IDENTIFICATION OF THE CELLULAR MECHANISMS RESPONSIBLE FOR THE GENERATION OF PARTICULAR HLA-DR EPITOPES

by

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ABSTRACT

HLA class II/peptide complexes (pHLA-II), organized into microdomains on the surface of antigen presenting cells (APCs) or on APC-secreted exosomes, engage CD4+ T cells for immune recognition. The association of the HLA-II alleles DRB1*0401/0404 with rheumatoid arthritis may be due to their propensity to present self-peptides for immune recognition. pHLA-II presentation on APCs is largely determined by HLA-DM, an intracellular chaperone, and its negative regulator, HLA-DO. Previously described DRB1*04-restricted epitopes (D11-0401, D13-0401, and D13-0404) were found dependent, sensitive, and resistant, respectively, to HLA-DM activity. The aims of this study were to determine whether (a) HLA-DO affects epitope expression; (b) cell surface microdomains concentrate these epitopes; and (c) exosomes express these epitopes. Key findings include: HLA-DO appears not essential, but its role in optimal epitope expression may be cell-context dependent; lipid raft disruption abrogated only the DM-dependent D11-0401epitope; exosomal expression of these epitopes was cell specific and independent of their cell surface expression. Altogether, this study has enhanced our knowledge of DM-dependent, -sensitive, and -resistant epitopes on rheumatoid arthritis-associated pHLA-DRB1*04 molecules.

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List of Abbreviations and Symbols

AEP	Asparagine endopeptidase
APC	Antigen presenting cell
ATP	Adenosine triphosphate
B-LCL	B lymphoblastoid cell line
BCA	Bicinchoninic acid
BD	Becton Dickinson
BL	Burkitt's lymphoma
BFA	Brefeldin A
BSA	Bovine serum albumin
CBI II	Cathepsin B inhibitor II
CD	Cluster of differentiation
CELISA	Cell enzyme-linked immunosorbent assay
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CIITA	Class II, major histocompatibility complex, transactivator
CLIP	Class II-associated invariant chain peptide
CLIP/DR	Class II-associated invariant chain peptide - human leukocyte antigen DR complex
CREB	cAMP response element-binding protein
D11-0401	DRB1*04:01 molecules with NFLD.D11 epitope
D13-0401	DRB1*04:01 molecules with NFLD.D13 epitope
D13-0404	DRB1*04:04 molecules with NFLD.D13 epitope
DC	Dendritic cell
DM	Human leukocyte antigen DM
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Human leukocyte antigen DO
DP	Human leukocyte antigen DP
DQ	Human leukocyte antigen DQ
DR	Human leukocyte antigen DR
DRA	Human leukocyte antigen DR alpha chain
DRB	Human leukocyte antigen DR beta chain
DRM	Detergent resistant membrane
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDM	Exosome-depleted media
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complexes required for transport
FACS	Fluorescence-activated cell sorting

FCDR	Flow cytometric assay of differential detergent resistance
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLAER	Fluorescent aerolysin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GILT	Interferon-inducible lysosomal thiol reductase
GPI	Glycosylphosphatidylinositol
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ii	Invariant chain
ILV	Intraluminal vesicle
IMDM	Iscove's Modified Dulbecco's media
kDa	Kilodalton
LAMP	Lysosome-associated membrane glycoprotein
LAT	Linker-for-activation of T cells
LFA	Lymphocyte function-associated antigen
LIP	Leupeptin-induced peptide
mAb	Monoclonal antibody
MBCD	Methyl-beta-cyclodextrin
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
MVB	Multivesicular bodies
NF-Y	Nuclear factor Y
ND	Not determined
NOD	Non-obese diabetic
NT	Not tested
OD	Optical density
pAPC	Professional antigen presenting cell
PBL	Peripheral blood
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
pHLA-II	HLA class II/peptide complex
pMHC-II	Peptide-major histocompatibility complex class II complex
PNH	Paroxysmal nocturnal hemoglobinuria
RFX	Regulatory factor X
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLIP	Small leupeptin-induced peptide
TAP	Transporter associated with antigen processing

TBS	Tris-buffered saline
TCR	T-cell receptor
TEM	Tetraspanin-enriched microdomain
TRIM	Tripartite motif-containing
xMHC	Extended major histocompatibility complex

List of Appendices

Co-authorship Statement

Chapter 1 is a general introduction containing the relevant immunological concepts and background related to the remaining chapters. Chapter 2 describes all methods performed in the subsequent experiments detailed in the following chapters. Chapters 3 and 4 are two separate manuscript preparations which contain original work performed by Christopher P. Corkum under the supervision of Dr. Sheila Drover at Memorial University of Newfoundland. Each manuscript chapter involves varying amounts of collaboration with other scientists and individuals both within and outside of Memorial University of Newfoundland. Chapter 5 is an integrated summary of the results presented in Chapters 3 and 4.

The work described in Chapter 3 contains a significant amount of collaboration with Dr. David R. Spurrell. For clarity of overall presentation and future publication, some of the data presented in this chapter was previously presented within his Ph.D dissertation at Memorial University of Newfoundland. However, the presentation of this prior data has been entirely re-formatted, edited, and in some instances re-analyzed for the current work. More specifically, the CELISA and flow cytometry experiments involving inhibition of cellular proteases (Figures 3.3.A, 3.3.C, 3.5.A, 3.6.A, 3.6.B, 3.6.C, 3.8.A, 3.9.B, and Appendix A) and the antibody blocking experiments (Figures 3.2.A, 3.4.D, and 3.7.B) were performed by D. R. Spurrell, in some cases with assistance from Diane Codner. The data presented in Figure 3.5.B was performed entirely by D. Codner. Transfection of DRB1*0401 into some of the B cell lines, including Ramos, Bjab, Bjab

V, and Bjab DO, was performed by Tracey Dyer (Figures 3.1.B, 3.1.C, 3.1.D, 3.1.E, and 3.4.C), as part of a summer internship under the supervision of the author and Dr. Drover. Finally, the experiment and results described in Figure 3.4.A was originally performed by Dr. Drover. The author is responsible for the remainder of the data presented in Chapter 3, including all flow cytometry experiments and western blotting work, generation of figures, re-analysis of data where indicated, and manuscript preparation.

The entirety of the work presented in Chapter 4, including experiment design and performance, data analysis, and manuscript preparation was performed by the author under the supervision of Dr. Drover.

Chapter 1

Introduction

1.1 The major histocompatibility complex

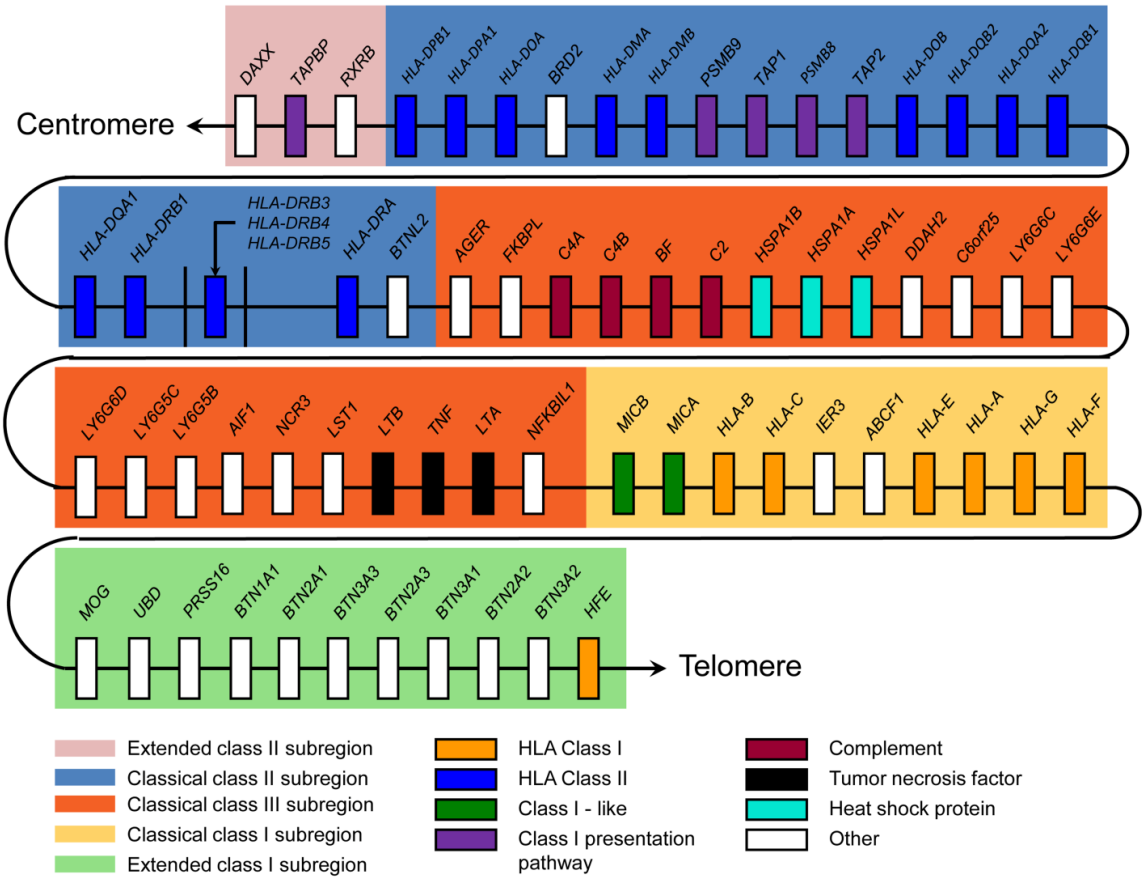
1.1.1 Genetic organization

The major histocompatibility complex (MHC) is a large genomic region in the mammalian genome that contains a tightly linked cluster of genes which encode several proteins that are involved in both innate and adaptive immunity. The human MHC, also referred to as the human leukocyte antigen (HLA) system, is located on the short arm of chromosome 6 and in mice on chromosome 17. More recently, the extended MHC (xMHC) has been described as shown in Figure 1.1, which includes gene clusters adjacent to the classical MHC (1). The xMHC contains upwards of 420 loci including both expressed genes and pseudogenes, making it one of the most gene-dense regions of the genome (1). Furthermore, several genes within the MHC are highly polymorphic in that multiple variants of each gene exist within the population. The human MHC contains several loci which encode structurally and functionally homologous proteins that are classified as HLA class I (HLA-I or class I), including HLA-A, -B, and -C, and HLA class II (HLA-II or class II), including HLA-DR, -DP and -DQ molecules. Both HLA-I and HLA-II molecules were first identified because of their role in mediating tissue rejection and acceptance in transplantation, but upon further analysis it was revealed that their main function involved antigen presentation to T lymphocytes (2).

In humans, the MHC is organized into three distinct gene-rich regions that are designated from telomere to centromere as class I, III, and II regions (1, 3, 4). Each region contains several genes encoding immunologically relevant proteins detailed below, but also includes additional genes whose function is unrelated to the immune response

Figure 1.1. Gene map of the extended human MHC.

A selected fragment of the extended major histocompatibility complex (xMHC) on the short arm of human chromosome 6 containing immunologically relevant genes is shown. The class I, II, and III subregions are described in the text. Some immunologically irrelevant genes have been excluded for ease of demonstration. Furthermore, pseudogenes have been omitted for presentation purposes. In total, the xMHC is comprised of 421 loci, of which 252 are classified as expressed genes, and 169 as pseudogenes and transcripts (1). The extended class I and II subregions are not described in detail, but include genes such as *death domain associated protein* (*DAXX*), *TAP binding protein* (*TAPBP*), *retinoid x receptor beta* (*RXRB*), *myelin oligodendrocyte glycoprotein* (*MOG*), *ubiquitin D* (*UBD*), *protease, serine 16* (*PRSS16*), members of the *butyrophilin* subfamily of genes (*BTN1A1, BTN2A1, BTN3A3, BTN3A1, BTN2A2, BTN3A2*), and *hemochromatosis* (*HFE*). Adapted from (1).


Centromere
DAXX
TAPBP
RXRB
HLA-DPB1
HLA-DPA1
HLA-DOA
BRD2
HLA-DMA
HLA-DMB
PSMB9
TAP1
PSMB8
TAP2
HLA-DOB
HLA-DQB2
HLA-DQA2
HLA-DQB1
HLA-DQA1
HLA-DRB1
HLA-DRB3
HLA-DRB4
HLA-DRB5
HLA-DRA
BTNL2
AGER
FKBPL
C4A
C4B
BF
C2
HSPA1B
HSPA1A
HSPA1L
DDAH2
C6orf25
LY6G6C
LY6G6E
LY6G6D
LY6G5C
LY6G5B
AIF1
NCR3
LST1
LTB
TNF
LTA
NFKBIL1
MICB
MICA
HLA-B
HLA-C
IER3
ABCF1
HLA-E
HLA-A
HLA-G
HLA-F
MOG
UBD
PRSS16
BTN1A1
BTN2A1
BTN3A3
BTN2A3
BTN3A1
BTN2A2
BTN3A2
HFE
Telomere
Extended class II subregion
Classical class II subregion
Classical class III subregion
Classical class I subregion
Extended class I subregion
HLA Class I
HLA Class II
Class I - like
Class I presentation pathway
Complement
Tumor necrosis factor
Heat shock protein
Other

ranging from cell growth, development, DNA repair and transcriptional regulation. The class I subregion contains genes encoding the alpha (α) chain of HLA-I molecules HLA-A, -B, and -C, as well as the HLA-I-like genes encoding class Ib molecules HLA-E, -F, -G, MICA, and MICB. The α chain of HLA-A, -B, and -C combine with a molecule termed beta-2-microglobulin (B2M), encoded outside the MHC on chromosome 15, forming a heterodimer whose function is to bind short peptides typically derived from endogenous antigens and present them to CD8+ T lymphocytes (5). By contrast, the functions of class Ib molecules is less well understood, but several studies demonstrate that they act as ligands for the activation and inhibition of natural killer cells, as well as perform important immunoregulatory functions (6-8). Several genes encoding members of the tripartite motif family (TRIM) are also located within the class I subregion, which are involved in a wide variety of cellular processes. Accumulating evidence suggests that several TRIM family members are important in the regulation innate immunity, as well as having a direct role in the restriction of viral infection in a variety of cell types (9, 10).

The class III region does not contain genes for any classical class I or II antigens; however, approximately 62, many whose physiological function is unknown, are found in this subregion (11). The genes for complement proteins C2, factor B, C4A and C4B are located at the centromeric end of the class III region. Other immunologically relevant genes in this region encode for members of the tumor necrosis factor family of cytokines including tumor necrosis factor-α, lymphotoxin A, and lymphotoxin B (12).

The class II region is located nearest to the centromere and contains genes encoding the classical HLA-II antigens HLA-DR, -DP, and -DQ as well as two non-classical class II antigens HLA-DM and -DO. Both classical and non-classical class II

antigens consist of an α and beta (β) chain which associate in the endoplasmic reticulum to form a functional dimer. DR, DP, and DQ bind short peptides and present them to CD4+ T lymphocytes (13). DM and DO function within the class II processing and presentation pathway and shape the repertoire of peptides presented by class II molecules (14). Also included in this region are genes encoding the proteins tapasin, TAP1, TAP2, LMP2, and LMP7. Similar to DM and DO in the class II pathway, tapasin, TAP1, and TAP2 contribute to the formation of mature peptide-MHC complexes (pMHC) in the class I pathway as discussed below (15). Both LMP2 and LMP7 are subunits of the immunoproteasome which is responsible for generating optimal peptides for binding to HLA class I molecules (16).

1.1.2 Polymorphism and nomenclature

Several MHC genes exhibit a high degree of polymorphism, particularly the class I and class II antigens, in that several allelic variants exist within the global population. There are currently 3,399 *HLA-A*, 4,242 *HLA-B*, and 2,950 *HLA-C* different alleles described (17). The class II genes exhibit a similar degree of polymorphism. Currently 911 and 644 variants have been identified for *HLA-DQB1* and -*DPB1* respectively and 69 and 43 alleles for *HLA-DQA1* and -*DPA1* respectively (17). HLA-DR exhibits considerably more polymorphism than DP and DQ with 1883 and 7 known alleles for *HLA-DRB1* and -*DRA* respectively (17). Since only 7 alleles are known for *DRA* (2 of which encode functional proteins), much of the DR polymorphism is a result of the *DRB1* gene. The DR region is further complicated due to the fact that a second DRB gene may

be expressed depending on the allele expressed at the *DRB1* locus. With the exception of the DRB1*01, *08 and *10 alleles, all haplotypes contain a second DRB allele in addition to *DRB1* (18). For example, the *DRB3* gene is expressed when one or two of the DRB1*11, *12, *13, *14, *17 or *18 alleles are expressed at the *DRB1* locus. The *DRB4* gene is expressed when one or two of the DRB1*04, *07 or *09 alleles are expressed at the *DRB1* locus. Finally, the *DRB5* gene is expressed when the allele DRB1*15 or *16 is expressed at the *DRB1* locus. Several different alleles for *DRB3*, *DRB4*, and *DRB5* exist within the population, albeit not nearly as polymorphic compared to *DRB1*. Currently, 77, 24, and 26 alleles have been described for *DRB3*, *DRB4*, and *DRB5* respectively (17). The extensive polymorphism of the MHC decreases the likelihood that two unrelated individuals express the same class I or II antigens. This genetic diversity confers protection against pathogens by a population as a whole by increasing the chances that an immune response against a given pathogen is generated in a proportion of the population and therefore eliminated.

Due to the complexity of the class II antigens, particularly the DR loci, the system of HLA nomenclature has evolved over the years. Initial nomenclature of HLA was based on the identification of different HLA alleles using serological techniques (19). However, with the advent of molecular biology, several more allelic subtypes were discovered which could be distinguished using the serologically defined specificities. For example, under the old classification system, two individuals may be identified as carrying the DR4 haplotype, however, they may carry very different alleles at the *DRB1*04* locus. The new nomenclature system is based on DNA sequences of alleles and is able to accommodate the inadequacies of the old system (20). Using HLA-DRB1*04:01 as an example, 'HLA'

indicates the HLA region; ‘DRB1’ indicates the particular HLA locus; ‘04’ indicates the group of alleles which encode the DR4 antigen; ‘01’ indicates the specific allele. Additional notation is included to identify mutations and differences in level of expression.

1.1.3 General function of HLA class I and II molecules

As stated above, both HLA class I and II molecules bind and present antigen-derived peptides to CD8+ and CD4+ T lymphocytes respectively. During the early stages of infection, an innate immune response is mounted against the pathogen. An important feature of this early response is that proteins derived from the pathogen are taken up and degraded by professional antigen presenting cells (pAPC) such as dendritic cells, and subsequently presented on their surface in complex with class I or class II molecules (21). Within the lymph nodes, dendritic cells expressing these MHC molecules in complex with antigenic peptides on their surface are able to interact with naive CD4+ (in the case of class II) or CD8+ (in the case of class I) T lymphocytes which bear the appropriate T cell receptor (TCR) leading to activation, proliferation, and differentiation of these cells, eventually mounting an adaptive immune response against the pathogen (21). In order for differentiated T lymphocytes to exert their effector functions as the adaptive immune response progresses, it is necessary that they recognize their corresponding pMHC on infected or accessory immune cells. Furthermore, both class I and class II molecules are crucial for the development of immune cells in the thymus acting as ligands for positive and negative selection of lymphocytes (22). Therefore, class I and II molecules are

responsible for generating the repertoire of circulating T lymphocytes, provide a vital link between innate and adaptive immunity, and at the same time are required for initiating the effector mechanisms of the adaptive immune response.

