odintsova et al. 2003). Moreover, CD82 and CD63 are shown to be present in MIICs and to interact with MHC II, HLA-DM and HLA-DO and with each other (Hammond et al. 1998). Therefore, it has been speculated that tetraspanins could play a role in MHC II

trafficking and maturation as well as influencing actual antigen presentation by sequestering MHC II-peptide complexes to areas of the cell membrane that are functionally more efficient (Kropshofer et al. 2002a;Unternaehrer et al. 2007). In this regard, mouse DCs deficient in CD37 or CD151 have both been demonstrated to be more stimulatory to T cells (Sheng et al. 2009). Interestingly, different mechanisms underpin this hyperstimulatory phenotype. CD151 does not appear to act through MHC II interaction but instead its knockout elevates DC's co-stimulatory activity. In contrast, CD37 interferes directly with MHC II-peptide presentation (Sheng et al. 2009). It is conceivable that CD37 sequesters MHC II-peptide complexes away from TERMS and hence impairs the formation of those speculated activation clusters. With this in mind we investigated the compartmentalisation of MHC II upon CD63 depletion. This was done by performing a flotation of cell lysates prepared from CD63low or control LCLs on a sucrose density gradient and comparing the distribution of MHC II molecules within that gradient. The overall conclusion from these experiments was that CD63 knockdown does not influence MHC II compartmentalisation. This conclusion could be confirmed in future work by chemical protein cross-linking and subsequent analysis of the resulting protein complexes by Western blotting using an antibody recognising MHC II.

4.6.3 MHC II, ICAM-1, actin and tubulin during immune synapse formation

Tetraspanins are known to regulate cytoskeletal rearrangements as demonstrated by their influence on integrin signalling and cell spreading after adhesion (Delaguillaumie et al. 2004;Feigelson et al. 2003;Goschnick et al. 2006;Lammerding et al. 2003). This led us to investigate the potential influence of CD63 on the

regulation of immunological synapse (IS) formation and structure. The interactions between cells in IS are considered as stable cell junctions requiring a specific protein distribution and cytoskeletal polarisation (Dustin et al. 1998). After an exploratory contact between the APC and T cell (mediated by ICAM1 and LFA1 on the surface of APC and T cell respectively) a specific immune recognition of antigen presenting MHC molecules by TCR occurs. Subsequent signalling leads to the formation of robust, LFA-1 dependent, adhesions of the T cell to the APC and a selective redistribution of membrane molecules. The specific concentric pattern of this contact area, the IS, is divided into a central supramolecular activation cluster (cSMAC) and a peripheral SMAC (pSMAC) (Monks et al. 1998b). Microfilaments as well as microtubules play a crucial role in the formation of the immunological synapse since LFA-1 for example is linked to the actin cytoskeleton via talin (Sampath et al. 1998). In the way the actin cytoskeleton organises and regulates the IS, a positive feedback signalling from the SMAC does influence actin polymerisation and contraction (Ardouin et al. 2003;Gil et al. 2002;Krause et al. 2000).

Tetraspanins CD81 (Mittelbrunn et al. 2002) and CD82 (Delaguillaumie et al. 2002) have been reported to localise to the IS, CD81 in particular is shown to cluster to the cSMAC. Since both these tetraspanins are taking part in the formation of TERMs, these observations indicate that TERMs may be redistributed to the IS in general. In agreement with this hypothesis is the observation that cross-linking of tetraspanins on the T cell surface causes co-stimulation. Antibodies specifically recognising and ligating CD9, CD53, CD81 or CD82 caused co-stimulation which is different from the mechanism known from the classical CD28-mediated co-stimulation pathway (Lagaudriere-Gesbert et al. 1997;Lebel-Binay et al. 1995;Tai et al. 1997;Witherden et

al. 2000). This antibody-mediated co-stimulation has been speculated to result from aggregation of tetraspanin microdomains which are components of the IS hence facilitating TCR engagement (Tarrant et al. 2003). This may not only be the case for the T cell side but also for the APC side of the IS since tetraspanins regulate MHC II clustering as well (Kropshofer et al. 2002b).