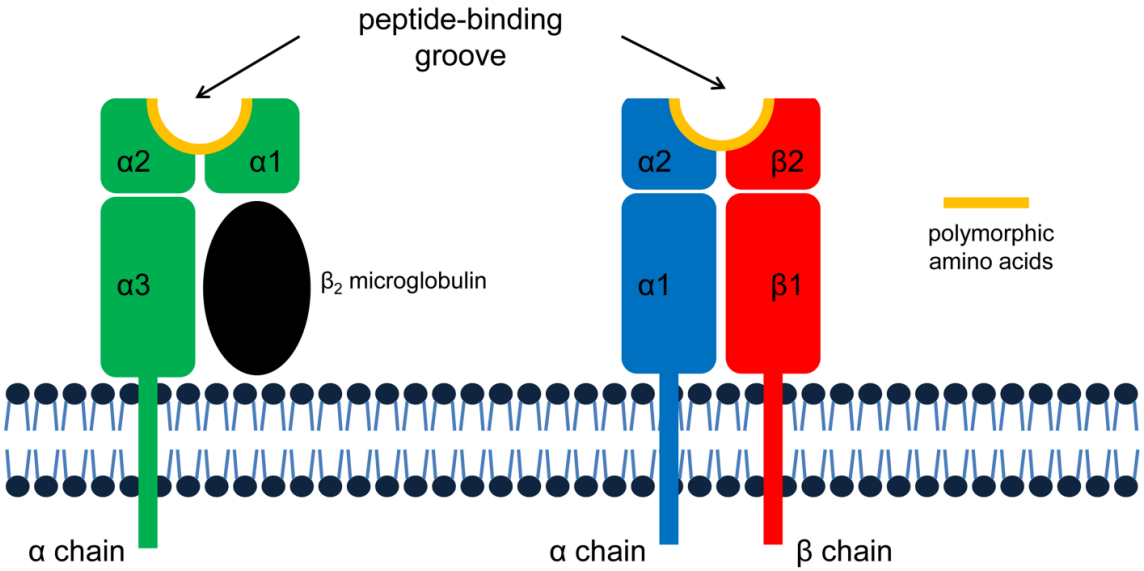
1.1.4 HLA class I structure and antigen processing pathway

HLA-A, -B, and -C are glycoproteins expressed by all nucleated cells within the body and are heterodimers consisting of a polymorphic 45 kDa α chain, encoded by the *HLA-A*, *-B*, and *-C* genes, and a nonpolymorphic 12 kDa protein B2M encoded outside the MHC on chromosome 15 (Figure 1.2). The α chain contains three domains α1, α2, and α3, with α1 and α2 forming a peptide binding groove that is distally oriented from the plasma membrane (23). Peptides 8 to 11 amino acids in length are able to interact with the peptide binding groove resulting in the formation of a stable pMHC that survives trafficking through the secretory pathway and prolonged display at the cell surface (25). The amino acid variability that exists as a result of the allelic polymorphisms is concentrated in the domains forming the peptide binding groove, more specifically, at residues which line the groove. Therefore, the repertoire of peptides able to bind this groove are entirely dependent on the sequence motif of the class I molecule.

HLA class I molecules are synthesized *de novo* in the endoplasmic reticulum (ER) where several co-chaperone molecule assist in assembly and peptide loading (26). After translocation to the ER, the class I α chain associates with the chaperones calnexin and ERp57 which facilitate binding of B2M. Binding of calnexin and ERp57 promote the correct folding of the free α chain and retains it in the ER. The α chain eventually

Figure 1.2. Schematic representation of the structure of HLA class I and HLA class II molecules.

HLA class I molecules are heterodimers consisting of a 45 kDa α chain and a 12 kDa protein B2M. The α chain contains three domains a1, α2, and α3, with α1 and α2 forming a peptide binding groove that is distally oriented from the plasma membrane. HLA class II molecules consist of a 35 kDA α and 29 kDa β chain forming a heterodimeric glycoprotein. Both the α and β chain contain a membrane-proximal domain termed a2 and b2 respectively. The membrane-distal domains of the α and β chains, termed α1 and β1 respectively, form a peptide binding groove. The amino acid residues that contribute to formation of the peptide binding groove of both class I and class II molecules are highly polymorphic in that the main differences in amino acid sequence of different class I and class II alleles are within this region. Adapted from (24).



HLA Class I **HLA Class II**

associates with B2M followed by formation of a peptide loading complex where calnexin-ERp57 is replaced by calreticulin-ERp57 and the entire complex associates with the transmembrane protein tapasin which in turn is associated with the peptide transporter TAP. This complex stabilizes the peptide receptive class I molecule so that peptides are able to bind (27). Tapasin also acts to optimize the repertoire of peptides bound to the class I by exchanging low affinity peptides in favor of peptides that bind with a higher affinity and thus are more stable upon egress of the pMHC-I from the ER (28, 29). TAP, functioning as an ATP-binding cassette transporter, allows transport of peptides approximately 8 to 16 residues in length into the ER from the cytosol for potential binding to MHC-I. Upon successful peptide binding, the co-chaperones and peptide loading machinery dissociate from the pMHC-I and the stable molecule is transported to the plasma membrane via the secretory pathway.

The majority of peptides available for binding to MHC-I are generated in the cytosol by the proteasome and then transported into the ER by the TAP complex as described above. Therefore, the source proteins of the peptides presented by MHC-I are primarily of intracellular origin and include endogenous and viral antigens. MHC-I peptides can also be generated in a proteasome-independent manner in the cytoplasm by proteases such as calpains, tripeptidyl peptidase II, leucine aminopeptidase, bleomycin hydrolase and puromycin-sensitive aminopeptidase and in the ER by ER-associated aminopeptidase or ER aminopeptidase 1 and 2 (30, 31).

Dendritic cells and macrophages are able to present antigens from their extracellular environment on MHC-I molecules in a process known as cross-presentation. Exogenous antigens taken up by endocytosis or phagocytosis can be transferred into the

cytosol for degradation followed by transport into the ER for MHC-I presentation. Alternatively, these antigens may be degraded by endosomal proteases and bind to MHC-I in recycling endosomes or the ER following back-fusion of endocytic vesicles. In either case, cross-presentation is an important mechanism in the immune surveillance of tissues where cancers and viruses can be detected even if they do not directly infect dendritic cells. Furthermore, this mechanism enables the immune system to monitor bacterially infected pAPC by allowing cytotoxic T cells to eliminate cells harboring intracellular bacteria within their phagosomes (31).

1.1.5 HLA class II structure and antigen processing pathway

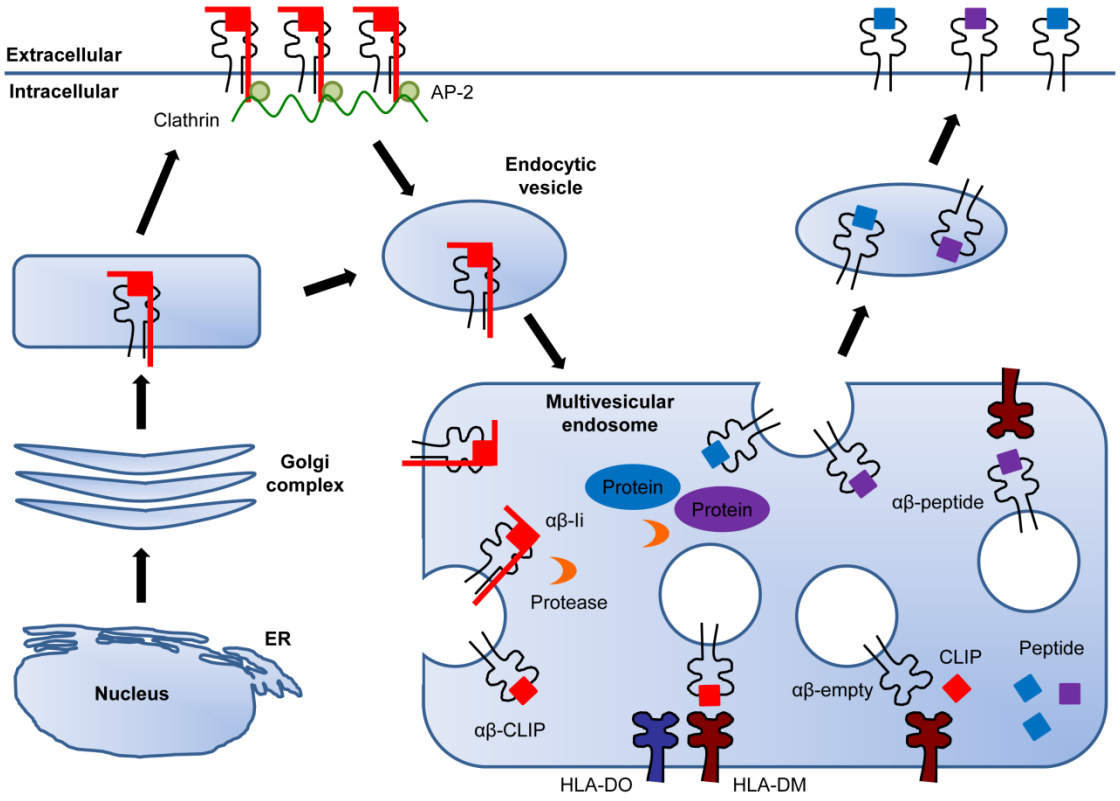
HLA class II antigens HLA-DR, -DP, and -DQ consist of a non-polymorphic 35 kDA α and polymorphic 29 kDa β chain forming a heterodimeric glycoprotein (Figure 1.2). Similar to the HLA-I α3 domain, HLA-II α and β chains contain a membrane-proximal domain designated α2 and β2 respectively. The membrane-distal domains of the α and β chains, termed α1 and β1 respectively, form a peptide binding groove similar to that of HLA-I molecules. Similar to MHC-I molecules, the amino acid variability that exists as a result of MHC-II allelic polymorphism is concentrated at residues which form the peptide binding groove. However, the MHC-II binding groove differs from the MHC-I groove in that a) it is formed by a combination of two chains instead of one and b) the terminal ends of the groove are open allowing longer peptides to bind, typically 13 to 25 amino acids in length.

Compared to HLA-I, the expression of the HLA-II antigens HLA-DR, -DP, and -DQ is more restricted, with constitutive expression largely confined to pAPC and thymic epithelial cells. This is due to the transcriptional control of class II genes by the class II MHC transactivator (CIITA) which also exhibits similar tissue restricted expression. CIITA is referred to as a transcriptional coactivator because it does not directly bind to DNA but instead initiates transcription of class II molecules by binding several transcription factors including RFX, CREB, and NF-Y which are bound to regulatory elements in the promoters of MHC-II genes. The expression of CIITA can be upregulated by IFN-γ, resulting in increased expression of MHC-II in immune cells and induction in endothelial and fibroblast cells. More recently, chromatin-modifying events such as histone acetylation, methylation, deacetylation, and ubiquitination have been suggested as additional regulators of class II and CIITA expression, suggesting that epigenetic events are additional influences in the MHC-II presentation pathway (32).

A general schematic of the HLA class II antigen processing pathway is shown in Figure 1.3. MHC-II α and β chains are synthesized in the endoplasmic reticulum, where three αβ dimers associate with three invariant chain (Ii) proteins forming nonameric $(\alpha\beta)_3Ii_3$ complexes (33). The function of Ii is twofold, (a) to stabilize MHC-II dimer formation and prevent premature binding of peptides to the peptide binding groove in the ER and during transport through the endocytic pathway and (b) to target MHC-II to lysosome-like late endosomal compartments (34). The Ii chain contains a di-leucine motif in the N-terminal cytoplasmic domain which is required for targeting Ii and associated MHC-II to late endosomal compartments (35, 36). This can occur by direct targeting after

Figure 1.3. Schematic representation of the HLA class II antigen processing and presentation pathway.

Newly synthesized MHC-II αβ heterodimers associate with the invariant chain (Ii) in the endoplasmic reticulum. After travelling through the Golgi apparatus, Ii-MHC-II complexes are either directly transported to late endosomal compartments or first to the plasma membrane where they are internalized by clathrin-mediated endocytosis. These late endosomal vesicles have a low pH and are enriched in several proteolytic enzymes and antigen presentation accessory molecules, and are commonly referred to as multivesicular endosomes, multivesicular bodies (MVB), or MHC-II loading compartments (MIIC) in pAPCs. Ii is sequentially degraded by proteolytic enzymes within MIIC leading to the generation of a fragment of Ii, termed class II-associated invariant chain peptide (CLIP), which remains in the peptide binding groove of MHC-II molecules. CLIP is released from the peptide binding groove by HLA-DM, which subsequently facilitates peptide binding resulting in the formation of a stable peptide-MHC-II complex. The activity of DM is regulated by HLA-DO. After stable peptide binding, mature MHC-II molecules are transported to the plasma membrane for presentation to T lymphocytes. Adapted from (44).


Extracellular
Intracellular
Clathrin
AP-2
Endocytic vesicle
Golgi complex
ER
Nucleus
Multivesicular endosome
Protein
Protein
αβ-Ii
Protease
αβ-peptide
αβ-CLIP
αβ-empty
CLIP
Peptide
HLA-DO
HLA-DM

exiting the trans-Golgi network, or by clathrin-mediated endocytosis from the plasma membrane (37-39). Following endocytosis, Ii-MHC-II complexes traffic through the endocytic pathway with the final destination being late endosomal-lysosomal antigen processing compartments which contain antigenic proteins and peptides (40).

Although peptide-MHC-II complexes (pMHC-II) can be produced at several locations throughout the endocytic pathway (41), the typical antigen-loading compartments are specialized late endosomal organelle-like vesicles which have a low pH and are enriched in several proteolytic enzymes (40, 42, 43). Electron microscopy initially revealed an abundance of MHC-II concentrated in these late endocytic vesicles, and so they were termed MHC- II peptide loading compartments, or MIICs (45-47). In pAPCs, MIICs are heterogeneous in their morphology, contain several internal complexes, and are all forms of multivesicular bodies, or MVBs (13, 43). MVBs are so named due to the presence of intraluminal vesicles (ILVs) which are formed by the inward budding of the vesicle's limiting membrane and contain several of the necessary co-molecules required for optimal antigen processing and presentation (48, 49). Proteins destined for degradation in lysosomes are also transported to these MHC-II-enriched late endosomes where they are cleaved into shorter peptides by the acidic proteolytic environment.

At the same time, some of these endolysosomal proteases degrade the Ii chain of Ii-MHC-II complexes, leaving a short peptide fragment termed the class II associated invariant chain peptide (CLIP) in the MHC-II peptide-binding groove (50). Ii is sequentially degraded from its full length, which differs in size due to several isotypes, to the intermediates LIP (22 kDa), SLIP (10 kDA), and eventually CLIP (51, 52).

Depending on the cell type, several proteases have been implicated in these cleavage steps. In pAPC, cathepsin S has been shown to be responsible for late stage Ii degradation of the SLIP intermediate to CLIP (53, 54), whereas in thymic epithelial cells cathepsin L is mainly responsible for this cleavage step (55-59). Furthermore, cathepsin S, not cathepsin L, can degrade Ii in non-professional APC such as epithelial cells (60). Less is known concerning the proteases responsible for full length and intermediate (LIP – SLIP) cleavages; however, evidence suggests that multiple proteases can contribute (61). An unidentified aspartic protease and asparaginyl endopeptidase (AEP) in mice can initiate Ii processing (62, 63). However, others have demonstrated that AEP is dispensable and that initial Ii processing is redundant and may depend on the MHC-II allele expressed by the cell (64). The redundancy in Ii processing is likely a reflection of the differential expression of proteases across APC types. However, in all cases the end result is the creation of a MHC-II-CLIP complex, which serves as the template for MHC-II peptide loading.

CLIP must be removed from the peptide-binding groove of MHC-II to allow the binding of antigenic peptides. This process is facilitated by the MHC-II-like molecule HLA-DM (DM), which is present in the MVB internal and limiting membranes, and interacts with MHC-II-CLIP complexes that have been sorted onto ILVs within these vesicles (13). DM acts as an enzyme to catalyze the exchange of CLIP for peptides within MIIC by facilitating CLIP release, stabilizing the peptide-receptive MHC-II, and enabling the stable binding of peptide species resulting in the formation of stable pMHC-II (65-67). HLA-DO, a second MHC-II-like molecule expressed in B lymphocytes, thymic medullary epithelial cells, and primary dendritic cells inhibits DM activity by directly

binding it and preventing it from interacting with MHC-II (65, 68, 69). Following formation of stable peptide-loaded MHC-II, they are transported from MVB in tubulovesicular endosomes to the plasma membrane where they interact with T cell receptors (TCR) on CD4+ T lymphocytes.

Once expressed on the cell surface, pMHC-II can be internalized through a clathrin-independent endocytosis pathway, resulting in either transportation to lysosomes for eventually degradation, or to early endosomes where they are recycled back to the plasma membrane (13). Although some pMHC-II are exclusively generated with MIIC as described above, other pMHC-II may be generated in these early endosomes in a process that is independent of nascent MHC-II synthesis, Ii-mediated transport, and DM peptide editing, and involves limited proteolytic processing (70-73). Furthermore, recycling pMHC-II may also return to conventional MIIC, resulting in the formation of new epitopes (74).

1.2 HLA class II antigen presentation

1.2.1 Role of HLA-DM and HLA-DO

As mentioned above, both DM and DO act together in pAPC to regulate the loading of peptides derived from foreign and self antigens onto MHC-II. DM acts as a catalytic enzyme that binds transiently to MHC-II complexes resulting in the destabilization hydrogen bonding networks causing the release of CLIP, then stabilizing MHC-II dimers in an open conformation allowing repeated binding and dissociation of peptides, ultimately resulting in accumulation of stable pMHC-II with high affinity

peptides (14, 75-77). Spontaneous dissociation of CLIP from MHC-II can also occur and is dependent on the binding affinity of CLIP for the particular MHC-II allele (78, 79). CLIP has been shown to dissociate more rapidly from DRB1*04:01 and DRB1*04:04 compared to DRB1*04:02 (78). Until recently, the precise mechanism by which DM binds MHC-II to facilitate peptide exchange was not clearly known; however, two recent studies which have identified the crystal structure of DM-MHC-II complexes provide some important clues (80, 81). Upon binding DR, DM does not appear to undergo any significant conformational changes when compared to crystal structures of DM alone (82-84). However, DM induces a dramatic conformational alteration in DR at residues 35 to 57 of the MHC-II α subunit, which is important in forming part of the floor of the peptide binding groove, as well as the P1 pocket (80). The P1 pocket and hydrogen bonds in this region are important for providing a stable interaction between peptide and the MHC-II molecule. These findings provide a direct molecular mechanism for the action of DM, where DM binding destabilizes pMHC-II by interfering with crucial peptide – MHC-II binding interactions (65).

The importance of DM in autoimmunity is demonstrated by several studies that have reported a significant role for DM activity in the development of type 1 diabetes (85, 86). Eliminating DM-function in the NOD mouse model for type 1 diabetes blocked the development of diabetes (86), suggesting that DM is responsible for the presentation of disease-initiating peptides. In contrast, other studies support a view where DM is responsible for editing out disease-causing peptides, and disease development is due to the generation of an epitope in recycling endosomes where DM activity is limited (87, 88).

As stated above, DM function is regulated by the class II-like molecule DO, which is expressed in B lymphocytes, thymic medullary epithelial cells, and primary dendritic cells (68, 69). DO acts as a competitive inhibitor of DM by directly binding it and preventing interaction of DM with MHC-II-CLIP and pMHC-II complexes (89-91). Interestingly, DO is unstable in the absence of DM and may require association with DM in order for egress from the ER (92). Expression of DO is modulated during B cell differentiation, where a reduction in DO expression is observed in germinal center B cells compared to naïve and memory B cells (93). Several studies have identified roles for DO in regulating antigen presentation and influencing the repertoire of MHC-II-bound peptides (94-98). DO-deficient cells present endocytosed antigens internalized by fluid-phase endocytosis more efficiently than in the presence of DO. Furthermore, presentation of antigens internalized by surface immunoglobulin was improved in the presence of DO. These results suggest that the function of DO is to focus presentation on antigens that have been internalized by surface immunoglobulin (94). More recently, over expression of DO in DCs in the NOD mouse model for type 1 diabetes blocked development of diabetes suggesting that by inhibiting DM function, DO prevents the presentation of self antigens by possibly maintaining a broad peptide repertoire (99). The importance of DO in regulating autoimmunity is perhaps most apparent in a recent study utilizing H2-O (DO in mouse) knockout mice (100). Whereas only a few limited changes were observed in H2-$O^{-/-}$ mice in previous studies (94, 95), Gu *et al*. demonstrated that DO knockout mice exhibit an autoimmune phenotype characterized by spontaneously development of higher titers of anti-nuclear antibodies and delayed antibody responses to model antigens,

suggesting that DO decreases immunity to self antigens while increasing immunity to foreign antigens (100).

Since both DM and DO have a profound effect on the repertoire of peptides presented by MHC-II molecules, much attention has concentrated on determining the effect of these molecules on the presentation of specific peptide sequences (65, 90, 101). Based on the effect of DM on presentation, peptide antigens or epitopes can be divided into 3 groups (102-104). For one group of peptides, termed DM-resistant, presentation can occur in both the presence and absence of DM. Since DM does not affect presentation of these antigens, DM-resistant epitopes are expressed on all MHC-II+ cells (102). In contrast, DM-sensitive epitopes are suppressed by DM, and are thus only expressed in the absence of DM. Under circumstances of DO co-expression with DM as in pAPC, the presentation of DM-sensitive antigens can be restored (102). Finally, there is a category of peptides that are presented only in cells expressing DM, termed DM-dependent epitopes (103). A main factor determining whether a particular epitope is DM-resistant, -sensitive, or -dependent might be the affinity of the peptide for the specific MHC-II molecule (103). In summary, both DM and DO contribute to the peptide repertoire presented by MHC-II and further research will clarify the physiological roles of these molecules in maintaining tolerance and development of autoimmunity.

1.2.2 Proteolytic processing of peptide antigens in the endocytic pathway

APCs use several cellular processes to capture external antigens and deliver them to antigen loading compartments for processing and presentation by MHC-II, including

macropinocytosis, receptor-mediated endocytosis, and phagocytosis (40). Internalized antigens eventually enter acidic late endocytic vesicles containing proteases and reductases responsible for antigen denaturation and proteolysis. Proteins contain multiple sequences that are able to bind MHC-II, but only a few are finally presented to T cells. In the hierarchy of epitope presentation, immunodominant epitopes are important for immunity to pathogens, whereas subdominant and cryptic epitopes are associated with autoimmunity (105). The expression of a specific pMHC-II epitope is dependent on various cellular factors including the route of antigen entry, mechanism of peptide loading, the intracellular location of degradation, and perhaps most importantly, proteolytic processing (61, 106-108). Multiple endocytic proteases have been shown to contribute to processing antigens for specific pMHC-II presentation, including cathepsins B, D, L, S, and AEP (109). These proteases are typically classified according to their proteolytic activity, the major species active within pAPC being cysteinyl and aspartyl and either endo-, carboxy, - or aminopeptidases (110). Evidence to date suggests that generation of some CD4+ T cell epitopes may require the action of specific proteases while other epitopes may exhibit less strict processing requirements (61, 106, 109, 111). This was initially suggested following studies where treatment of pAPCs with inhibitors of endolysosomal proteases could either enhance or inhibit the presentation of epitopes (112). Redundancy in antigen processing is supported by the observations that knock-out mice for specific members of the cathepsin family of proteases have normal immune cell numbers and similar peptide repertoires in terms of complexity compared to wild type mice (113-115). Furthermore, several studies have highlighted instances where one protease may compensate for lack of another (61, 106, 109, 116). For example, analysis

of peptides eluted from MHC-II I-Ab-expressing embryonic fibroblast cells expressing either cathepsin S or L revealed that the majority of peptides are presented irrespective of the cathepsin expressed (117). However, this study also identified a subset of peptides that were positively or negatively regulated depending on the cathepsin expressed, suggesting that individual proteases may contribute to the generation of specific peptides for MHC-II presentation (117).

Several studies have investigated the effect of cathepsins on the generation of specific epitopes (57, 61, 106, 110, 116, 118). For example, cathepsin B and S were shown to mediate the degradation of ^{125}I-labeled $F(ab)_2$ fragments, whereas cathepsin D and L were dispensable (119). Additional studies have investigated the role of cathepsins in the presentation of the immunodominant epitope from tetanus toxoid, with cathepsin D, E, and B implicated in generating the appropriate antigenic peptides (120-122). Furthermore, analysis of the protease processing requirements of hen egg lysozyme (HEL) demonstrated a reduction in expression of two H-2^b-restricted T cell epitopes in the absence of cathepsin S in murine B cells (123). Endolysosomal proteases other than members of the cathepsin family, such as AEP and the IFN-γ inducible thiol reductase (GILT), can also contribute to the generation of class-II epitopes (124-128). Other studies propose that cytoplasmic processing events may also be required for the formation of some MHC-II epitopes (30, 129).

Endolysosomal proteases can also contribute to the destructive processing of peptide epitopes, where lysosomal proteolysis of a protein can perturb the generation of some peptides epitopes (130, 131). For example, presentation of an I-A^b-restricted immunoglobulin M epitope was inhibited by cathepsin S and L expression (117).

Expression of a DRB1*15:01-retricted myelin basic protein epitope (MBP_{85-99}) was reduced in the presence of cathepsin G in human B lymphocytes, suggesting that this protease contributes to the destructive processing of the immunodominant epitope (132). Whereas the previous study demonstrated that AEP was dispensable in the processing of MBP, a second study demonstrated that this epitope is more efficiently presented in the absence of AEP due to a destructive cleavage by AEP within MBP_{85-99}(60). AEP is highly expressed in thymic APC (133) and cathepsin G in peripheral DC (134), suggesting tissue-specific processing of the same antigen can occur and thus may contribute to the development of tolerance or autoimmunity (109, 135).

Whereas the majority of peptides destined for MHC-II presentation are localized and processed in endocytic vesicles as described above, approximately 10-30% of the peptides bound to MHC-II are derived from nuclear or cytoplasmic proteins (136, 137). Within APC, the delivery of proteins and peptides from the cytoplasm and nucleus to the endosomal network is mediated by autophagy (137). Macroautophagy is a process where cytoplasmic material, including organelles, is engulfed by membranes resulting in the formation of autophagosomes which can fuse with lysosomal antigen-processing endosomes to form autophagolysosomes (138, 139). Chaperone-mediated autophagy, where cytoplasmic chaperones including Hsc70 and Hsp90 in conjunction with MIIC transmembrane protein marker LAMP-2A selectively deliver peptides to late endosomes, also contributes to the MHC-II peptide repertoire (140). Furthermore, cytoplasmic antigens can be captured by microautophagy for transport into endosomes, although the contribution of this process to the antigen presentation is less clear compared to the other forms of autophagy (141). The importance of autophagy in the generation of specific

MHC-II epitopes is highlighted by the finding that autophagy can cause the generation of citrullinated proteins and peptides by peptidylarginine deiminase (142). The presentation of citrullinated peptides by MHC-II has been linked to the pathophysiology of autoimmune rheumatoid arthritis (RA) (143). Macroautophagy-deficient dendritic cells are also unable to activate herpes simplex virus-specific CD4+ T cells, suggesting that autophagy may be required for generation of antigen-specific MHC-II epitopes in the periphery (144).

1.2.3 MHC-II trafficking and presentation at the plasma membrane

The precise mechanisms that regulate transport of peptide-loaded MHC-II from antigen-loading compartments to the plasma membrane are poorly understood and most likely differ between different types of APC (13). Kleijmeer *et al*. found that maturation of DCs resulted in a loss of ILVs from MVBs, suggesting that pMHC-II containing intraluminal vesicles back-fuse with the limiting membrane of MVB (145). However, this study did not rule out the degradation of ILVs or their release as exosomes. Once redistributed to the limiting membrane of MVBs, pMHC-II traffic into tubularvesicular endosomal structures which fuse with the plasma membrane (146-148). Movement of these tubular endosomes from MIIC to the plasma membrane involves several molecules including Rab GTPases, actin-dependent motor proteins, and cytoskeletal proteins (149).

At the cell surface, a substantial proportion of pMHC-II cluster within distinct membrane microdomains including lipid rafts and tetraspanin-enriched microdomains (150, 151). Lipid rafts are cholesterol-rich stable lipid domains found in all cell types

which provide membrane organization in a less structured plasma membrane. Constitutive association of pMHC-II with lipid rafts has been detected in B cells, DCs, monocytes, macrophages, and thymic epithelial cells (152). Tetraspanin-enriched microdomains (TEMs), or tetraspanin webs, have also been shown to be responsible for the clustering of pMHC-II (153).Incorporation of pMHC-II into membrane microdomains is hypothesized to be functionally important for T cell activation in cases where there are low levels of a specific pMHC-II on the surface of APC (154-156). Activation of antigen-specific CD4+ T cells requires T cell receptor-mediated recognition of cognate MHC-II loaded with a specific peptide. The repertoire of peptides eluted from MHC-II on APC is complex (157, 158), but T cells are somehow able to find their rare cognate pMHC-II. Studies estimate that CD4+ T cells require as little as 200 specific pMHC-II complexes per APC to be activated (159-161), and a more recent study demonstrates that as few as 10 pMHC-II are able to stimulate a T cell response (162). Given that APCs contain upward of 10^5 pMHC-II molecules on the plasma membrane at a given time, the ability of a T cell to recognize enough individual cognate pMHC-II to induce activation is difficult to imagine. It has thus been proposed that identical pMHC-II complexes are clustered into lipid rafts during transport to the cell surface of APC thus making TCR recognition of relevant pMHC-II more efficient (152, 163).

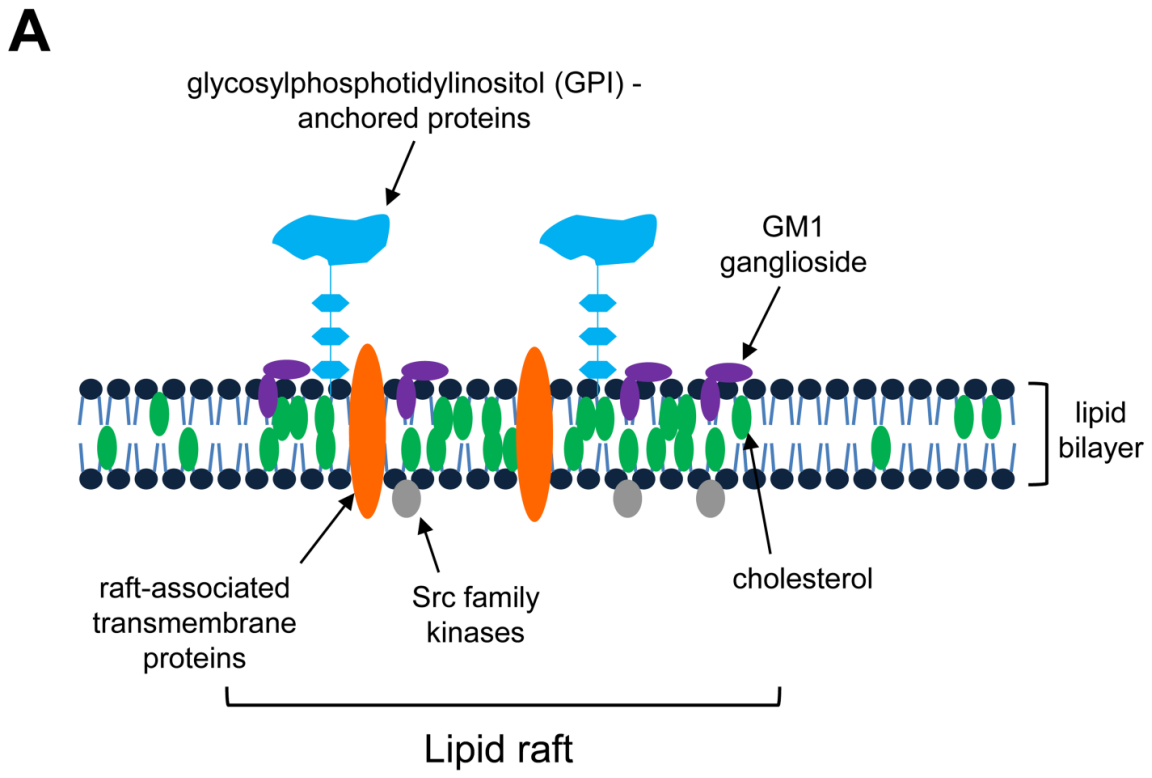
1.2.4 Role of membrane microdomains

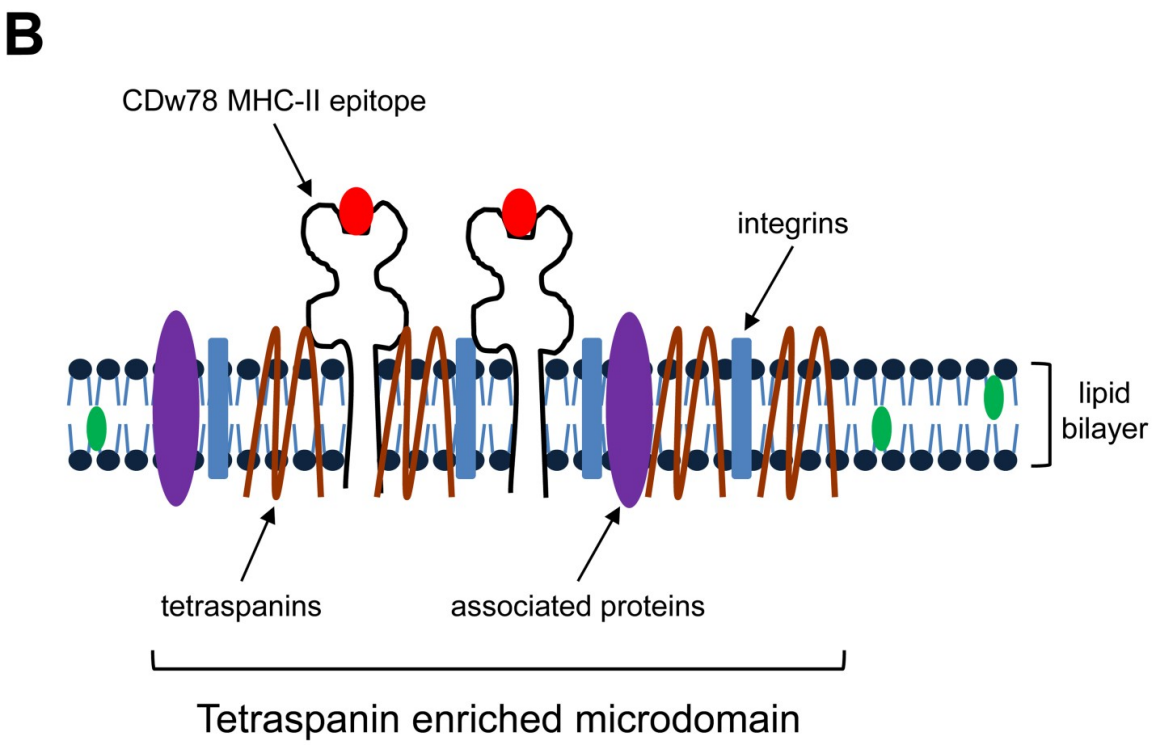
1.2.4.1 Lipid rafts

Initial descriptions of the biological membranes described a membrane where all lipid and protein molecules were free to diffuse throughout the structure independent of other molecules in what was regarded as the fluid mosaic model (164). This model has since evolved and it is now generally accepted that membranes are not homogenous, but instead contain distinct and specialized membrane microdomains that are essential in a variety of cellular processes (165). Perhaps the most well studied membrane microdomains are lipid rafts, also known as detergent-insoluble glycolipid-enriched complexes (DIGs) or detergent-resistant membranes (DRMs) (166, Figure 1.4A). The alternative name DRMs is based on their insolubility in mild nonionic detergents such as Triton X-100 at low temperatures, which is likely a product of their highly organized structure and increased concentration of cholesterol (166). Lipid rafts are enriched in cholesterol, sphingomyelin, and glycosphingolipids (167, 168), resulting in a highly ordered structure compared to the adjacent cell membrane. They are also enriched in glycosyl-phosphotidylinositol (GPI)-anchored proteins and certain transmembrane proteins depending on cell type (169, 170). Lipid rafts are involved in a variety of cellular processes including signal transduction, endocytosis, vesicular trafficking, and cell adhesion (171, 172). A well described function of lipid rafts in the immune response is their role in the recruitment and concentration of intracellular signaling molecules in the case of T cell activation (173). Several proteins responsible for regulating T cell activation including the Src family kinase Lck and the adapter protein LAT are

Figure 1.4. Schematic diagram of (A) a lipid raft and (B) a tetraspanin-enriched microdomain in the cell plasma membrane.

(A) Lipid rafts are enriched in cholesterol, sphingomyelin, glycosphingolipids, glycosyl-phosphotidylinositol (GPI)-anchored proteins, and certain transmembrane proteins depending on the cell type (167-170). **(B)** Tetraspanin-enriched microdomains (TEMs) are characterized by their enrichment in tetraspanin proteins (179). Several members of the integrin family of membrane proteins are known to associate with tetraspanin proteins and are concentrated in TEMs (180). Depending on the cell type, certain cell-specific membrane proteins have been found to cluster in TEMs, including MHC-II molecules in APCs (179, 181, 182). Most relevant to this study, TEMs were also shown to cluster on the cell surface of APC with a select set of peptide-MHC-II containing the CDw78 epitope (153).


A
glycosylphosphotidylinositol (GPI) - anchored proteins
GM1 ganglioside
lipid bilayer
raft-associated transmembrane proteins
Src family kinases
cholesterol
Lipid raft


B
CDw78 MHC-II epitope
integrins
lipid bilayer
tetraspanins
associated proteins
Tetraspanin enriched microdomain

concentrated in lipid rafts along the intracellular face of the plasma membrane and several studies have demonstrated the importance of these lipid rafts in T cell activation (174, 175). Early studies utilizing electron microscopy and confocal microscopy revealed that pMHC-II were not uniformly distributed on the cell surface, but instead are found in small clusters (176, 177). The first indication which implicated the involvement of lipid rafts in this clustering was the observation that antibody cross-linking of pMHC-II induced association of MHC-II with Triton X-100 insoluble lipid rafts (178). An additional study by Hiltbold *et al.* confirmed pMHC-II association with rafts since pMHC-II clusters co-localized with cholera toxin B subunit, a typical lipid raft marker that binds GM1 glycosphingolipids (150). Further evidence which suggests clustered pMHC-II represent lipid raft-associated pMHC-II is that treatment with methyl-beta-cyclodextrin (MBCD), a chemical that disrupts protein association within lipid rafts by sequestering cholesterol from the plasma membrane (183), caused a redistribution of pMHC-II from their clustered formation (156).

Additional studies by Roche and colleagues and other laboratories have resulted in several important findings regarding the importance of lipid rafts in antigen presentation (150, 152, 154, 184). A substantial fraction of MHC-II is constitutively associated with lipid rafts in APC, including Epstein-Barr virus (EBV)+ and EBV- B cell lines (154, 185, 186), primary B cells (187), monocytes (188), and DCs (155, 156, 189, 190), indicating that MHC-II association with lipid rafts is not restricted to particular cell lines or types. For example, approximately 50% of surface MHC-II was found to reside in membrane rafts in both human and murine B cells (154). MHC-II molecules also colocalize with conventional raft markers, including GM1-ganglioside, in Triton X-100-resistant

membranes on the surface of intact B cells (150). Disruption of rafts with MBCD results in an approximate 60% reduction in the total amount of MHC-II associated with rafts (154). In the same study, treatment with MBCD inhibited the ability of B cells to stimulate antigen-specific T cells in conditions of low antigen dose. Since MBCD did not disrupt the formation of SDS-stable MHC-II dimers or total MHC-II surface expression, this suggests that lipid rafts concentrate pMHC-II at the plasma membrane of APC prior to T cell contact thereby allowing efficient antigen specific T cell activation at low antigen availability (154). APC lipid rafts containing pMHC-II traffic to the immunological synapse during the initial stages of T cell interaction with an APC and relevant pMHC-II are eventually recruited to the central region of the synapse while irrelevant pMHC-II are excluded (150, 191). In summary, these results support the hypothesis the lipid rafts function to concentrate specific pMHC-II at the plasma membrane to facilitate T cell activation.

Despite these studies evaluating the functional role of raft-associated pMHC-II, less is known about what controls the incorporation of pMHC-II into these microdomains. Both palmitoylation and ubiquitylation are two important post-translational modifications that modulate protein targeting to membrane microdomains (192). However, deletion of the cytoplasmic domain of both the α and β chains does not affect recruitment of MHC-II into lipid rafts, suggesting raft recruitment is mediated through interaction with additional molecules or proteins (187). Pulse-chase biosynthetic radiolabeling and protein transport inhibiting experiments have demonstrated that MHC-II become associated with lipid rafts prior to peptide binding, possibly as early as the Golgi apparatus, and up to 60% of these MHC-II remain raft-associated during Ii degradation, peptide loading, and transport to the

plasma membrane (184, 193). Interestingly, one of these studies suggested that MHC-II-bound peptides may modulate the affinity of MHC-II to membrane rafts by inducing conformational changes (193). Some pMHC-II epitopes may exhibit an inherent affinity for membrane lipid rafts, as evidenced by the observation that some anti-pMHC-II mAbs recognize a subset of lipid-raft resident pMHC-II (186). However, the molecular mechanisms governing this possible association are currently not fully understood.

Additional findings suggest that MHC-II may associate with lipid rafts during transport onto ILVs within MVB (152, 194). ILVs contain several of the molecules associated with lipid rafts including cholesterol, sphingomyelin, and gangliosides (195-197). Furthermore, the pMHC-II present on exosomes is detergent insoluble suggesting that exosome-, and thus ILV-associated MHC-II is largely present in lipid rafts (195). Peptide loading is likely to occur on ILV-associated MHC-II, and it has been suggested that fusion of ILV with the MVB limiting membrane occurs prior to transport of mature pMHC-II to the plasma membrane (145). A hypothetical model has been proposed where ILVs containing raft-associated MHC-II containing similar antigenic peptides fuse with the limiting membrane of MVB, followed by outward budding of the MVB and formation of a transport vesicle which eventually docks and fuses with the plasma membrane, thereby delivering raft-associated pMHC-II into the plasma membrane (152). This model is supported by the observation that pMHC-II generated in antigen-loading compartments arrive at the plasma membrane of DCs in small microclusters which resemble lipid rafts (156). While there is much left to be known about the role of lipid rafts in antigen presentation, if the above model holds true, it will represent a mechanism for APCs to temporally and spatially coordinate the presentation of relevant antigens.