Regarding the APC's IS, most research so far focused on DCs. But the process of formation and maintenance of the IS seems to be very similar in B cells. Apart from MHC-TCR interaction, ICAM-1-LFA-1 binding is necessary for the formation of the IS between APC and T cell (Davis 2009). DCs (Barreiro et al. 2007) as well as B cells (Carrasco et al. 2004;Carrasco and Batista 2006a) are shown to recruit ICAM-1 to the IS to form the APC pSMAC opposite to the T cell pSMAC exhibiting the LFA-1. In terms of cell morphology and the influence of actin in IS formation abundant interactions between the actin cytoskeleton and the SMAC regulate efficiency of the IS (Al-Alwan et al. 2001;Billadeau & Burkhardt 2006;Riol-Blanco et al. 2009). For B cells in particular, it was demonstrated that Rap GTPase-regulated localised actin polymerisation (Lin et al. 2008) as well as F-actin dependent spreading of the B cell (Fleire et al. 2006) is required for B cell IS formation. Interestingly, a difference between the DC IS and the B cell IS seems to exist in terms of microtubule organisation and the microtubule-organising-centre (MTOC) polarisation. The MTOC is the site of microtubule nucleation in eukaryotic cells and one of its main functions is the organisation of the mitotic and meiotic spindle apparatus separating the chromosomes during cell division. In most animal cells during interphase the MTOC is usually located near the nucleus, and generally associated closely with the Golgi apparatus. MTOC-associated microtubules are therefore also of importantance for

membrane bound transport, as the motor proteins kinesin and dynein move along microtubules, allowing vesicles to be directed to or from the endoplasmic reticulum and Golgi apparatus.

For DCs, MTOC polarisation towards the contact area with T cells is well established and is thought to regulate the transport of cargo, including MHC and co-stimulatory molecules (Boes et al. 2002;Keller et al. 2007;Vyas et al. 2007) and control targeted delivery of cytokines at the IS (Pulecio et al. 2010). For B cells on the other hand, only little data exist demonstrating that the B cell's MTOC is not particularly oriented to the IS upon contact with T cells (Kupfer et al. 1986).

We investigated the structure of the LCL-T cell IS by immunostaining using antibodies against MHC II, ICAM-I and tubulin as well as phalloidin to label actin. Confocal microscopy allowed us to analyse protein distribution on the surface and in the cytoplasm, to analyse the actual IS for protein content, to study the localisation of MVBs and the polarisation of actin or tubulin fibres. As far as the distribution of MHC II, ICAM-1 and actin is concerned, we observed similar patterns of staining as described in the literature. However, in contrast to observations made by Kupfer et al. in LCL we found that tubulin filaments and the MTOC appeared to orientate somewhat towards the contact site of the interacting immune cells. While this is an interesting observation, more detailed analyses using specific antibodies, detailed quantification and statistical analyses of MTOC localisation in the different areas of the LCL-T-cell interface would be needed to allow more solid conclusions to be drawn. Especially considering the formation of the IS being a very dynamic process (Krummel and Cahalan 2010;Lee et al. 2002;Yokosuka and Saito 2009) future experiments exploring the kinetics of IS formation by life cell imaging over time

would be of interest. Regarding CD63, our data suggest that the depletion of CD63 does not have any influence on the localisation, distribution and clustering of any of these analysed proteins. We did not find any differences between the control and the CD63 low LCLs when they were pre-mixed with CD4+ T cells. We do point out however that further investigations would be needed before a firm conclusion can be drawn from this work.

4.6.4 LMP-1 distribution during immune synapse formation

The same approach was used to study T cell activation in the context of LCLs expressing the EBV-derived membrane protein LMP-1 (latent membrane protein 1). LMP-1 has multiple functions during EBV infection including mimicking CD40 signalling and hence maintaining B cell activation, affecting cell-cell contacts, cytokine and chemokine production as well as antigen presentation (Brooks et al. 2009;Middeldorp and Pegtel 2008a).