1.2.4.2 Tetraspanin-enriched microdomains

In addition to associating with lipid raft microdomains, pMHC-II also bind a class of membrane proteins termed tetraspanins (151, 181, 182), which form larger membrane complexes called tetraspanin-enriched microdomains (TEMs) or the tetraspanin web (198, 199). As their name suggests, TEMs are enriched in tetraspanins, a family of proteins containing four transmembrane domains (Figure 1.4B). A conserved feature of tetraspanin proteins is the presence of a small extracellular loop and large extracellular loop that connects four transmembrane domains (200). These extracellular domains mediate specific protein-protein interactions with laterally associated proteins within the plasma membrane, ultimately resulting in the formation of a larger scaffolding complex. The cytoplasmic regions of tetraspanins contain palmitoylation sites which contribute to the clustering of tetraspanin molecules and mediate associations with cytoskeletal and signaling molecules (201, 202). Tetraspan microdomains are important in several diverse cellular processes including signal transduction, cell proliferation, cell adhesion, cell fusion, cell migration, and host-pathogen interactions (203, 204).

Membrane clustering of TEMs involves several protein-protein interactions that can be classified as primary, secondary or tertiary (199, 205). All three types of tetraspanin interactions contribute to the formation and preservation of TEM structure and function (206). Primary interactions involve the association of tetraspanins with other non-tetraspanin molecules. Tetraspanin molecules can form different primary interactions in different cell types. For example, the tetraspanin CD81 associates with CD19 in B cells, forming part of the coreceptor for antigen recognition by surface immunoglobulin (207). However, CD81 associates with CD4 and CD8 in T cells (208). Tetraspanins

expressed in immune cells are also well known to directly bind a variety of integrins (180). Secondary interactions include associations of tetraspanins with other members of the tetraspanin family and require the palmitoylation of tetraspan cytoplasmic residues to maintain such interactions (209). Whereas primary and secondary interactions involve direct binding of tetraspanins, tertiary interactions involve the indirect association of tetraspanins with additional proteins in TEMs as a result of the clustering of several proteins (205). Similar to lipid rafts, TEMs are enriched in membrane cholesterol and are relatively detergent insoluble (210). Furthermore, the different types of tetraspanin interactions described above can be alternatively classified based on their susceptibility to disruption by different detergents (211).

Tetraspanins are expressed in a variety of cell types and modulate several distinct cellular processes including but not limited to endocytosis, exocytosis, adhesion, migration, signaling, and intracellular protein transport (207). Nearly all cells of the immune system express tetraspanins including CD9, CD37, CD53, CD63, CD81, CD82, and CD151 (179, 207). There are several reports which demonstrate the ability of MHC-II to associate with different tetraspanin family members (153, 179, 182, 212). The endolysosomal tetraspanins CD81 and CD82 co-immunoprecipitate with MHC-II in human B cell lines (182, 198, 212). CD63 associates with peptide-loaded MHC- II within intracellular vesicles of immature DCs, whereas other tetraspanins including CD9, CD53, and CD81 associate with MHC-II at the plasma membrane (181). Furthermore, MHC-II associates with CD9 and CD38 in human monocytes (188), and with CD9, CD53, CD81, and CD82 in human DCs (212).

Tetraspanins were also reported to cluster on the cell surface of APC with a select set of MHC-II containing the CDw78 epitope identified by the mAb FN1 which interacts only with clustered MHC-II epitopes. Immunoprecipitation with this antibody in mild detergents resulted in the enrichment of tetraspanins and DM, compared with an antibody recognizing the entire pool of DM molecules (153). Moreover, these tetraspanin-associated CDw78+ clusters of MHC-II were shown contain a select set of peptides that were functionally enhanced in their ability to activate T cell responses (151, 153, 213). These findings supported the idea that TEMs facilitate pMHC-II clustering into distinct membrane microdomains separate from lipid rafts (153, 214). However, an additional study found no correlation between expression of CDw78 and expression of tetraspanins and instead concluded that rather than defining a unique tetraspanin-associated subset of MHC-II, CDw78 is instead a conformational epitope generated during intracellular trafficking of MHC-II (212). It has been suggested by some that the distinction between lipid raft microdomains and TEMs is artificial and non-existent *in vivo* (152), supported by several studies which have indicated that tetraspanins can be isolated in detergent-insoluble fractions in a cholesterol-dependent manner along with lipid rafts (215, 216).

More recently, Unternaehrer *et al.* have suggested that the ability of distinct forms of mouse MHC-II to cluster is largely dependent on the direct association of MHC-II with the tetraspanin CD9 (217). Conversely, a follow-up study demonstrated that deletion of CD9 and CD81 had no effect on this clustering of MHC-II, and that association with lipid rafts was responsible for this clustering (190). Moreover, an additional study demonstrated that silencing of CD9, CD63, and CD81 actually enhanced MHC-II expression (218). Given the inconsistency in studies which have evaluated tetraspanin

association with MHC-II, the concept that TEMs function to cluster unique functionally-relevant subsets of pMHC-II at the surface of APC is controversial, and the functional role of TEMs in antigen presentation remains uncertain.

1.3 Exosomes

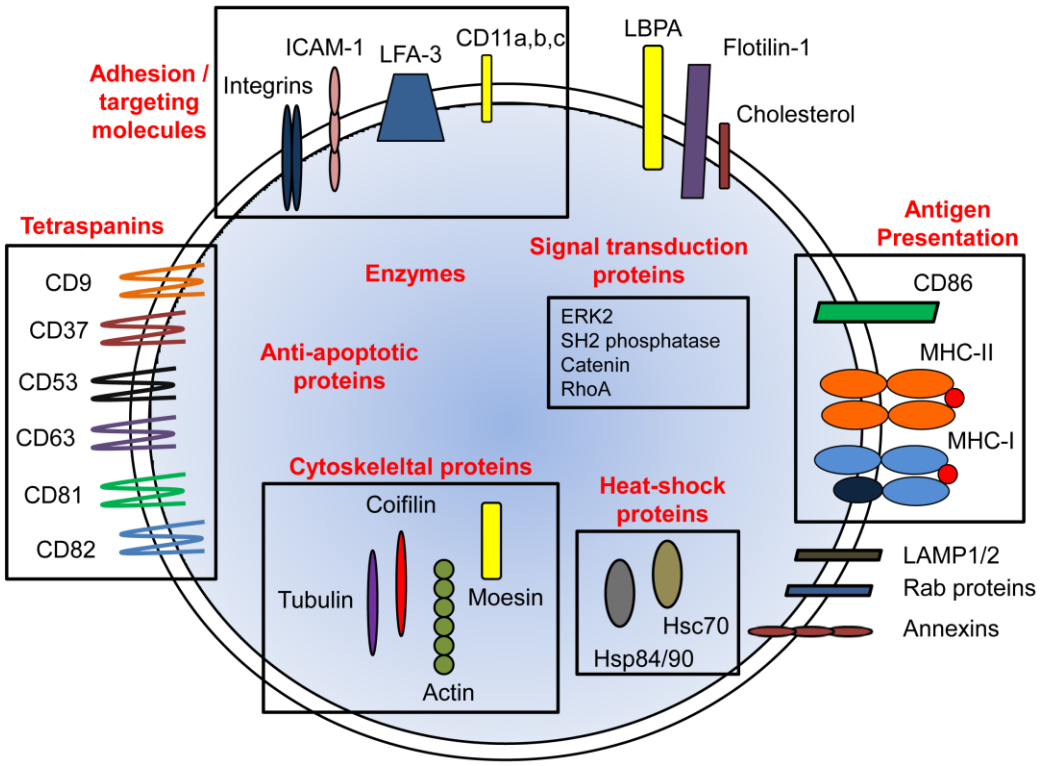
Antigenic presentation of peptides by MHC-II to CD4+ T cells can also occur in the absence of cell-cell contact between an APC and T cell via a mechanism involving membrane vesicles termed exosomes (219-221). As previously mentioned, the typical mode of MHC-II presentation involves the creation of mature pMHC-II on the ILVs within MIICs followed by back-fusion of ILVs with the MIIC limiting membrane and subsequent transport to the cell surface. In some instances, an entire MVB fuses directly with the plasma membrane, releasing its entire contents including ILVs into the extracellular milieu and the secreted ILVs are referred to as exosomes (222). The term exosome was first used to describe microvesicles that were secreted by neoplastic cell lines (223). However, exosomes were not widely studied until EBV-transformed B cells were shown to secrete exosomes that could induce antigen-specific MHC-II-restricted T cell responses (219). These results were further extended to dendritic cells (220, 221). In addition to professional APCs, exosomes are secreted by a wide variety of cell types such as T cells, mast cells, intestinal epithelial cells, and tumor cells (224). The possibility that exosomes are involved in several pathological conditions including the spread of pathogens (225, 226) and modulation of immune responses (227, 228) has stimulated further research on these vesicles.

Exosomes can be distinguished from other membrane vesicles based on their size, density in sucrose, sedimentation value, intracellular origin, and protein and lipid composition (229). Exosomes have been purified from a variety of biological sources including plasma (230), serum (231), urine (232), milk (233), and several body tissue fluids (234-236) suggesting that these vesicles are actively secreted from cells *in vivo*. The protein composition of exosomes is dependent on the cell type which it has been secreted from, but all exosomes share some ubiquitous protein markers. Proteomic analysis of exosomes purified from cultured cells and biological fluids have identified a broad range of exosomal markers including adhesion molecules (integrins, intercellular adhesion molecule 1, lymphocyte function-associated antigen 3, CD11 a, b, and c) signaling molecules (kinases, Src homology 2 domain-containing proteins, phosphatases, catenins), membrane trafficking and MVB formation proteins (lysosomal-associated membrane proteins 1 and 2, Rab GTPases, annexins), cytoskeletal proteins (tubulin, coifilin, actin, moesin), lipid raft-associated molecules (lysobisphosphatidic acid, flotilin-1, cholesterol), tetraspanins (CD9, CD37, CD53, CD63, CD81, CD82), and antigen presentation molecules (CD86, MHC-I, MHC-II) as shown in Figure 1.5 (229, 237). Given the variety of molecules which are carried by exosomes, these vesicles have been implicated in a variety of cellular processes (229).

Since the initial discovery that EBV-transformed B cells secrete significant amounts of specific pMHC-II on exosomes after incubation with intact protein which can in turn stimulate antigen specific T cell responses (219), much work has concentrated on understanding the role of exosomes in antigen presentation and their ability to trigger immune responses. Exosomes themselves can function as a source of antigen for dendritic

Figure 1.5. Diagram of the protein composition of exosomes compiled from various cell types.

Adapted from (237).


Adhesion / targeting molecules
ICAM-1
LFA-3
CD11a,b,c
Integrins
LBPA
Flotilin-1
Cholesterol
Tetraspanins
CD9
CD37
CD53
CD63
CD81
CD82
Enzymes
Signal transduction proteins
ERK2
SH2 phosphatase
Catenin
RhoA
Anti-apoptotic proteins
Antigen Presentation
CD86
MHC-II
MHC-I
Cytoskeleltal proteins
Coifilin
Tubulin
Moesin
Actin
Heat-shock proteins
Hsc70
Hsp84/90
LAMP1/2
Rab proteins
Annexins

cells to present to T cells. For example, exosomes purified from tumor cell lines contain tumor antigens that can induce activation of antigen-specific T cells in the presence of DCs (238). As previously stated, exosomes can also function as APCs and present antigen on their own when they express pMHC-II on their surface. APC-derived exosomes can directly activate CD4+ T cell lines (219, 239) and primary CD4+ T cells *in vitro* and *in vivo*, respectively (220, 240, 241). Furthermore, exosome-derived pMHC-II can be transferred to recipient DCs to generate peptides to load onto their own MHC molecules (242) or may remain intact and be expressed on the cell surface of recipient DCs for the activation of allogeneic T cells (243). Exosomes purified from mature rather than immature DCs are more efficient at inducing T cells activation suggesting that mature DC co-stimulatory molecules on exosomes may assist in T cell activation (239, 242). Plasmacytoid DCs are relatively poor activators of naïve CD4+ T cells due to their limited phagocytic activity, but after capturing and internalizing exosomes from the environment, they become effective T cell stimulators (244).

Interestingly, exosomes purified from human monocyte-derived DCs, various cell lines, and human plasma were found to contain novel MHC-I structures as detected by conformational-dependent antibodies, suggesting that exosomes may contain unique MHC complexes or epitopes for recognition by T cells that are not present on the surface of APCs (245). Given their ability to function as APCs and activate specific T cell responses, preliminary studies investigating the therapeutic potential for exosomes to be used as a possible alternative to DC-based immunotherapy have been performed, especially in the case of anti-tumor immunotherapy (246-250). Further studies are

required in order to understand the role of exosomes *in vivo* and their importance in the immune response to pathogens and immune tolerance.

1.4 Peptide-dependent anti-MHC-II antibodies

The generation of antibodies against MHC-II molecules has proven to be useful tools in the study of fundamental processes in antigen presentation. One of the first identified peptide-MHC-II specific antibodies, termed Y-Ae, recognizes self Eα peptide bound to mouse I-A^b molecules (251). Initial studies using this antibody provided the first evidence for differential expression of pMHC-II between the thymic medulla and cortex, supporting the hypothesis that different ligands are involved in the positive and negative selection of T lymphocytes (252). Since its description, several additional anti-pMHC-II antibodies have been described (253-256). For example, antibodies recognizing specific pMHC-II complexes have been useful in quantifying the abundance of these complexes in a variety of cell types. Using the Y-Ae antibody, the Eα peptide was found to be bound to 12% of all I-A^b molecules on APCs (256). Another mAb termed 30-2, which binds a partially degraded intermediate of Ii in the context of I-A^b, was also used to show similar levels of surface expression of this self pMHC-II complex when compared to Y-Ae (254). The mAb Aw3.18 which recognizes murine I-A^k molecules bound to the peptide residues 48-62 of the exogenous antigen hen egg lysozyme, was used to determine the fraction of I-A^k molecules loaded with this peptide after culture of APC with exogenous antigen (253). The mAb UL-5 A1 recognizes a conformational epitope formed by DRB1*01:01molecules containing HLA-A2 derived peptides, and binds to pMHC-II in a

similar fashion as T cells (257). The use of these pMHC-II specific mAbs in these early studies resulted in key findings that contributed to our early understanding of the peptide repertoire expressed by antigen presenting cells. It has been well documented that pMHC complexes can exist in multiple conformations as a result of their traffic through the endocytic pathway and eventual binding of peptide (14, 258). The initial detection and description of these conformational changes was aided by the use of anti-MHC mAbs that were sensitive to these shifts in conformational states (259-264). One such example is the mAb 25-9-17 (265) which was shown to bind I-A^b molecules on the surface of B cells. 25-9-17 recognized I-A^b molecules containing CLIP but failed to recognize I-A^b loaded with a peptide derived from the Eα chain of I-E suggesting bound peptides induce subtle changes in MHC-II conformation (266).

Additional studies using the anti-HLA-DR3 mAb 16.23 and mutant B cell lines revealed that HLA-DR3 molecules can exist in different conformational states (267, 268). Mutagenized B lymphoblastoid cells selected for loss of the 16.23 epitope were unable to process and present protein antigen, contained SDS-instable MHC-II dimers, and lost the expression of two DR3 determinants (267, 269). The role of HLA-DM in the MHC-II pathway had not yet been uncovered; however, it was not long until the absence of HLA-DM was attributed to the defective antigen presenting phenotype in these mutant B lymphoblastoid cell lines (270, 271). Formation of the 16.23 epitope was later shown to be influenced by interaction with HLA-DM and the Ii (272, 273). These conformational differences are important because they can affect T cell reactivity. For example, it was shown that T cells could differentiate between the 16.23+ or – conformations of DR3 (273). Others have also found that T cells can selectively recognize distinct pMHC-II

conformations which result from different antigen processing and peptide loading pathways (274, 275). Given that these findings were partly based on the detection of conformers using anti-MHC-II mAbs, one cannot discredit the value of these mAbs as a valuable research tool in uncovering particulars of antigen presentation.

1.5 Antibody-defined epitopes on HLA-DRB1*04 molecules

1.5.1 NFLD.D1, NFLD.D2, NFLD.D10

Several mouse monoclonal antibodies that recognize different epitopes on HLA-DRB1*04 molecules have been previously produced and characterized in our laboratory (276-281). These antibodies were generated using class-II-negative murine L-cell fibroblasts transfected with human HLA-DR molecules as immunogens. The NFLD.D1 mAb recognizes an epitope in the β_2 domain of all DRβ1*04 molecules (278). Both a leucine at position 180 and a threonine at position 181 are critical for this epitope (282). The NFLD.D2 mAb binds DRB1 molecules near the peptide binding groove which have the amino acid sequence QKRAA or QRRAA from position 70 to 74. The epitope for NFLD.D2 is influenced by peptide in the peptide-binding groove and other amino acids at positions 28, 67, and 86 (278, 279). An additional mAb, NFLD.D10, recognizes an epitope on DRB1 molecules near the peptide binding groove, with amino acids at positions 70 and 73 playing a critical role in mAb binding (279). These mAbs have been useful in studying the expression of HLA-DRB1*04 in professional and non-professional antigen presenting cells (278-282).

1.5.2 NFLD.D11 and NFLD.D13

Two other mAbs, which are the main focus of this thesis, are NFLD.D11 and NFLD.D13. Compared with the mAbs described above, their epitopes are more restricted in their cellular expression. NFLD.D11 was previously shown to bind an epitope on DRB1*04:01 and 04:13, but not 04:02, 04:04, 04:05, 04:06, 04:07, 04:08, 04:10, or 04:11 molecules, in EBV-transformed B cell lines (78, 280). These results demonstrated that both a lysine at position 71 in pocket 4 and a glycine or valine at position 86 in pocket 1of the DRB1*04 molecule are important for NFLD.D11 binding. Further investigation demonstrated that expression of this epitope requires co-expression of HLA-DM in the context of DRB1*04:01, as this epitope is not expressed in B cells lacking DM but is restored after reconstitution of DM expression (280). In addition, the epitope was not expressed by the DM- B cell line 9.5.3 0401, but abundantly expressed by its DM+ parent cell line 8.1.6 0401 (78). Further investigation showed that binding of NFLD.D11 to its epitope can be prevented by blocking with antibodies which bind near the peptide binding groove including NFLD.D2 and NFLD.D10 (283). Given the DM-requirement for presentation of this epitope, it is similar to and can be classified as a DM-dependent epitope as previously described (102).

The epitope recognized by NFLD.D11, hereafter referred to as D11-0401, requires DM for expression, but DM alone is not sufficient for expression, suggesting that formation of this epitope is contingent on other cellular factors (280). Furthermore, several cells which express both DM and DRB1*04:01 lack expression of D11-0401 including IFN-γ stimulated synovial, epithelial, and breast cancer cells (281), as well as

the Burkitt's B cell lymphoma line Daudi and T cell line Jurkat transfected with DRB1*04:01, suggesting that normal and EBV-transformed B cells contained the correct combination of cellular factors responsible for D11-0401 expression (280).

Similar to NFLD.D11, the NFLD.D13 mAb binds an epitope on DRB1*04:01 molecules in B cell lines (283). However, whereas NFLD.D11 binding to DRB1*04:01 requires co-expression of DM, NFLD.D13 binds to DRB1*04:01 only in the absence of DM (78). For example, the NFLD.D13 epitope on DRB1*04:01, hereafter referred to as D13-0401, is abundantly expressed by the DM- B cell line 9.5.3 0401, whereas the DM+ parent cell line 8.1.6 0401 lacks expression of this epitope (78). D13-0401 is also expressed on other DM- B cell lines including 5.2.4 0401, SJO Dw4, BLS Dw4, and T2 Dw4 (283). Similar to D11-0401, the D13-0401 epitope is near the peptide binding groove of DRB1*04:01 because binding can be prevented by blocking with antibodies that bind near this region including NFLD.D2 and NFLD.D10 (283). Since the presence of DM prevents the presentation of the D13-0401 epitope, it can be classified as DM-sensitive, as described for other peptide epitopes (102-104).

Since the 8.1.6 and 9.5.3 cell lines were important in characterizing the D11-0401 and D13-0401 epitopes in previous studies, both cell lines were routinely used in the current study. For this reason, a brief summary of the derivation of these cell lines, as well as an additional DM- cell line, 5.2.4, is shown in Figure 1.6. As previously reported, 9.5.3 0401 lacks expression of DM compared to its parent 8.1.6 0401, while both retain expression of intact HLA-DR molecules (Figure 1.7).

NFLD.D13 also recognizes an additional epitope on DRB1*04:04, but not 04:05, 04:08, 04:10, 04:13, 01:01, 01:02, or 14:02 molecules, in EBV-transformed B cell

Figure 1.6. Derivation of the cell lines 8.1.6, 5.2.4, and 9.5.3 from the B lymphoblastoid cell line T5-1 and mapping of gene deletions.