Discussions with another research group raised the possibility that the enhanced T cell stimulation exhibited by CD63 depleted LCLs could be due to the unique expression in LCLs of the EBV-encoded protein LMP1. Of interest to us were the findings that LMP1 co-localised with CD63 (Verweij et al. 2011). Since LMP1 also contains a motif that has immunosuppressive properties towards T cells (Dukers et al. 2000) this raised the possibility that CD63 localises LMP1 into the IS whereupon LMP1 decreases T cell activity. If this was the case then depletion of CD63 could decrease the amount of LMP1 in the IS which in turn would allow such cells to stimulate T cell more effectively. Three pieces of experimental data argue against this model. First, using immunofluorescence microscopy as described above we could not see any

difference in LMP-1 localisation at the immunological synapse following CD63 depletion. Furthermore we did not detect any change in the expression level of LMP-1 upon CD63 knockdown in LCLs or LCL-derived exosomes. Second, we observed increased T cell stimulation upon knocking down CD63 in class II positive HeLa cells that lack LMP-1.

4.6.5 LCL's co-stimulatory capacity upon CD63 knockdown

CD63 on T cells was previously shown to function as an activation-linked co-stimulatory element in its own right in the context of DC encounter (Pfistershammer et al. 2004). Furthermore, Sheng et al. demonstrated that CD151 influences T cell stimulation by inhibiting murine DC's co-stimulation of T cells (Sheng et al. 2009). Therefore, we also had to test whether CD63 knockdown in LCLs may have an effect on co-stimulatory capacity. An assay designed to determine the co-stimulatory capacity of APCs showed that the co-stimulatory activity of control and CD63low LCLs towards CD4+ T cells was the same. However, as stated in section 4.3, the CD4+ T cell clones used in our study, in common with all T cell clones, required little if any co-stimulatory signalling in order to be activated. In this regard, knocking down CD63 expression in HeLa CIITA cells was of particular importance as it did allow us to investigate the influence of CD63 on T cell stimulation in a non-professional, EBV-negative APC. The fact that CD63low HeLa CIITA cells express the same level of MHC II but exhibited a strong hyperstimulation of the CD4+ T cell clone c38 compared to control HeLa cells supported our previous results. CD86 was not detected in HeLa CIITA cells (data not shown) and no data so far report the expression of any other co-stimulatory proteins by this cell line. Therefore, the

observed increase in T cell stimulation by CD63low HeLa cells agrees with the outcome of our co-stimulation assay conducted with CD63low LCLs. CD63 appears to regulate T cell stimulation via MHC II-antigen presentation and not via co-stimulation.

Since knockdown of genes is considerably easier in HeLa cells compared to LCLs I made use of the HeLa CIITA system to perform some preliminary experiments on the effect of other tetraspanins on T cell stimulation. I was able to knock down both CD81 and CD151 in this cell line. Both knockdowns caused an enhanced stimulation of CD4+ T cell clones. Here, it has to be considered that these proteins, particularly CD151, may execute different regulatory functions in human cells (HeLa) than in mouse DCs. In this regard, CD151 is conceivable, apart from influencing the co-stimulatory pathway as demonstrated by Sheng et al., to regulate T cell stimulation by modulating integrin mediated signalling in HeLa cells. CD151 is shown to directly interact with particular integrins (Berditchevski 2001;Sterk et al. 2000) and to regulate their activity through TERMs. Association with CD151 is critical for the recruitment of these integrins to TERMs, where they can cooperate with other TERM-associated transmembrane proteins (Stipp et al. 2003a). Thus, CD151 can affect the functionality of integrins, for example, by modulating ligand-binding properties (Hemler 2005) or regulating internalisation of associated integrins (Liu et al. 2007;Winterwood et al. 2006). Due to the involvement of CD151-associated integrins in a number of processes, CD151 indirectly influences adhesion-dependent signalling and post-adhesion events such as cell migration, cell motility, granule polarisation (Liu et al. 2009), co-stimulating cytoskeletal processes that bring T cell signalling proteins to the IS (Wulfing and Davis 1998) and regulating the integrity of the IS and the duration of T cell contacts for optimal functional responses (Semmrich et al. 2005).