Cell lines were derived from their parent by ethyl methane sulfonate mutagenesis followed by immunoselection with the indicated antibody and complement lysis (267). The vertical bars indicate approximate locations of HLA-I and HLA-II genetic loci. Additional genes located in this region of the MHC, as shown in Figure 1.1, have been omitted for ease of demonstration. Adapted from (271).

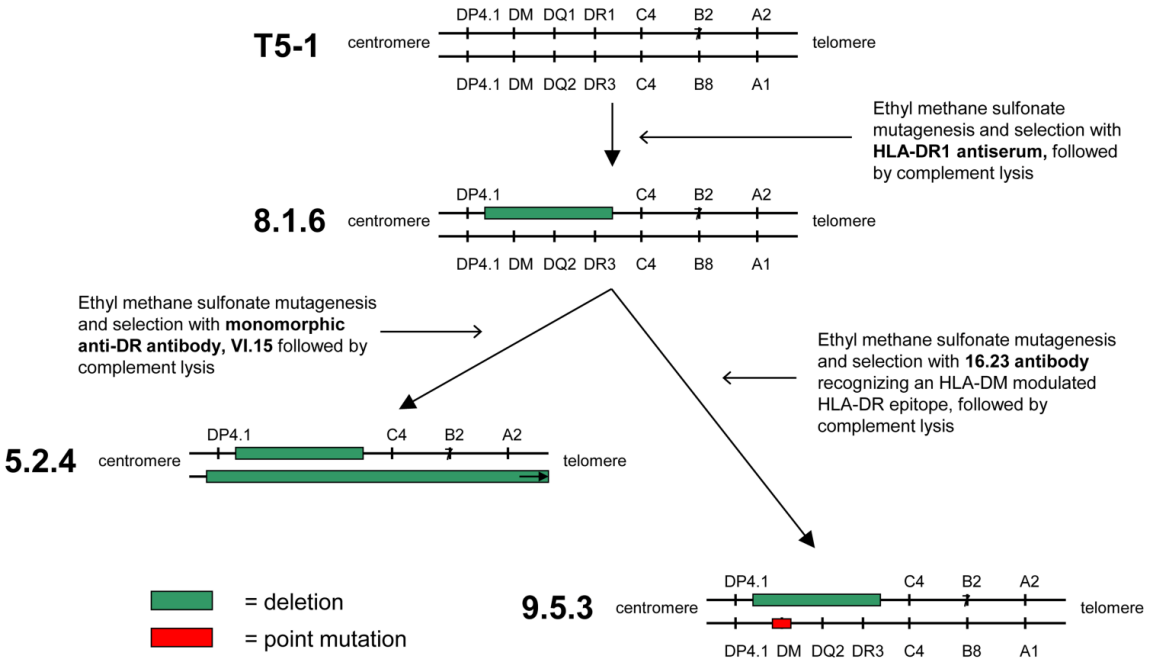
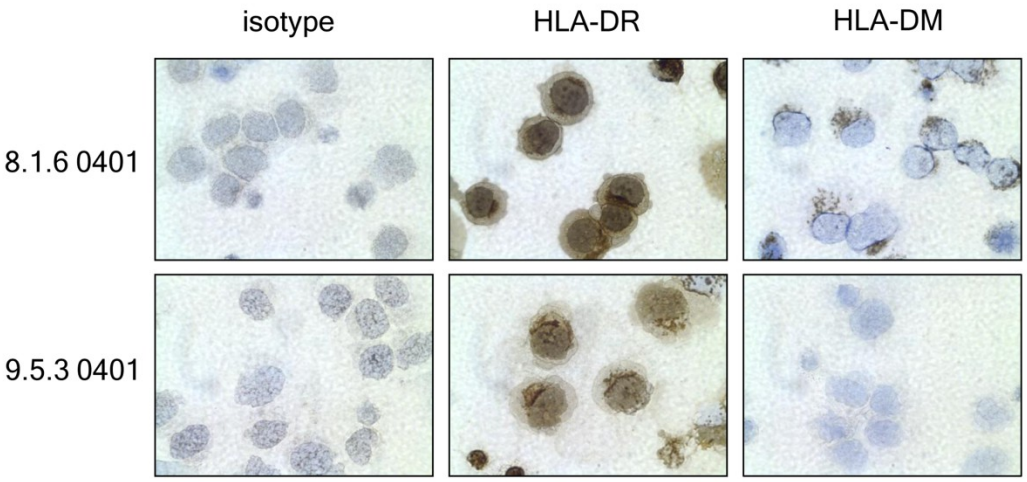

T5-1
centromere
DP4.1 DM DQ1 DR1 C4 B2 A2
telomere
DP4.1 DM DQ2 DR3 C4 B8 A1
Ethyl methane sulfonate mutagenesis and selection with **HLA-DR1 antiserum,** followed by complement lysis
8.1.6
centromere
DP4.1 C4 B2 A2
telomere
DP4.1 DM DQ2 DR3 C4 B8 A1
Ethyl methane sulfonate mutagenesis and selection with **monomorphic anti-DR antibody, VI.15** followed by complement lysis
Ethyl methane sulfonate mutagenesis and selection with **16.23 antibody** recognizing an HLA-DM modulated HLA-DR epitope, followed by complement lysis
5.2.4
centromere
DP4.1 C4 B2 A2
telomere
= deletion
= point mutation
9.5.3
centromere
DP4.1 C4 B2 A2
telomere
DP4.1 DM DQ2 DR3 C4 B8 A1

Figure 1.7. The cell line 9.5.3 lacks expression of DM compared to its parent cell line 8.1.6.

The cells lines 8.1.6 and 9.5.3 (both transfected with DRB1*04:01) were stained for HLA-DM and HLA-DR using immunocytochemistry. Cells were removed from cell culture and washed in PBS. Cell preparations were made using a cytocentrifuge by centrifuging 50 000 cells/prep at 1600 rpm for 5 minutes. Slides were allowed to dry at room temperature for a couple of hours and were then fixed in ice cold acetone at -20^{o}C for 10 minutes followed by a 1 hour drying period at room temperature. Following fixation, cells were rehydrated with PBS, rinsed with wash buffer (0.5% BSA, 0.05% Tween-20 in PBS), and incubated in H_2O_2 for 10 minutes. Cells were then washed 3 x 5 minutes with wash buffer and blocked for 1 hour with 15% goat serum in PBS. Cells were incubated for 1 hour at room temperature with primary antibodies anti-DR (L243), anti-DM (MaP.DM1), and an isotype control diluted in wash buffer, followed by 3 x 5 minute washes as above and 30 minute incubation with a peroxidase-conjugated secondary antibody. Cells were washed again as above and stained with ImmPACT 3, 3`-diaminobenzidine peroxidase substrate (Vector) for 4 minutes and counterstained with Mayers' hematoxylin for 1 minute. Slides were viewed using a Leica stereomicroscope and images were captured using a SPOT RT CCD Cool Camera. Images shown are 100x.


isotype
HLA-DR
HLA-DM
8.1.6 0401
9.5.3 0401

lines. Comparing the amino acid sequence of these DRB1 molecules, four residues that contribute to formation of the NFLD.D13 epitope, hereafter referred to as D13-0404, were deduced: tyrosine at position 37, aspartic acid at position 57, arginine position 71, and valine at position 86 (283). Interestingly, all four of these positions are contact points for either T cell or peptide recognition, which suggest that this epitope is also near the peptide-binding groove of DRB1*04:04, similar to D11-0401 and D13-0401 on DRB1*04:01.

Unlike D11-0401 and D13-0401, it is not clear whether DM alters expression of this epitope. As with NFLD.D11, mAbs known to bind near the peptide binding groove such as NFLD.D2 and NFLD.D10 were able to block binding of NFLD.D13 to its epitope on DRB1*04:04 molecules (283).

Given the allele-dependent and cell-restricted expression of the D11-0401, D13-0401, and D13-0404 epitopes, additional work by Spurrell D.R. examined other factors contributing to formation of these epitopes (283). It was observed that D11-0401, D13-0401, and D13-0404 formation required newly synthesized DRB1*04 molecules. In addition to being expressed at the cell surface, both D11-0401 and D13-0404 were observed in the endosomal pathway and co-localized with markers for peptide loading compartments. Given that these epitopes are modulated by DM, are located near the peptide binding groove, and reside intracellularly within MIICs, he further hypothesized that cellular-restricted peptides or specific peptide processing events contribute to the formation of these epitopes. Both D11-0401 and D13-0401 were found to require a subset of cysteine proteases for epitope formation, indeed suggesting that specific peptides contribute to formation of these epitopes (283).

1.6 Rationale and objectives

As previously described, the formation of the DRB1*04:01-restricted D11-0401 epitope requires expression of the peptide-editor HLA-DM, and is thus DM-dependent (78, 280, 283). Conversely, the D13-0401 epitope is DM-sensitive as it is only expressed on DRB1*04:01 molecules in the absence of DM (78, 283). Interestingly, this epitope is similar to the D13-0404 epitope present only on DRB1*04:04 molecules. Prior to my thesis work, Spurrell, D.R. and Drover, S. demonstrated that these epitopes mapped to the peptide-binding groove, suggesting that peptides directly contribute to epitope formation. Additional findings supporting this hypothesis were (a) the cell-specific expression profile of these epitopes (278-280); (b) D11-0401 and D13-0404 co-localized with markers of MIICs (283); and (c) inhibition of a particular subset of cellular cysteine proteases abrogated D11-0401 and D13-0401 expression (283). The hypothesis that bound peptide contributes to the formation of these epitopes is further investigated in this thesis. Since these DM-modulated epitopes are expressed on DRB1 alleles carrying the shared epitope (SE) that is strongly associated with the development of RA (284, 285), and DM has been suggested to play a role in RA (286-288), further investigation into the formation of these epitopes may provide insight into the mechanism of SE-associated autoimmunity.

Although the DM requirements of these epitopes were partially understood when this thesis work began, the role of HLA-DO to the formation of these epitopes was unknown. HLA-DO can affect DM-mediated peptide loading and has also been shown to alter the presentation of antigen-specific epitopes (68, 97, 289, 290). Since D11-0401

requires DM for formation and is likely influenced by the bound peptide, we questioned whether HLA-DO also influences this epitope. Similarly, we examined the role of DO in the presentation of the DM-sensitive D13-0401 epitope. Understanding the antigen processing events that govern the formation of these epitopes may give insight into the formation of DM-resistant, DM-sensitive, and DM-dependent antigenic epitopes and their role in DRB1*04-associated autoimmune disease.

Although the previous study identified D11-0401 complexes on exosome-like vesicles using electron microscopy, analysis of these epitopes on purified exosome populations was not done (283). Other evidence to suggest this included colocalization of these epitopes with exosomal tetraspanin markers CD63 and CD82 within MVBs, the sire of exosome biogenesis (283). EBV-transformed B lymphocytes are known to constitutively secrete exosomes that contain functionally competent pMHC-II complexes on their surface (219). Recently, novel MHC-I epitopes have been detected on exosomes derived from DCs, suggesting that unique MHC epitopes not expressed on the cell surface may be present on exosomes for recognition by the immune system (245). For these reasons, we investigated the expression of D11-0401, D13-0401, and D13-0404 on B-LCL-derived exosomes.

The tetraspanins CD63 and CD82 are also integral components of TEMs, which are able to concentrate particular epitope-defined pMHC-II complexes in the plasma membrane (153). These TEM-associated pMHC-II complexes were associated with DM and contained a select set of antigenic peptides (153). After preliminary experiments indicated that apparent disruption of TEMs caused a decrease in expression of D11-0401 and D13-0404 (283), we hypothesized that these epitopes may represent a distinct subset

of DRB1*04 molecules that associate with TEMs similar to other previously described epitopes (153, 217). We also investigated the role of another type of membrane microdomain, lipid rafts, in the presentation of these epitopes, since distinct pMHC-II molecules have been shown to associate with these microdomains (156, 163). The possibility that the DRB1*04 epitopes described here may represent distinct pMHC-II complexes associated with particular membrane microdomains would further our understanding of expression of DM-sensitive, DM-dependent, and DM-resistant epitopes and implications for T cell recognition.

Objectives:

1. To determine the role that HLA-DO has in regulating the expression of the D11-0401, D13-0401, and D13-0404 epitopes in B-LCL.
2. To further investigate if endogenous cell-specific peptides are involved in forming D11-0401, D13-0401, and D13-0404.
3. To characterize the expression of the D11-0401, D13-0401, and D13-0404 epitopes on B-LCL-derived exosomes.
4. To determine if lipid rafts and TEMs contain specific subsets of pMHC-II expressing the D11-0401, D13-0401, and D13-0404 epitopes.

Chapter 2

Materials and Methods

2.1 Cell lines, reagents, and antibodies

The human B cell lines SAVC (DRB1*04:01), MT14B (DRB1*04:04), Boleth (DRB1*04:01), PF97387 (DRB1*04:01), WT51 (DRB1*04:01), GM2219 (DRB1*04:01), and BM92 (DRB1*04:04) were obtained through the 10th International Histocompatibility Workshop (291). The DRB1-transfected human B cell lines 8.1.6 0401, 9.5.3 0401, 5.2.4 0401, 5.2.4 0401 DM, 5.2.4 0404, and 5.2.4 0404 DM were kindly provided by Dr. E. D. Mellins (Stanford University) and are previously described in detail (78, 270). The B cell lines BJAB.DO2 (BJAB DO) and BJAB.V2 (BJAB V) were kindly provided by Dr. P. Roche (National Institutes of Health) and are described elsewhere (68). Both B cell lines BJAB and RAMOS were kind gifts from Dr. J. Thibodeau (University of Montreal). Ramos, BJAB, BJAB V, and BJAB DO were transfected with DRB1*04:01 using a previously described method (292) and successful transfection of DRB1*04:01 was confirmed by flow cytometric analysis of surface expression. DRA*01:01 and DRB1*04:01 transfected cells lacking DM expression (T2.Dw4, BLS-1.Dw4, SJO.Dw4) and DM-expressing cell lines (T2.Dw4DM, BLS-1.Dw4 x .174, SJO.Dw4 x .174) are described elsewhere in detail (280). T2.Dw4 was provided by Dr. W.W. Kwok (Benaroya Research Institute at Virginia Mason) while T2.Dw4DM was a kind gift from Dr. P. Cresswell (Yale University School of Medicine). All cell lines were maintained in either RPMI-1640 or Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 2 mM L-

glutamine (Gibco), and incubated in a humidified air chamber containing 5% CO_2 at 37^{o}C.

HLA-DR expression was analyzed using the following monoclonal antibodies (mAbs): L243, which binds a conformational epitope on DR dimers near the peptide groove (293, 294); NFLD.D1 specific for an epitope in the beta-2 (β_2) domain of DRB1*04 (278); NFLD.D11, specific for an allele-specific DM-dependent epitope on DRB1*04:01 dimers; NFLD.D13, cross reactive for a DM-sensitive epitope on DRB1*04:01 dimers and a DM-resistant epitope on DRB1*04:04 DR dimers (78, 103, 280). Other mAbs used in this study included mAbs against HLA-DO (DOB.L1), HLA-DM (MaP.DM1), CLIP (cerCLIP), Ii (LN2), CD71 (M-A712), CD45 (HI30), ICAM-1 (HA58), CD55 (phycoerthryin (PE)-conjugated IA10), CD40 (5C3), CD86 (IT2.2), CD82 (50F11), and LAMP-1 (H4A3) from BD Pharmingen; GAPDH (6C5), HLA-DM (Tal 18.1), HLA-DRB (Tal 14.1), Ii (PIN.1), CD59 (MEM-43), and CD82 (TS82b) from Abcam; and CD63 (CLB-180) from Cedarlane. The mAb clone W6/32 was used to detect MHC-I. Isotype control mAbs were locally prepared or obtained from BD Pharmingen, eBioscience, or Abcam depending on the application. Secondary antibodies included peroxidase-conjugated goat anti-mouse $F(ab)_2$ IgG and IgM for cell enzyme-linked immunosorbent assay, PE-conjugated goat anti-mouse IgG Fc_γ and PE-conjugated goat anti-mouse IgM μ chain for flow cytometry, horse radish peroxidase (HRP)-conjugated goat anti-mouse $F(ab`)_2$ IgG Fc_γ and HRP-conjugated goat anti-mouse IgM μ chain for immunoblotting (Jackson Immunoresearch Laboratories, Inc.), and Alexa Fluor 488-

conjugated goat anti-mouse IgG1 F_c fragment for confocal microscopy (Life Technologies).

2.2 Flow cytometry

Freshly harvested cells were washed twice with wash buffer (phosphate buffered saline (PBS) containing 0.2% FCS and 0.02% sodium azide) and adjusted to 1 x 10^7 cells/ml for staining. 5 x 10^5 cells per stain were incubated with the indicated primary antibody diluted in a total volume of 100 µl wash buffer for 30 minutes at 4^{o}C. Incubation with an isotype control was performed in parallel. Cells were then washed twice with wash buffer and incubated with 100 µl of the appropriate fluorochrome-conjugated secondary antibody diluted in wash buffer for 30 minutes at 4^{o}C in the dark. After 2 washes as described above, cells were fixed with 1% paraformaldehyde (PFA) diluted in PBS and analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson). Analysis of flow cytometric data was performed using FlowJo 7.6 software (FlowJo, LLC).

For assessment of intracellular antigens by flow cytometry, cells were fixed with 2% PFA in PBS for 15 minutes at room temperature, washed with media followed by a wash with PBS, then permeabilized with 0.2% Tween-20 diluted in PBS for 10 minutes at room temperature. After permeabilization, cells were stained as described above for surface staining, except that both primary and secondary antibodies were diluted in permeabilization buffer.

For flow cytometric analysis of exosome-coated beads, 10 µl of exosome-labeled beads was incubated with the indicated primary antibody diluted in wash buffer (PBS containing 0.5% bovine serum albumin (BSA)) in a total volume of 50 µl for 30 minutes at 4^{o}C. Incubation with an isotype control antibody was performed in parallel. Beads were then washed twice in wash buffer and incubated with the appropriate fluorochrome-conjugated secondary antibody diluted in wash buffer in a total volume of 50 µl for 30 minutes at 4^{o}C. Beads were washed two more times and resuspended in wash buffer for analysis. Fluorescence was analyzed on the single beads only by gating on the single bead population.

2.3 Cell enzyme-linked immunosorbent assay

Cell surface expression of DR molecules and epitopes was measured in some experiments using a modified enzyme-linked immunosorbent assay, termed cell enzyme-linked immunosorbent assay (CELISA), as previously described in detail (295, 296). Cells were fixed in 2% PFA diluted in PBS, washed with media followed by a wash with PBS, and seeded in a 96 well round-bottom plate at 2.5 x 10^4 cells per well. Cells were incubated for 1 hour at room temperature with the indicated primary antibody or isotype controls. Cells were washed 3 times with CELISA wash buffer (PBS containing 0.5% BSA and 0.05% Tween-20) and incubated for 1 hour at room temperature with the appropriate peroxidase-conjugated secondary antibody. After 3 washes as described above, cells were incubated for 30 minutes in the dark with o-phenylenediamine dihydrochloride substrate (Sigma) for colorimetric detection. The reaction was stopped by

the addition of sulfuric acid and results were analyzed using a Multiscan spectrophotometer (Biorad) using a 490nm filter. Background optical density (OD) values obtained from isotype controls were subtracted from each test OD and values from triplicate samples were averaged. Where indicated, CELISA results are presented as the fold change in expression of the particular molecule or epitope, calculated by dividing the mean OD of treated cells by the mean OD of control cells, where a value equal to 1 indicates no change in expression.

2.4 Western blot analysis

Whole cell lysates were prepared in either Triton X-100 lysis buffer (PBS pH 8.0, 1% Triton X-100, 0.5 M ethylenediaminetetraaccetic acid (EDTA)) or 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate **(**CHAPS) lysis buffer (PBS pH 8.0, 1% CHAPS, 0.5 M EDTA) containing the inhibitors aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin A (1 μg/ml), and phenylmethylsulfonyl fluoride (10 μg/ml) (Sigma). The protein content of lysates was determined using the bicinchoninic acid assay (Thermo Fisher Scientific). Proteins were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis on 8 – 12 % gels (SDS-PAGE) under non-reducing or reducing conditions where indicated, followed by western blotting. Nitrocellulose membranes were blocked with blocking buffer (tris buffered saline (TBS) containing 0.05% Tween-20 and 5% milk powder) for 1 hour at room temperature and incubated overnight with primary antibodies diluted in blocking buffer at 4^{o}C. Membranes were thoroughly washed with TBS containing 0.05% Tween-20. Blots were subsequently

probed with the appropriate HRP-conjugated secondary antibody diluted in blocking buffer for 1 hour at room temperature, followed by thorough washing as above, and detection with Immobilon Western Chemiluminescent HRP substrate (Millipore).

2.5 Lipid raft labeling and confocal microscopy

Cells from culture were washed in serum-free culture media and incubated with antibody against the molecule of interest for 30 minutes on ice. The cells were then washed three times in PBS containing 2% FCS, followed by a 30 minute incubation on ice with the appropriate fluorophore-conjugated secondary antibody. Plasma membrane lipid rafts were detected using the Vybrant Alexa Fluor 555 Lipid Raft Labeling Kit (Invitrogen) according to the manufacturer's instructions. Cells were incubated with Alexa Fluor 555-conjugated cholera toxin subunit B (CT-B) for 10 minutes at 4^{o}C, followed by three washes as above. Cells were then incubated with anti-CT-B antibody for 15 minutes at 4^{o}C. All cells were washed twice with PBS containing 2% FCS, fixed with 4% PFA, and mounted on slides in Vectashield anti-fade mounting media (Vector Labs). Slides were viewed using an Olympus FluoView 1000 confocal laser scanning microscope (Olympus).

2.6 Fluorescent aerolysin (FLAER) assay

To measure the surface expression of GPI-anchored proteins on cells using flow cytometry, we performed the unique diagnostic FLAER assay which utilizes an Alexa

Fluor 488-conjugated version of a non-lysing, mutated form of proaerolysin (297). Proaerolyisn is a bacterial toxin secreted by *Aeromonas hydrophila.* After converting to its active form, aerolysin binds to the GPI moiety of GPI-linked molecules resulting in the formation of channels in the cell membrane causing cell lysis (298, 299). The FLAER assay was initially generated for clinical use to aid in the diagnosis of paraoxysmal nocturnal hemoglobinuria (PNH) (300, 301). PNH is an acquired hematopoietic stem cell disease characterized by a mutation in the phosphatidyl-inositol glycan gene resulting in a deficiency of GPI-linked proteins in a clone of hematopoietic cells (302).

The FLAER assay was performed as previously described (297). Approximately 2 x 10^5 cells were washed with PBS and resuspended in 50 µl PBS containing 2% FCS. Either 25 µl of a FITC-conjugated isotype control antibody (BD Pharmingen) or 5 µl of FLAER (Protox Biotech working solution (diluted 1:10 in PBS from stock) was added to the cells and incubated for 15 minutes at room temperature. Finally, 50 µl of PBS containing 2% FCS was added to the cells and the samples were immediately analyzed using a BD FACSCalibur flow cytometer.

2.7 Epitope blocking assay

The location of D11-0401, D13-0404, and D13-0401 epitopes on DRB1*04:01 and DRB1*04:04 molecules was ascertained using several mAbs with known epitopes to block NFLD.D11 and NFLD.D13 binding measured using CELISA and flow cytometry. For CELISA, cells were washed in PBS, plated in 96 well plates, and incubated for 1 hour at room temperature with either anti-DRB1*04 (NFLD.D1), anti-CLIP/DR (cerCLIP), or

anti-DR (L243) at several concentrations (4 – 500 μg/ml). Cells were washed 3 times with PBS containing 0.5% BSA followed by a 1 hour incubation at room temperature with either NFLD.D11 for SAVC cells or NFLD.D13 for MT14B cells. The remainder of the assay was carried out as described above for CELISA.

For flow cytometry, cells were first incubated with anti-DR (L243) or an isotype control antibody for 30 minutes on ice followed by 2 washes with flow cytometry wash buffer. Cells were incubated with NFLD.D11, NFLD.D13, or isotype control antibodies for 30 minutes on ice followed by 2 washes with flow cytometry wash buffer. Cells were then incubated with a goat anti-mouse IgM μ chain-specific PE-conjugated secondary antibody for 30 minutes on ice in the dark. Finally, cells were fixed in 2% PFA dissolved in PBS.

2.8 Protease inhibitor treatment

B-LCL were treated for 18 hours in culture with 25 μM leupeptin (Sigma), 25 μM pepstatin A (Sigma), 100 μM cathepsin B inhibitor II (CBI II, Ac-Leu-Val-lysinal) (Calbiochem), or 50μM calpeptin (Calbiochem) as previously described (30, 280). Control cells were similarly treated with an equivalent volume of the protease inhibitor diluent (PBS or dimethyl sulfoxide). The protease inhibitors had minimal effect on cell viability evaluated by trypan blue exclusion. Following inhibitor treatment, cells were harvested from culture and flow cytometry was performed to determine surface expression of DR epitopes. Alternatively, cells were fixed with 2% PFA for 15 minutes at 4^{o}C, washed with culture media and PBS, and analyzed by CELISA.

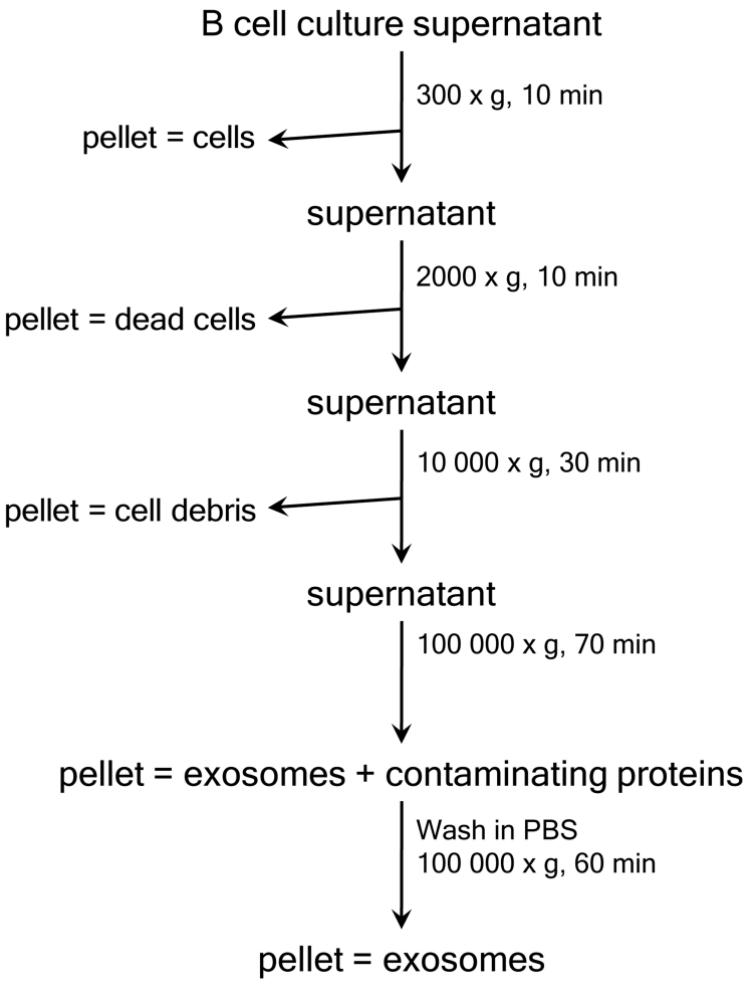
2.9 Exosome purification

Exosomes were purified from SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 cultures by differential ultracentrifugation as previously described in detail (303) and outlined in Figure 2.1. The procedure involves successive centrifugations at increasing speeds to eliminate dead cells and large cellular debris leading to the collection of a pellet containing small vesicles corresponding to exosomes. Prior to cell culture, RPMI-1640 medium supplemented with 10% heat-inactivated FCS was depleted of contaminating FCS-derived exosomes by overnight centrifugation at 100, 000 x g at 4^{o}C in a Sorvall Discovery 100SE ultracentrifuge (Thermo Fisher Scientific), after which the supernatant was filtered sterilized using a vacuum-connected 0.22 µm filter attached to a sterilized bottle. Exosome-depleted media (ED-media) was stored at 4^{o}C up to 4 weeks until required for exosome purification. Prior to cell culture, media was supplemented with 2 mM L-glutamine**.**

Cells were cultured under standard conditions in 50 ml of complete media until they reached approximately 70% of their maximum concentration in suspension. Cells were then centrifuged for 10 minutes at 300 x g at 4^{o}C, and resuspended in 50 ml of ED-media and cultured for 48 hours. Cells were then centrifuged for 10 minutes at 300 x g at 4^{o}C to separate exosomes from the pelleted cells. Making sure not to disturb the cell pellet, the supernatant containing exosomes was collected and transferred to a 50 ml conical tube and centrifuged for 20 minutes at 2000 x g at 4^{o}C. The resulting supernatant was transferred to ultracentrifugation tubes and centrifuged for 30 minutes at 10, 000 x g at 4^{o}C with a Sorvall TH-641 rotor (Thermo Fisher Scientific) in a Sorvall Discovery

Figure 2.1. Purification of exosomes from B-LCL.

Exosomes were isolated from cell culture supernatants of the B-LCL SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 by differential ultracentrifugation as described in detail in the Materials and Methods and as illustrated in the flow diagram. Adapted from (303).


B cell culture supernatant
300 x g, 10 min
pellet = cells
supernatant
2000 x g, 10 min
pellet = dead cells
supernatant
10 000 x g, 30 min
pellet = cell debris
supernatant
100 000 x g, 70 min
pellet = exosomes + contaminating proteins
Wash in PBS
100 000 x g, 60 min
pellet = exosomes

100SE ultracentrifuge. The resulting supernatant was transferred to new ultracentrifugation tubes and subsequently centrifuged for 70 minutes at 100, 000 x g at 4^{o}C, after which the supernatant was carefully removed with a pipette as not to disturb the pelleted exosomes. Exosomes were resuspended in 1 ml PBS and pooled from several tubes into a single ultracentrifugation tube and centrifuged for 60 minutes at 100, 000 x g at 4^{o}C to wash the purified vesicles. Finally, the supernatant was completely removed and exosomes were resuspended in 100 µl PBS and cryopreserved at -80^{o}C.

2.10 Electron microscopy analysis of exosomes

Purified exosomes were visualized by whole mount electron microscopy as previously described (303). Exosomes were fixed in 2% PFA diluted in PBS and deposited on formvar-carbon coated electron microscopy grids and allowed to absorb for 20 minutes. The grids were then washed with PBS and incubated with 1% glutaraldehyde (Sigma) for 5 minutes. Finally, grids were washed 8 times with distilled water and whole-mounted exosomes were imaged using a 1200EX transmission electron microscope (Jeol Ltd).

2.11 Attachment of exosomes to latex beads for flow cytometric analysis

To determine the expression of DR epitopes and associated proteins on the surface of exosomes, the vesicles were attached to latex beads and analyzed by flow cytometry as previously described (303). Five micrograms of exosomes, determined using the

bicinchoninic acid assay, was incubated with 10 µl surfactant-free aldehyde/sulfate, 4% weight/volume 3.9 µm latex beads (Interfacial Dynamics) for 15 minutes at room temperature to allow nonspecific absorption of the vesicles to the latex beads. The total volume of the latex bead – exosome mixture was brought to 1ml with PBS and incubated on a rotator wheel for 2 hours at room temperature. Free binding sites on the latex beads not bound by exosomes were saturated by adding 110 µl of 1M glycine (Sigma) and incubating for 30 minutes at room temperature to prevent nonspecific absorption of reagents to the beads during downstream steps. The exosome-labeled beads were then centrifuged for 3 minutes at 4000 rpm at room temperature. The supernatant was discarded and the bead pellet was washed three times with 1 ml PBS containing 0.5% BSA. After the final wash, exosome-labeled beads were resuspended in 0.5 ml PBS containing 0.5% BSA for analysis of protein expression by flow cytometry as described above.

2.12 Disruption of plasma membrane microdomains

To determine whether the DRB1*04 epitopes associate with membrane microdomains, lipid rafts and TEM were disrupted using MBCD and saponin respectively, as previously described (153). To disrupt lipid rafts, cells were washed with serum-free culture media and cultured for 10 minutes at 37^{o}C initially with 2.5, 5, or 10 mM MBCD (Sigma) dissolved in serum-free culture media. MBCD disrupts protein association with lipid rafts by depleting cholesterol from the plasma membrane of cells (304-306). After MBCD treatment, cells were washed in serum-free culture media at

room temperature and fixed in 2% PFA in PBS for 15 minutes at 4^{o}C to prevent the reassembly of rafts. Fixation was stopped by washing with 100 mM glycine diluted in PBS followed by a second wash with PBS. FCS was omitted from all buffers to prevent incorporation of cholesterol into cholesterol-depleted cells (307). Cells were then analyzed by flow cytometry for surface expression of relevant molecules and compared to untreated control cells. All concentrations of MBCD selectively disrupted expression of raft-associated proteins without compromising cell health and viability measured by forward vs side scatter plot analysis in flow cytometry and examination of cellular morphology by microscopy. Subsequent experiments were performed using 5 mM MBCD treatment unless indicated otherwise.

Saponin was previously shown to selectively disrupt tetraspanin-tetraspanin interactions (153, 210). Cells from culture were washed in PBS, fixed in 2% PFA in PBS for 15 minutes at room temperature, and further washed with culture media and PBS. Fixed cells were then incubated with 0.1% saponin (Sigma) diluted in PBS with 2% FCS for 20 minutes at 4^{o}C. Cells were washed twice with flow cytometry wash buffer. Cells were then analyzed by flow cytometry for surface expression of relevant molecules and compared to untreated control cells.

2.13 Isolation of detergent resistant membranes

Detergent resistant membranes (DRMs) containing lipid rafts were isolated using sucrose density gradient ultracentrifugation as previously described (308). Freshly harvested cells (approximately 5.5 x 10^{7}) were lysed in Triton X-100 lysis buffer (PBS

pH 8.0, 1% Triton X-100, 0.5 M EDTA) containing the inhibitors aprotinin (1 µg/ml), leupeptin (1 µg/ml), pepstatin A (1 µg/ml), and phenylmethanesulfonyl fluoride (10 µg/ml) for 1 hour at 4^{o}C on a rotator. The lysate was mixed with an equal volume of 80% sucrose dissolved in lysis buffer and overlaid with 35% sucrose and 5% sucrose dissolved in lysis buffer. The samples were then centrifuged at 280, 000 x *g* for 18 hours at 4^{o}C with a Beckman SW 55 Ti rotor (Beckman Coulter) in a Sorvall Discovery 100SE ultracentrifuge. Fractions approximately 400 µl in volume were carefully collected by pipetting from the top of the gradient. The second, third and fourth fractions corresponded to the visible 5 / 35% sucrose interface where DRMs float after ultracentrifugation in a sucrose gradient (309). To solubilize the DRMs for SDS-PAGE and western blotting analysis, 1% n-octylglucoside was added to each gradient fraction and incubated for 1 hour at 4^{o}C on a rotator. For SDS-PAGE, 10 µl of each gradient fraction was loaded per lane run under non-reducing conditions. Fractions were stored at -80^{o}C.

Chapter 3

Endosomal chaperones and proteases contribute to the formation of allele specific epitopes on HLA-DRB1*04 molecules

3.1 Preamble and objectives

As described in the rationale (Section 1.6), it was previously shown that D11-0401, D13-040, and D13-0404 epitopes were differentially modulated by DM, mapped to the peptide binding groove, and were peptide-dependent (78, 278-280, 283). Since DO is a negative regulator of DM and has a profound effect on the peptide repertoire presented by MHC-II to T cells (14, 68, 75-77, 97, 289, 290), we hypothesized that presentation of the DM-dependent D11-0401 and DM-sensitive D13-0401 epitopes is modulated by DO. It should be noted that this was not addressed in any preceding published or unpublished work and thus represents new information about these epitopes.

As described in the co-authorship statement, the role of cytoplasmic and endogenous proteases in generating these epitopes was previously explored using various inhibitors and the cell based assay, CELISA (283). Here we expanded the study to include western blotting analysis of the antigen processing pathway in each cell line, as well as new experiments using flow cytometry. For completeness and clarity and aspirations to prepare this chapter for a manuscript for publication, some of the previous work is included in the following results as previously detailed in the co-authorship statement.

The specific objectives addressed in this chapter are:

1. To compare the expression of DRB1*04 epitopes in B cell lines differing in their expression of HLA-DO.

2. To further investigate whether cell-restricted peptides contribute to formation of D11-0401, D13-0401, and D13-0404 by examining the effect of protease inhibitors on the formation of these epitopes in B cell lines.

3.2 Results

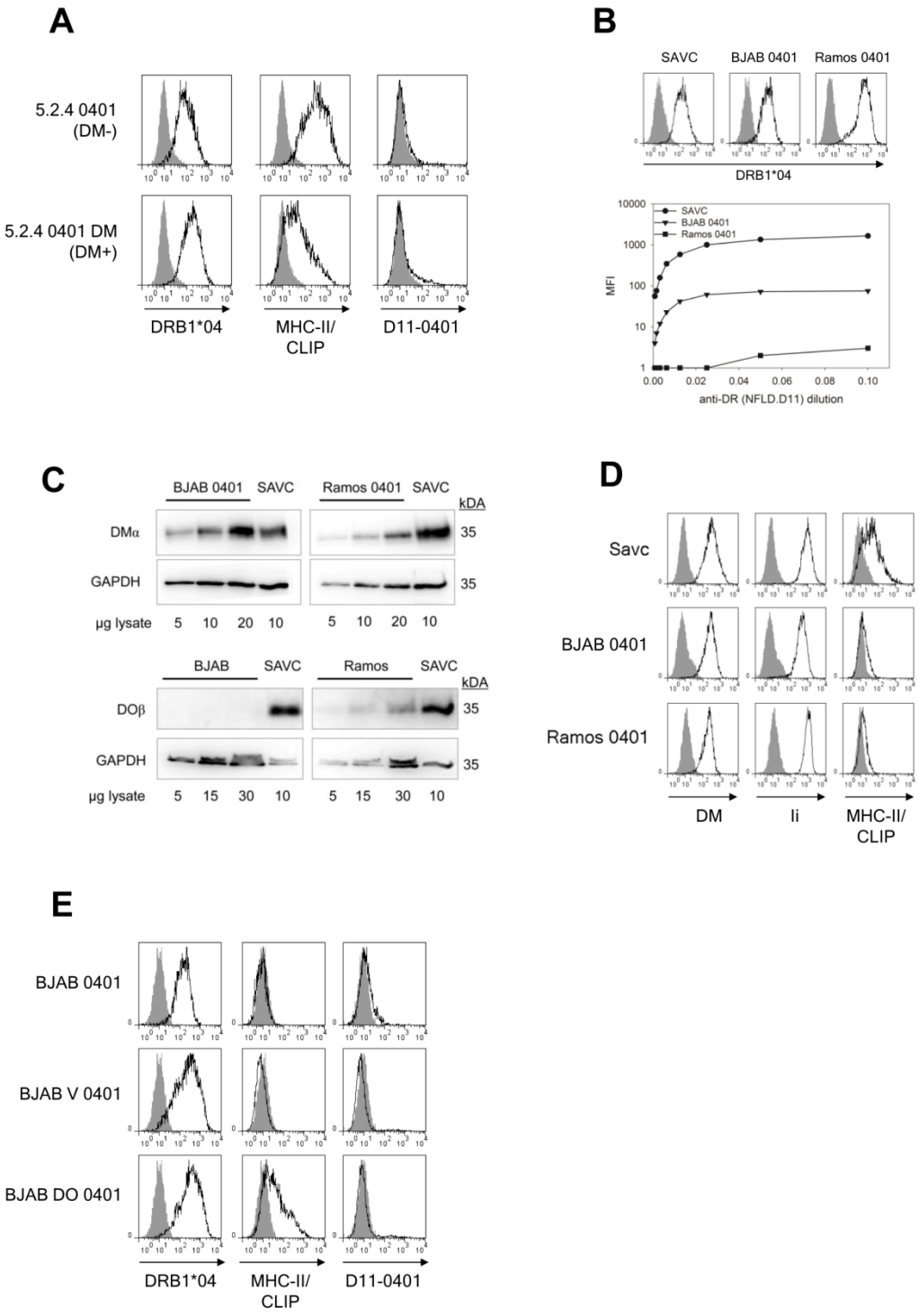
3.2.1 DM-dependent DRB1*04:01 epitope is cell context but not DO-dependent

The DM-dependency of the allele-specific D11-0401 epitope on DRB1*04:01+ peripheral blood B lymphocytes and Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines (B-LCL) was demonstrated by its absence on DM- DRB1*04:01+ antigen presentation mutant B cell lines and restoration in DM-reconstituted cells (78, 280, 310). Given the well documented modulatory effects of DO on DM-mediated peptide loading (65, 311) and the DM-requirements of the D11-0401 epitope (78, 280), we queried whether the DO molecule modulated D11-0401 expression. Thus, we analyzed D11-0401 expression on DM- DO- 5.2.4 0401 cells, also derived from 8.1.6 but lacking both copies of the *DOB* gene (271, 312). As expected, no D11-0401 was detected on 5.2.4 0401, but surprisingly it was poorly reconstituted in the DM transfected cells (Figure 3.1A). This was not due to reduced DRB1*04:01 since both DM- and DM+ cells expressed equivalent amounts, nor due to deficient DM since severely reduced surface MHC-II/CLIP on 5.2.4 0401 DM is indicative of functional DM in these cells (Figure 3.1A).