On the APC's side, integrins such as LFA-1 or VLA-4 are mainly known to be responsible for processes such as cell migration and homing but also for antigen recognition (Arana et al. 2008) and also antigen presentation or T cell activation as they mediate the formation of the IS (Carrasco and Batista 2006b). For HeLa cells however, there are no data yet about an involvement of integrins in mediating T cell stimulation. Additionally, it has to be taken into consideration that we observed a heterogeneous MHC II expression in both CD81 low and the CD151 low HeLa CIITA cell lines, which makes interpretations of these findings difficult and further investigation into this phenomenon necessary to allow any solid conclusions.

4.6.6 Quantification of exosome release following CD63 knock down

Exosome trafficking and release is a complex intracellular process involving highly organised pathways (Keller et al. 2006;Thery et al. 2002b). Biogenesis of exosomes starts with clathrin- or non-clathrin dependent endocytosis and the formation of early endosomes. These endosomes then develop into late endosomes, also called MVBs in the context of APCs, incorporating intraluminal vesicles (ILVs), which correspond to exosomes once MVBs fuse back with the surface cell membrane and release their vesicles in an exocytic manner (Lakkaraju and Rodriguez-Boulan 2008;Schorey and Bhatnagar 2008;Van et al. 2006). ILVs are generated by segregation of cargo molecules within the limiting membrane of endosomes, followed by inward budding and the release of vesicles into the endosome's lumen. ESCRT (endosomal sorting complex required for transport) is known to play a role in this process. ESCRT-0, -I, -II recognise and associate with ubiquitinated proteins in the endosomal limiting membrane, possibly followed by ESCRT-III-mediated membrane budding (Hurley

2008;Williams and Urbe 2007). However, it has also been demonstrated that some proteins found on ILVs are being released via exosomes by an ESCRT-independent manner (Fang et al. 2007;Trajkovic et al. 2008). One of these pathways requires ceramide, a lipid exhibiting many structural and physical properties that may facilitate the generation of vesicles (Bianco et al. 2009;Trajkovic et al. 2008;Zhang et al. 2009). Ceramide mediated oligomerisation and stabilisation of exosomal membrane domains may lead, if beyond a critical size, to domain-induced budding (Baumgart et al. 2003) and may well be partitioned by tetraspanins.

Due to the complexity, these processes of endosome formation and exosomes release can be affected at many different stages causing a quantitative alteration of exosome secretion. In blood cells, generally, stimuli causing an increase in intracellular calcium levels regulate the secretion of microvesicles including exosomes (Hugel et al. 2005). Such stimuli include the activation of thrombin receptor on platelets (Heijnen et al. 1999), Toll-like receptor 4 by lipopolysaccharides (LPS) on DCs (Obregon et al. 2006) or the cognate interaction with T cell clones (Buschow et al. 2009) on immature DCs. Moreover, the activation of $P2X_7$ receptor (purinergic receptor P2X, ligand-gated ion channel, 7) by ATP on neutrophils and monocytes (MacKenzie et al. 2001) or diacylglycerol kinase-α on T cells (Alonso et al. 2005;Alonso et al. 2007;Alonso et al. 2011) has been shown to regulate exosome release whereas p53 influences this process on tumour cells (Lehmann et al. 2008). Regulation of exosome secretion does also depend on the microenvironmental pH (Parolini et al. 2009), premature senescence (Lehmann et al. 2008), dynein dysfunction (Kimura et al. 2009) or the overexpression of v-SNARE proteins, molecules involved in trafficking of endosomes to lysosomes (Fader et al. 2009).