D11-0401 expression was analyzed on Burkitt lymphoma (BL) cell lines, which are DM+ but DO-deficient (93, 313, 314), potentially explaining lack of D11-0401 expression on the BL cell line, Daudi-Dw4 (280). Similarly, BL cell lines BJAB and Ramos transfected with DRB1*04:01 barely expressed D11-0401 despite DRB1*04:01 levels comparable to the control cell SAVC (Figure 3.1B). Western blot and flow

Figure 3.1. Expression of the DM-dependent D11-0401 epitope is cell-context dependent.

(A) Restoration of DM in the DO- 5.2.4 0401 cell line does not reconstitute the D11-0401 epitope. Surface expression of DRB1*04 (NFLD.D1), MHC-II/CLIP (cerCLIP), and D11-0401 was analyzed by flow cytometry on DM- and DM+ 5.2.4 0401 B cell lines. Isotype controls are shown in grey. **(B)** DRB1*04 surface expression on BJAB and Ramos transfected with DRB1*04:01 (BJAB 0401 and Ramos 0401) was compared to SAVC using flow cytometry. Isotype controls are shown in grey. D11-0401 expression was plotted against increasing dilutions of NFLD.D11 used to stain cells for flow cytometry (0.1 = 1:10 dilution). Results are representative of three independent experiments. **(C)** Western blot analysis of DMA (Tal 18.1) and DOB (DOB.L1) expression in whole cell lysates of BJAB 0401 and Ramos 0401 compared to SAVC. Results are representative of two experiments. **(D)** Intracellular expression of DM (MaP.DM1), Ii (LN2), and surface expression of MHC-II/CLIP (cerCLIP) was determined by flow cytometry. Isotype controls are shown in grey. **(E)** D11-0401 is not modulated by transfection of DO in BJAB 0401 cells. Surface expression of DRB1*04 (NFLD.D1), MHC-II/CLIP (cerCLIP), and D11-0401 was analyzed by flow cytometry on BJAB 0401, BJAB 0401 transfected with DO (BJAB DO 0401), and BJAB transfected with a vector control (BJAB V 0401). Isotype controls are shown in grey. Results are representative of two experiments.


A
5.2.4 0401 (DM-)
5.2.4 0401 DM (DM+)
DRB1*04
MHC-II/ CLIP
D11-0401
B
SAVC
BJAB 0401
Ramos 0401
DRB1*04
MFI
anti-DR (NFLD.D11) dilution
C
BJAB 0401
SAVC
Ramos 0401
kDA
DMα
GAPDH
µg lysate
BJAB
DOβ
D
Savc
BJAB 0401
Ramos 0401
DM
Ii
MHC-II/ CLIP
E
BJAB 0401
BJAB V 0401
BJAB DO 0401
DRB1*04
MHC-II/ CLIP
D11-0401

cytometric analysis demonstrated that DM expression was higher in BJAB 0401 than in Ramos 0401, but reduced compared to SAVC (Figures 3.1C, 3.1D). Consistent with previous reports (93, 313, 314), DOB was not detected in BJAB and was deficient in Ramos (Figure 3.1C). Although all cells expressed similar amounts of Ii, MHC-II/CLIP complexes were drastically reduced on BL lines compared to SAVC, indicating deficient DO and functional DM in these cells (Figure 3.1D).

To determine whether the D11-0401 epitope could be reconstituted in BL cells, we analyzed its expression on BJAB cells transfected with both DO and DRB1*04:01. As shown in Figure 3.1E, D11-0401 expression remained deficient on both BJAB DO 0401 and BJAB V 0401 despite equivalent DRB1*04:01 expression. Increased MHC-II/CLIP levels on BJAB DO 0401 compared to BJAB V 0401 and BJAB 0401 indicated functional DO in this cell line (Figure 3.1E). Taken together, these results suggest that DO does not contribute to formation of the D11-0401 epitope, but as will be discussed later, it is possible that these cells are missing other factors that generate the appropriate peptides bound to the DBR1*04:01 molecules which affects D11-0401 expression.

3.2.2 Endolysosomal and cytoplasmic cysteine proteases contribute to formation of the D11-0401 epitope

The aforementioned and published data suggest the D11-0401 epitope is created by peptides stably bound to the groove of DRB1*04:01 molecules (280). This idea was further supported using antibody blocking assays, where NFLD.D11 binding to SAVC is inhibited by mAb L243, which blocks peptide-specific T-cell responses (34-35).

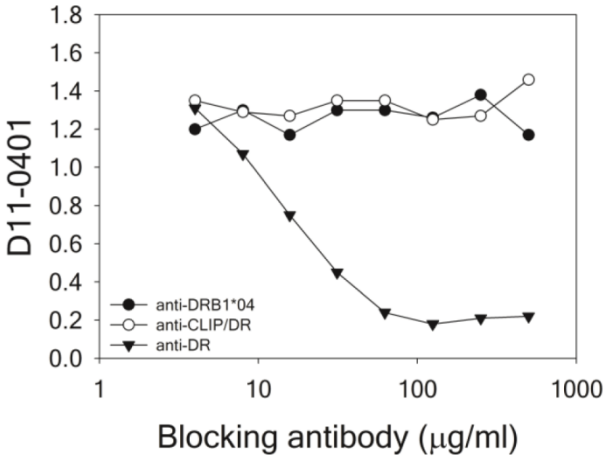
However, it was not inhibited by the pan-DRB1*04 β_2 domain specific mAb NFLD.D1 (Figures 3.2A, 3.2B), or by cerCLIP, an anti-MHC-II/CLIP mAb (Figure 3.2A). While these results suggest peptides other than CLIP are involved in the D11-0401 epitope, we cannot prove a restricted set of peptides contributes to its topology due the unsuitability of NFLD.D11 for pMHC-II immunoprecipitation and analysis of eluted peptides.

To investigate the peptide source and intracellular antigen processing mechanisms contributing to D11-0401, SAVC cells were treated or not with pepstatin A, an inhibitor of aspartyl proteases; leupeptin, a broad inhibitor of cysteine proteases; CBI II, a cathepsin B inhibitor; or calpeptin, an inhibitor of cytoplasmic calpain proteases for 18 hours, followed by analysis using CELISA. No inhibitor significantly altered total DR or DRB1*04:01; however, D11-0401expression was reduced by at least half in cells treated with leupeptin, CBI II, or calpain, but not reduced in cells treated with pepstatin A (Figure 3.3A). This suggests that antigen processing by endosomal and cytoplasmic cysteine proteases but not aspartyl proteases, contribute to generating peptides requisite for the D11-0401 epitope. Western blot analysis of non-reduced samples from whole cell lysates treated with cysteine protease inhibitors as described above confirmed large amounts of SDS-stable DR and DRB1*04 dimers in treated and untreated cell, as well as increased DR monomers in cells treated with leupeptin and calpeptin (Figure 3.3B). Furthermore, SAVC cells treated with both leupeptin and CBI II or calpeptin, or CBI II and calpeptin resulted in greater than 95% decrease in D11-0401 (Figure 3.3C). By contrast, SAVC treated with combinations of pepstatin A with each cysteine inhibitor showed no further D11-0401 decrease compared to single inhibitor treatments, indicating that aspartyl protease-derived peptides do not contribute to the D11-0401 epitope (Figure

Figure 3.2. D11-0401 is located on the peptide binding groove of DRB1*04:01 molecules.

(A) D11-0401 expression measured by CELISA on SAVC cells, after prior incubation with the indicated concentrations of blocking mAbs: NFLD.D1, specific for an epitope on the β_2 domain of all DRB1*04 molecules; cerCLIP, MHC-II/CLIP complexes; L243, specific for a conformational epitope near the peptide binding groove on all DR molecules. D11-0401 expression is given as OD values. **(B)** D11-0401 expression measured by flow cytometry on SAVC cells, previously incubated with the blocking mAb L243 (anti-DR) or with an isotype control mAb. Filled histograms represent isotype control staining. Unfilled histograms represent D11-0401 expression.

A


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D11-0401
anti-DRB1*04
anti-CLIP/DR
anti-DR
1
10
100
1000
Blocking antibody (μg/ml)

B

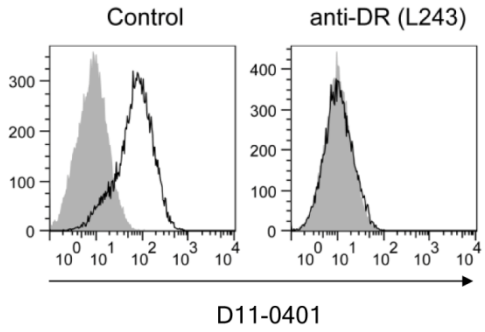
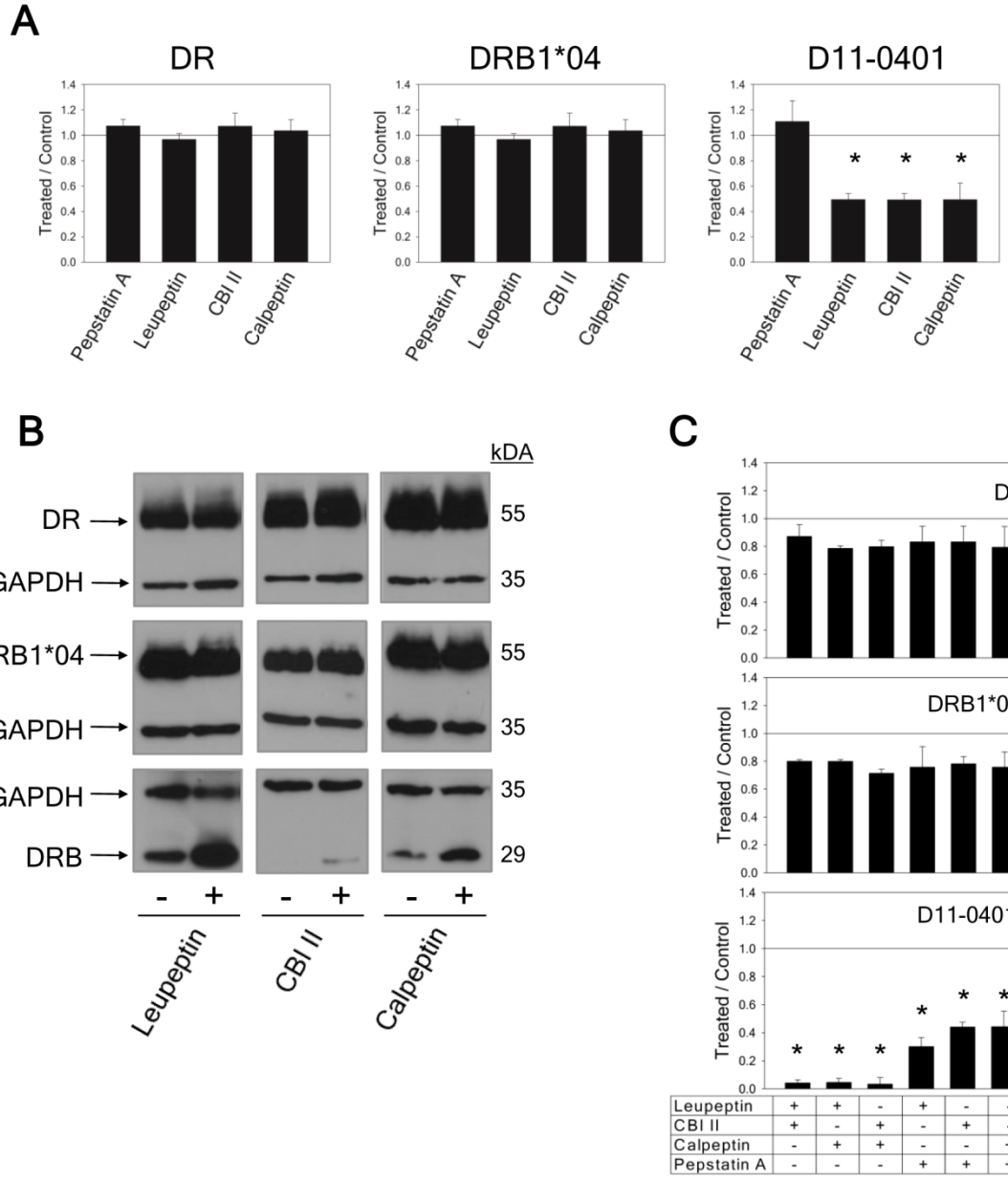

Control
anti-DR (L243)
300
200
100
0
400
300
200
100
0
10^0 10^1 10^2 10^3 10^4
D11-0401

Figure 3.3. Generation of the D11-0401 epitope is partially abrogated by cysteine protease inhibitors.

(A) SAVC cells treated with 25μM pepstatin A, 50μM calpeptin, 25μM leupeptin, 100μM CBI II, or with a diluent control and were analyzed for pan-DR (L243), DRB1*04:01 (NFLD.D1), and D11-0401 expression by CELISA. Results are presented as the fold change in expression (treated/control), calculated by dividing the mean OD of inhibitor-treated cells by the mean OD of control cells. A value equal to 1 (solid line) indicates no change in expression. Values are means of at the least three experiments with bars representing standard error ($n \geq 3$). Significant changes in expression due to protease inhibition were determined by comparing with total DR using a paired t-test, where an asterisk denotes $p < 0.05$. **(B)** The effect of cysteine protease inhibitors on formation of SDS-stable DR dimers in SAVC cells. Whole cell lysates prepared from SAVC cells treated with indicated cysteine protease inhibitors or diluent control were analyzed by SDS-PAGE (20 μg/lane) under non-reducing conditions and western blotting to detect SDS-stable DR dimers (L243), DRB1*04 dimers (NFLD.D1) or DRB monomers (Tal 14.1). GAPDH served as a loading control. Results are representative of two experiments. **(C)** SAVC cells treated with combinations of indicated protease inhibitors were analyzed by CELISA for DR, DRB1*04, and D11-0401 expression as described in A.


A
DR
DRB1*04
D11-0401
Treated / Control
Pepstatin A
Leupeptin
CBI II
Calpeptin
B
kDA
DR
GAPDH
DRB1*04
GAPDH
GAPDH
DRB
55
35
55
35
35
29
- + - + - +
Leupeptin
CBI II
Calpeptin
C
DR
DRB1*04
D11-0401
Leupeptin + + - + - -
CBI II + - + - + -
Calpeptin - + + - - +
Pepstatin A - - - + + +

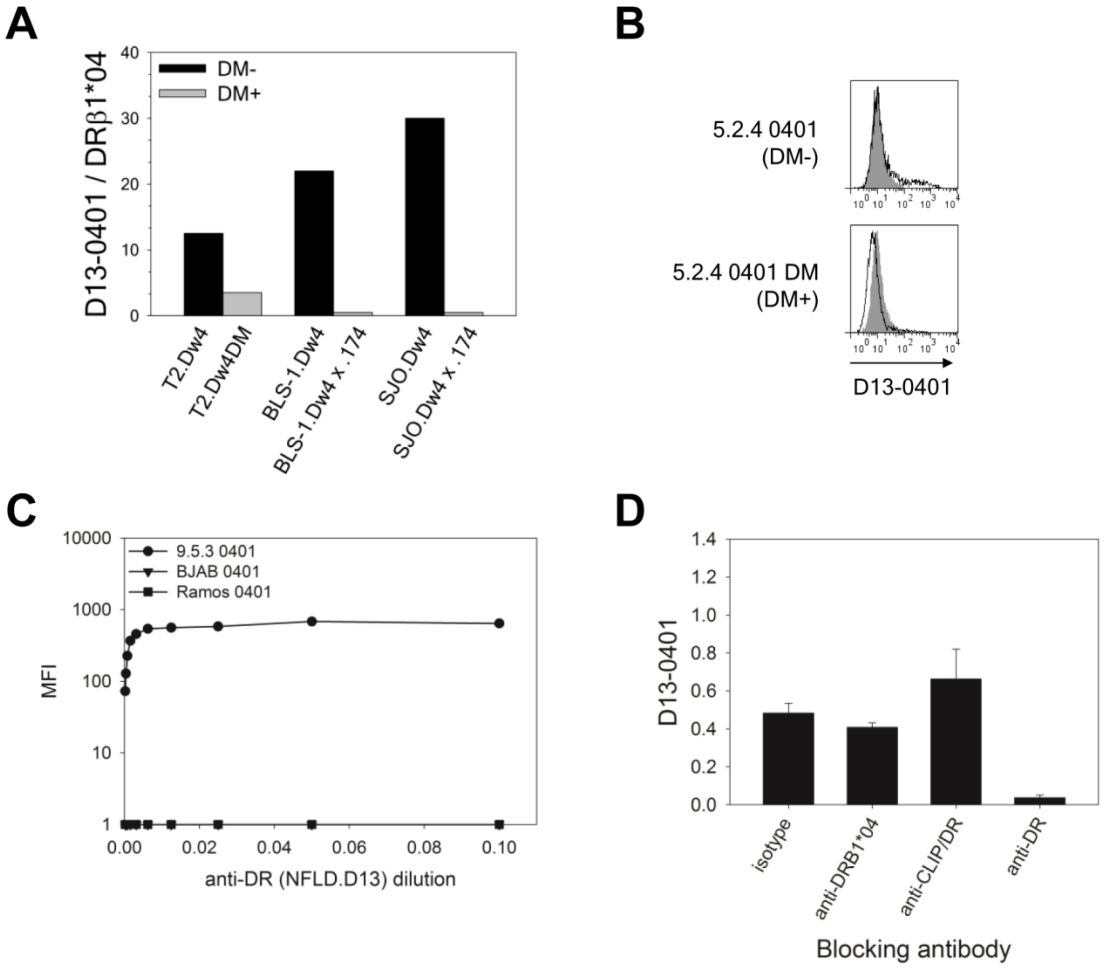
3.3C). Since cellular expression of mature pMHC-II complexes and DRB1*04:01 were minimally affected by protease inhibitor combinations, it is likely that D11-0401 loss was specific and not due to a general effect on DR molecules. Similar experiments using lactacytsin, a proteasome inhibitor; wortmannin, an autophagy inhibitor; bafilomycin, an endosomal acidification and late autophagy inhibitor; and chloroquine, an endosomal acidification inhibitor, did not significantly alter presentation of D11-0401 epitope (Appendix A). Altogether, these results support the idea that endogenous proteins cleaved by cysteine proteases within both endolysosomal and cytoplasmic compartments are the source of peptides forming the D11-0401 epitope. Alternatively and later examined in more detail, inhibition of cellular proteases may adversely affect the entire DM-mediated pathway of peptide loading, thus preventing formation of the DM-dependent D11-0401 epitope.

3.2.3 Expression analysis of the DM-sensitive D13-0401 epitope

We previously showed that a cross reactive epitope on DRB1*04:01 (D13-0401) and DRB1*04:04 (D13-0404) molecules, was strongly expressed on DM- DO+ 9.5.3 0401 cells but lost on the DM+ DO+ parental cell line 8.1.6 0401 (78). Consistent with these results, D13-0401 was expressed on the mutant cell lines T2.Dw4, BLS-1.Dw4, and SJO.Dw4 (all DM- DO-), and abrogated by DM restoration in these cells either by transfection or by gene complementation (Figure 3.4A). In addition, weak D13-0401 expression observed on DM- DO- 5.2.4 0401 cells was lost after DM restoration (Figure 3.4B). Not surprisingly, DM+ BL cell lines BJAB 0401, BJAB 0401 DO, and Ramos

Figure 3.4. Cell surface expression of the D13-0401 epitope on DRB1*04:01 molecules occurs in the absence of DM**.**

(A) DM- and DM-restored mutant B cell lines transfected with DRB1*04:01 were analyzed for D13-0401 by flow cytometry. DM was restored by either transfection or cell fusion with the DM-expressing cell line .174 as previously described (280). D13-0401 binding is expressed as a percentage of total DRB1*04 surface expression (NFLD.D1). **(B)** Weak D13-0401 expression on DO- 5.2.4 0401 is further diminished by DM restoration. Surface expression of D13-0401 was analyzed by flow cytometry on DM- and DM-restored 5.2.4 0401 cells. Isotype controls are shown in grey. **(C)** BJAB 0401 and Ramos 0401 do not express D13-0401. D13-0401 surface expression on BJAB 0401 and Ramos 0401 was compared to its expression on 9.5.3 0401 cells using flow cytometry. D13-0401 expression was plotted against increasing dilutions of NFLD.D13 used to stain cells for flow cytometry (0.1 = 1:10 dilution). Results are representative of three experiments. **(D)** D13-0401 is located on the peptide binding groove of DRB1*04:01 molecules. CELISA was used to determine NFLD.D13 binding to the D13-0401 epitope on 9.5.3 0401 cells after prior incubation with the blocking mAbs: anti-DRB1*04 (NFLD.D1), anti-MHC-II/CLIP (cerCLIP), anti-DR (L243), or an isotype control. D13-0401 expression is shown as OD values. Bars indicate standard deviation from triplicate measurements (n=1).