In this context, tetraspanins expressed within the endosomal system are predominantly found in MVBs and lysosomes and particularly CD37, CD53, CD63, CD81 and CD82 are shown to be highly enriched on ILVs and exosomes, respectively (Escola et al. 1998). In the perspective of our study, CD63 has been reported to associate with PI 4-kinase in platelets where it influences phosphoinositide-dependent signalling and platelet spreading (Israels and McMillan-Ward 2005). Importantly, CD63 is suggested to function as a transport regulator (Pols & Klumperman 2008) and it is conceivable that CD63 somehow takes part in the formation, trafficking or release of exosomes. CD63 is thought to be linked with the generation of exosomes in at least two ways. Firstly, CD63 bears a YXXØ consensus motif required for endocytosis at the plasma membrane. This targeting motif is important for association with adaptor proteins (APs) and the recruitment of a clathrin coat (Bonifacino and Traub 2003). AP-2 associates with CD63 and regulates CD63's clathrin-mediated endocytosis from the plasma membrane (Janvier and Bonifacino 2005;Peden et al. 2004;Rous et al. 2002). Secondly, CD63 is speculated to be located towards ILVs by a pathway involving ceramide (Trajkovic et al. 2008) and in accordance with this, CD63 is found to be up to 7 times enriched in ILVs as compared to the endosomal limiting membrane and hence is highly enriched in exosomes as compared to whole cells (Escola et al. 1998). However, knocking down CD63 in monocyte-derived macrophages shows that this tetraspanin is not required for the integrity of MVBs and lysosomes in these cells (Ruiz-Mateos et al. 2008). Furthermore, CD63-knockout mice did not exhibit any obvious abnormalities in late endosomal/lysosomal compartments (Schroder et al. 2009). Although a role of other tetraspanins in the biogenesis of MVBs is not known, recently it has been reported that elevated expression of particular tetraspanins can change the protein composition of exosomes.

Overexpression of CO-029/TSPAN8 in a pancreatic cancer cell line regulates recruitment of other tetraspanins and integrins into exosomes (Gesierich et al. 2006;Nazarenko et al. 2010b). Also CD82 and CD9 have been shown to influence the exosomal protein composition by facilitating recruitment of β-catenin, which plays a role in the Wnt- and E-cadherin signalling pathways (Chairoungdua et al. 2010). In addition to this, it has been demonstrated that CD9 knockout DCs produced less exosomes when compared to wild-type DCs (Chairoungdua et al. 2010). In relation to our data, this observed tetraspanin-dependent regulation of exosome secretion in DCs may represent a general mode of action of tetraspanins in APCs by which they influence antigen presentation.

We showed in this study for the first time that CD63 exhibits a regulatory function in exosome release in LCLs. Our data clearly show that knocking down CD63 increased the release of exosomes in an LCL compared to the control LCL. This increase in exosomes secreted to the extracellular environment was quantified by measuring exosomal protein amount in exosome preparations derived from both these LCLs and confirmed by western blotting for MHC II. Actin is non-specifically sequestered by exosomes during their formation and was used as a loading control. Noteworthily, the level of CD63 knockdown as well as the hyperstimulatory capacity towards CD4+ T cells was higher for the LCL CD63low-b compared to CD63low-a. The increase in exosome release however, is somewhat lower for CD63low-b compared to the CD63low-a lymphoblastoid cell line. This indicates that the mechanism by which CD63 takes part in antigen presentation and T cell stimulation is more complex than just influencing the process of exosome formation and release. Nonetheless, this increase in exosome secretion following CD63 depletion is a promising candidate for

being the underlying mechanism responsible for the observed increase of CD4+ T cell activation we observed. If this is indeed the case, then how specifically does increased exosome release translate into increased CD4+ T cell activation? We perfomed preliminary experiments aimed to investigate two potential mechanisms. Firstly, we investigated the possibility that after their release to the extracellular milieu exosomes bind back onto the surface of the same or neighbouring LCL cells. If this was the case, then the increased amount of surface bound exosomes, each abundantly expressing MHC II-peptide complexes, would increase the stimulatory active surface area of CD63low LCLs available for T cell recognition in turn increasing recognition by T cells. Flow cytometry experiments seeking to detect an increased amount of exosomes weakly bound to the surface of LCLs did not show any difference between CD63low and control cells. Secondly, since LCL-derived exosomes are shown to directly stimulate T cells, the elevated secretion of exosomes could directly lead to an enhanced T cell activation (Fig. 4.1).

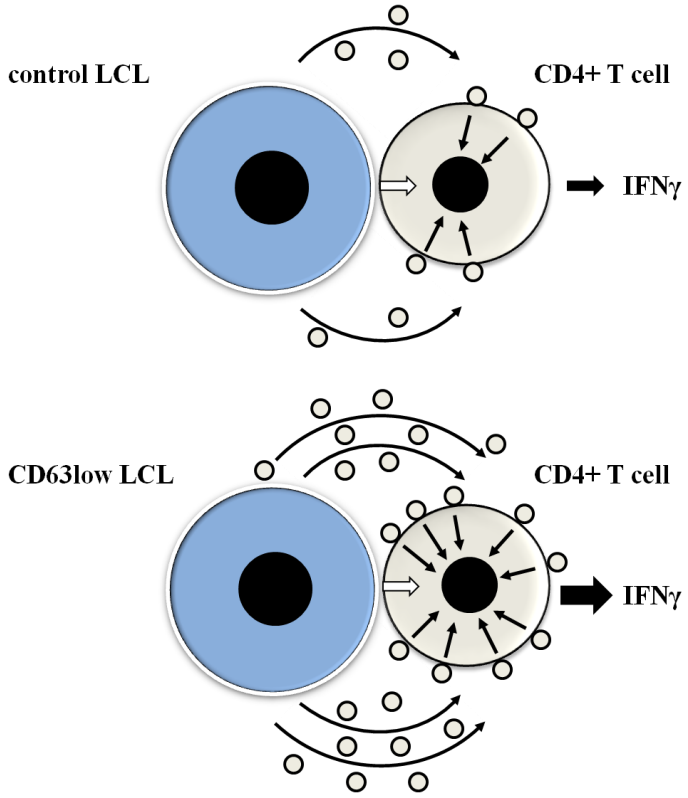

Figure 4.1: Model for how CD63 depletion causes CD4+ T cell hyperstimulation by increasing exosome secretion
We have shown that exosomes are able to directly interact with and stimulate CD4+ T cell clones. In this model CD63 depletion leads to enhanced T cell stimulation by increasing the secretion of stimulatory exosomes.

We tried to test this hypothesis by exogenously adding purified exosomes to LCL-T cell co-cultures. This was thought to mask the enhanced stimulation seen for the CD63low LCL. In one preliminary experiment that was performed, the CD63low LCL still stimulated the CD4+ T cell clone to a higher degree than the control LCL. Additional experiments, which could not be undertaken due to time pressure, would be needed in order to accept or reject the hypothesis. Since this theory explains the observed hyperstimulation by a quantitative change of exosome secretion rather than a selective qualitative change in antigen presenting molecules, it would be intriguing to investigate in more detail the MHC I dependent CD8+ T cell stimulation. More functional assays would be needed to substantiate our finding of CD63low LCLs not

being directly hyperstimulatory towards CD8+ T cells. Additionaly, CD63low exosomes purified from LCLs could be tested for their capability of stimulating CD8+ T cells antigen specifically.

One way to test this hypothesis independently from the LCL system would be determining the exosome release by HeLa CIITA cells in the context of CD63 depletion.

Independently from the cell line used in the assay, blocking of exosome secretion by the addition of exosome-release blocking adjuvants would possibly provide meaningful insights. Amiloride for example is an inhibitor specific for H+/Na+ and Na+/Ca2+ ion channels resulting in an intracellular change in Ca2+ concentration and has been shown to inhibit exosome secretion in certain tumour cells *in vivo* and *in vitro* (Chalmin et al. 2010). Controlling exosomes release of LCLs in functional assays analysing T cells stimulation would enable us to specify the role of exosomes in T cell activation in the context of CD63.

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