A
D13-0401 / DRβ1*04
DM-
DM+
T2.Dw4
T2.Dw4DM
BLS-1.Dw4
BLS-1.Dw4 x .174
SJO.Dw4
SJO.Dw4 x .174
B
5.2.4 0401
(DM-)
5.2.4 0401 DM
(DM+)
D13-0401
C
9.5.3 0401
BJAB 0401
Ramos 0401
MFI
anti-DR (NFLD.D13) dilution
D
D13-0401
isotype
anti-DRB1*04
anti-CLIP/DR
anti-DR
Blocking antibody

0401 also did not express D13-0401 (Figure 3.4C). Taken together, these results suggest that the D13-0401 epitope is negatively modulated by DM regardless of the presence of DO, but may well depend on whether the appropriate peptides are generated in these cell lines.

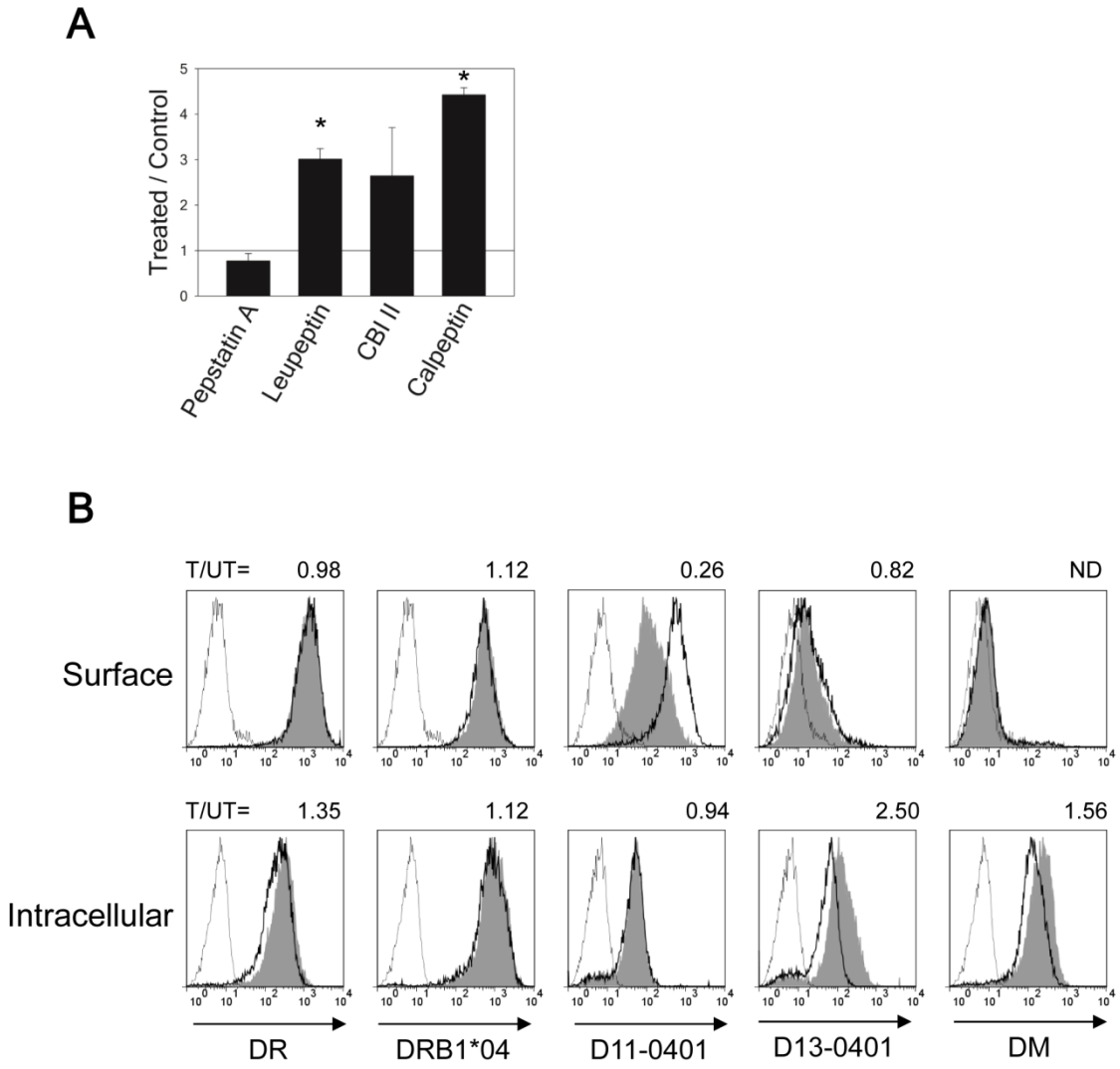
Since DM- cells express abundant MHC-II/CLIP complexes, we initially suspected the D13-0401 epitope was formed by CLIP bound to DRB1*04:01. However, its weak expression on 5.2.4 0401 cells which contains abundant MHC-II/CLIP complexes shown in Figure 3.1A suggests otherwise. Furthermore, the epitope was not blocked by cerCLIP or NFLD.D1, while it was fully blocked by L243 (Figure 3.4D), suggesting that peptides other than CLIP, but similar in terms of their DM-sensitivity are responsible for its formation and cell surface expression.

3.2.4 Endolysosomal and cytoplasmic cysteine proteases are necessary for D13-0401 expression in DM- cells, but contribute to its loss in DM+ cells

Although the D13-0401 epitope is clearly sensitive to expression of DM transgenes in antigen presentation mutants, it was variably expressed on SAVC cells. In particular, we noted substantially increased D13-expression in leupeptin-treated cells analyzed using CELISA (Figure 3.5A). However, this assay uses PFA-fixed cells and wash buffer containing Tween-20 detergent which may result in membrane permeabilization, thus allowing detection of intracellular epitopes. To clarify this, we analyzed D13-0401 and D11-0401 expression on leupeptin-treated SAVC cells using surface and intracellular flow cytometry. Leupeptin treatment minimally affected surface

Figure 3.5. Leupeptin treatment differentially modulates cell surface and intracellular DRB1*04 epitopes in SAVC cells.

(A) SAVC cells were analyzed for D13-0401 expression by CELISA after treatment with protease inhibitors or diluent control. Results are presented as the fold change in expression (treated/control), calculated by dividing the mean OD of inhibitor-treated cells by the mean OD or MFI of control-treated cells, where a value equal to 1 (solid line) indicates no change in expression. Presneted values are the mean of at the least three independent experiments with bars representing standard error ($n \geq 3$). Significant changes in expression due to protease inhibition were determined by comparing with total DR using a paired t-test, where an asterisk denotes $p < 0.05$. **(B)** SAVC, treated with 25μM leupeptin (filled histograms) or with diluent (unfilled histograms), were analyzed by flow cytometry for surface and intracellular expression of DR (L243), DRB1*04 (NFLD.D1), D11-0401, D13-0401, and DM (Map.DM1). Broken line histograms represent isotype controls. Leupeptin-treated / diluent control-treated values (T/UT) were calculated as: (MFI of leupeptin-treated cells – MFI of isotype control) / (MFI diluent-control cells – MFI isotype control). Results are representative of two independent experiments.

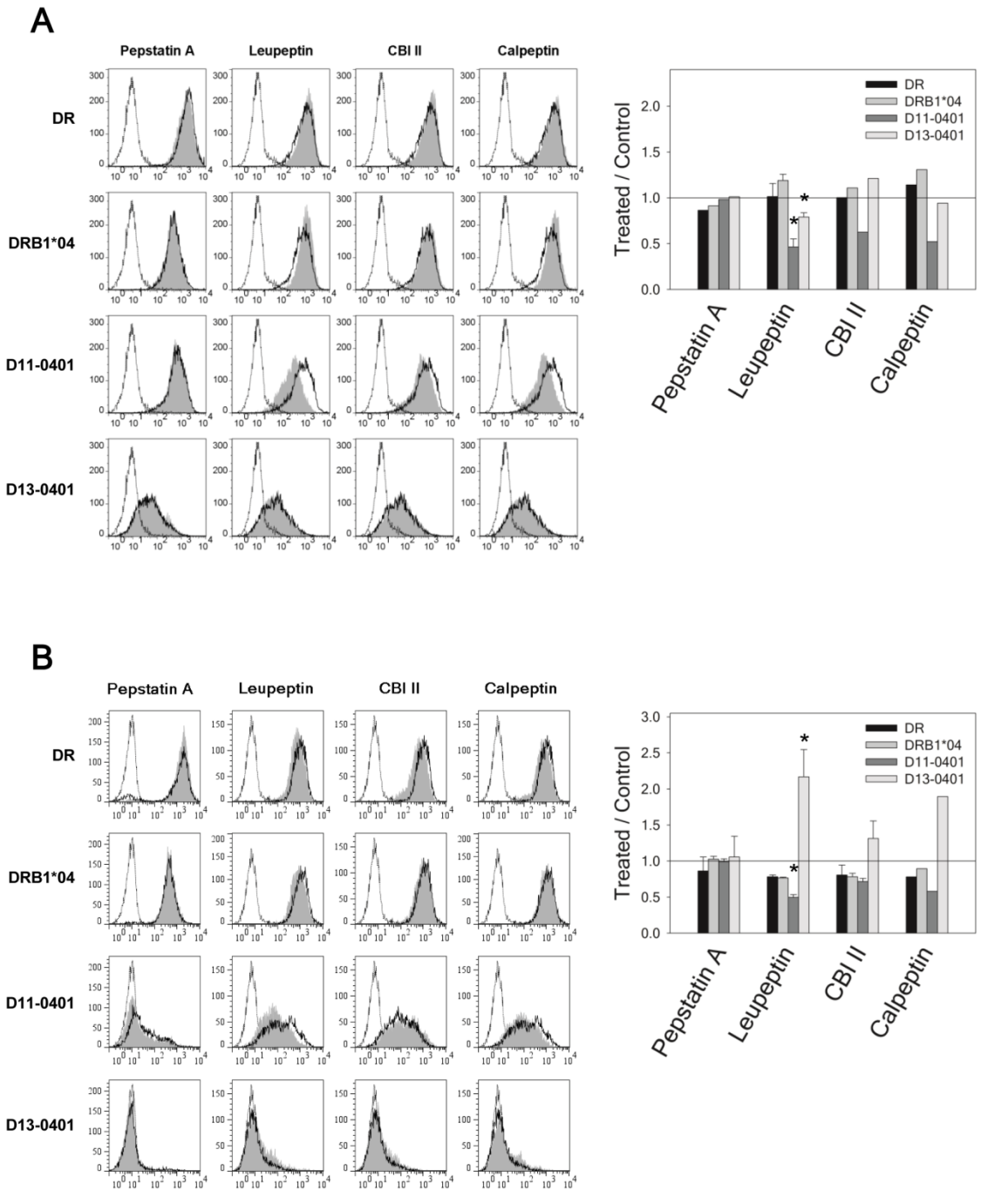

A
Treated / Control
0
1
2
3
4
5
*
*
Pepstatin A
Leupeptin
CBI II
Calpeptin
B
Surface
Intracellular
T/UT=
0.98
1.12
0.26
0.82
ND
T/UT=
1.35
1.12
0.94
2.50
1.56
DR
DRB1*04
D11-0401
D13-0401
DM

peptide-DR conformers, DRB1*04:01 and DM, but resulted in severe loss of surface D11-0401, and slightly diminished D13-0401 (Figure 3.5B). In contrast, intracellular flow cytometric analysis revealed a 2.5-fold increase in intracellular D13-0401 expression in leupeptin-treated cells, no appreciable change in D11-0401, and increased expression of pDR conformers and DM (Figure 3.5B). These results indicate that 1) the DM-sensitive D13-0401 epitope is present intracellularly in DM+ SAVC cells, but its formation is inhibited by cysteine proteases; 2) leupeptin-mediated increase in intracellular D13-0401 is not observed on the cell surface likely due to increased amounts of DM which prevent surface expression of DRB1*04:01 molecules bearing the D13-0401 epitope; and 3) leupeptin treatment diminishes surface expression of DM-dependent D11-0401 despite increased intracellular DM levels.

To validate the effect of cellular proteases in generating the DM-sensitive and DM-dependent DRB1*04:01 epitopes, SAVC, 8.1.6 0401, and 9.5.3 0401 cells were treated with protease inhibitors and analyzed for D11-0401 and D13-0401 using surface flow cytometry. Consistent with CELISA results, no inhibitor treatment significantly affected expression of total DR or DRB1*04 in all 3 cell lines (Figures 3.6A, 3.6B, 3.6C), while leupeptin, CBI II, and calpeptin, but not pepstatin A, significantly reduced D11-0401 surface expression on SAVC (Figure 3.6A). D13-0401 expression was variable on SAVC, but not significantly altered by any treatment (Figure 3.6A). 8.1.6 0401cells treated with leupeptin or calpeptin similarly showed a noticeable decrease in D11-0401, while CBI II had little effect (Figure 3.6B). D13-0401, not normally expressed on the surface 8.1.6 0401 cells, was somewhat elevated on the cysteine protease inhibitor-treated cells (Figure 3.6B). In contrast, treatment of 9.5.3 0401 cells with the same cysteine

Figure 3.6. Expression of DM-dependent D11-0401 and DM-sensitive D13-0401 epitopes requires similar proteases.

SAVC **(A)**, 8.1.6 0401 **(B)**, and 9.5.3 0401 **(C)** cells were treated with 25μM pepstatin A, 50μM calpeptin, 25μM leupeptin, 100μM CBI II (filled histograms), or with the appropriate diluent control (unfilled histograms) and surface expression of DR (L243), DRB1*04 (NFLD.D1), D11-0401, and D13-0401 was analyzed by flow cytometry. Broken line histograms represent isotype controls. Results from one to four independent experiments are shown in the bar plots (n = 1-4), where values represent the mean fold change in expression due to protease inhibitor treatment (treated/control) calculated as: (MFI of inhibitor-treated cells – MFI of isotype control) / (MFI diluent-control cells – MFI isotype control), where a value equal to 1 (solid line) indicates no change in expression. Error bars indicate standard error**.** Significant changes in expression due to protease inhibition were determined by comparing with total DR using a paired t-test, where an asterisk denotes $p < 0.05$. **(D)** The effect of cysteine protease inhibitors on formation of SDS-stable DR dimers in 8.1.6 0401 and 9.5.3 0401 cells. Whole cell lysates prepared from 8.1.6 0401 and 9.5.3 0401 cells treated with cysteine protease inhibitors or a diluent control were analyzed by SDS-PAGE (20 μg/lane) under non-reducing conditions and western blotting to detect SDS-stable DR dimers (L243), DRB1*04 dimers (NFLD.D1), and DRB monomers (Tal 14.1). Detection of GAPDH served as a loading control.



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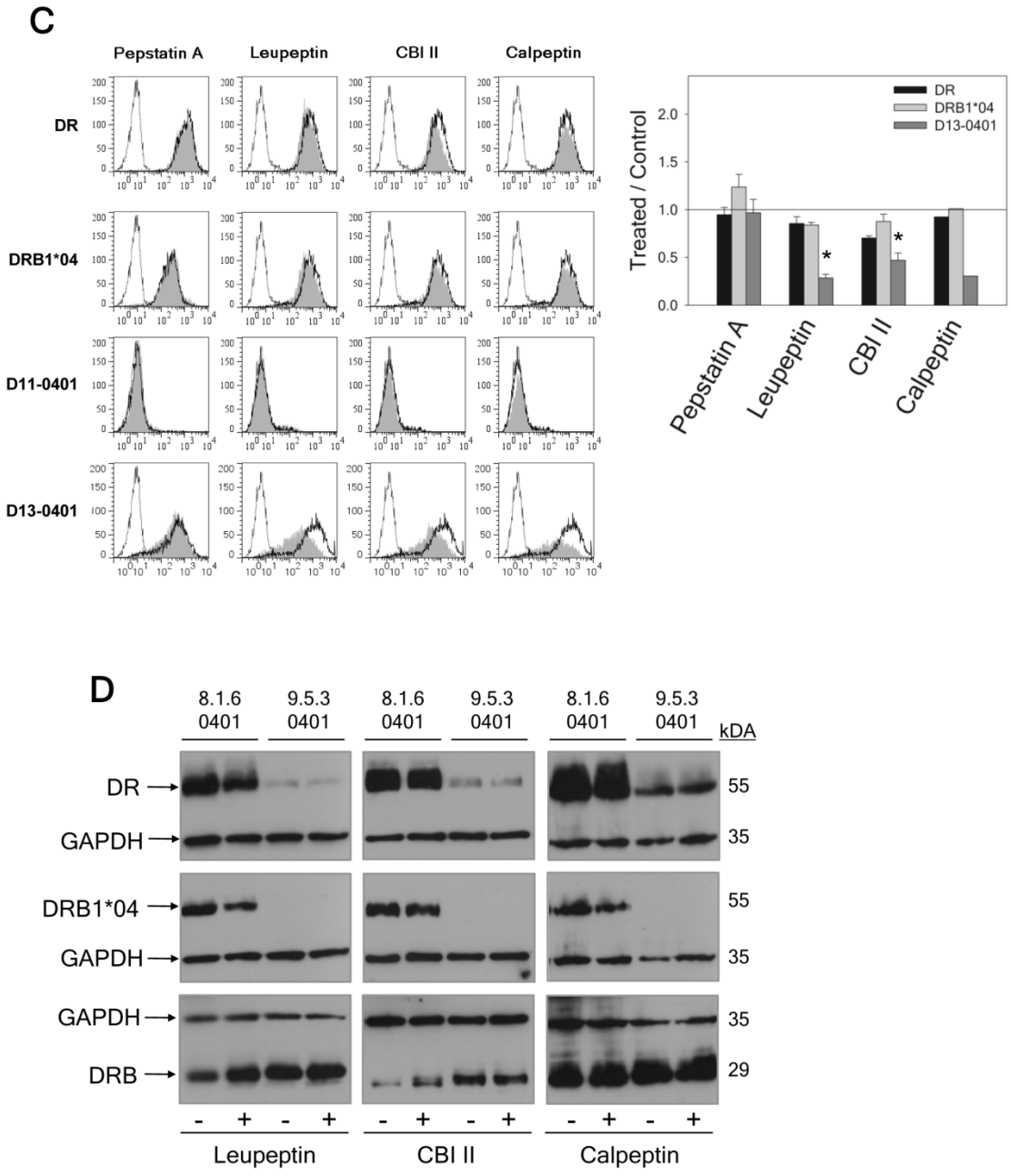

C
Pepstatin A
Leupeptin
CBI II
Calpeptin
DR
DRB1*04
D11-0401
D13-0401
Treated / Control
DR
DRB1*04
D13-0401
Pepstatin A
Leupeptin
CBI II
Calpeptin
D
8.1.6 0401
9.5.3 0401
kDA
DR
GAPDH
DRB1*04
DRB
55
35
29
Leupeptin
CBI II
Calpeptin

protease inhibitors resulted in severe loss of D13-0401 despite no significant reduction in cell surface DRB1*04 surface expression (Figure 3.6C). Thus, endosomal and cytoplasmic cysteine proteases contribute to expression of D11-0401 and loss of D13-0401 epitope in DM+ cells, while the same proteases are required for D13-0401 expression in DM- cells.

The sensitivity of D13-0401 to DM and cysteine proteases suggests this epitope is formed by less stable immature DRB1*04:01/peptide complexes. We therefore compared SDS-stable DR dimers in 8.1.6 0401 and 9.5.3 0401 cells, treated with or without the cysteine protease inhibitors. The amount of total SDS-stable DR and DRB1*04 dimers were noticeably decreased in lysates from leupeptin-treated 8.1.6 0401 cells along with a concomitant increase in DRB monomers (Figure 3.6D). The effects of CBI II and calpeptin on DR and DRB1*04 dimers in 8.1.6 0401cells were not perceptible, but DRB monomers were increased by CBI II treatment (Figure 3.6D). Consistent with previous reports (270), 9.5.3 0401 exhibited considerably reduced amounts of endogenous SDS-stable DR dimers and increased DRB monomers compared to 8.1.6 0401, while protease inhibitor treatment had minimal effect (Figure 3.6D). No SDS-stable DRB1*04 dimers were observed in 9.5.3 0401 indicating that DRB1*04:01 molecules in these DM- cells primarily exist in a SDS-unstable conformation. The amounts of DM were not appreciably reduced in lysates from inhibitor-treated 8.1.6 0401 cells indicating that the loss of D11-0401 and gain of D13-0401 is not a result of alterations in DM expression (Figure 3.9A). In summary, the above results suggest that endosomal and cytoplasmic cysteine proteases differentially effect formation and surface expression of D13-0401 in presence or absence of DM.

3.2.5 Expression analysis of the DM-resistant D13-0404 epitope

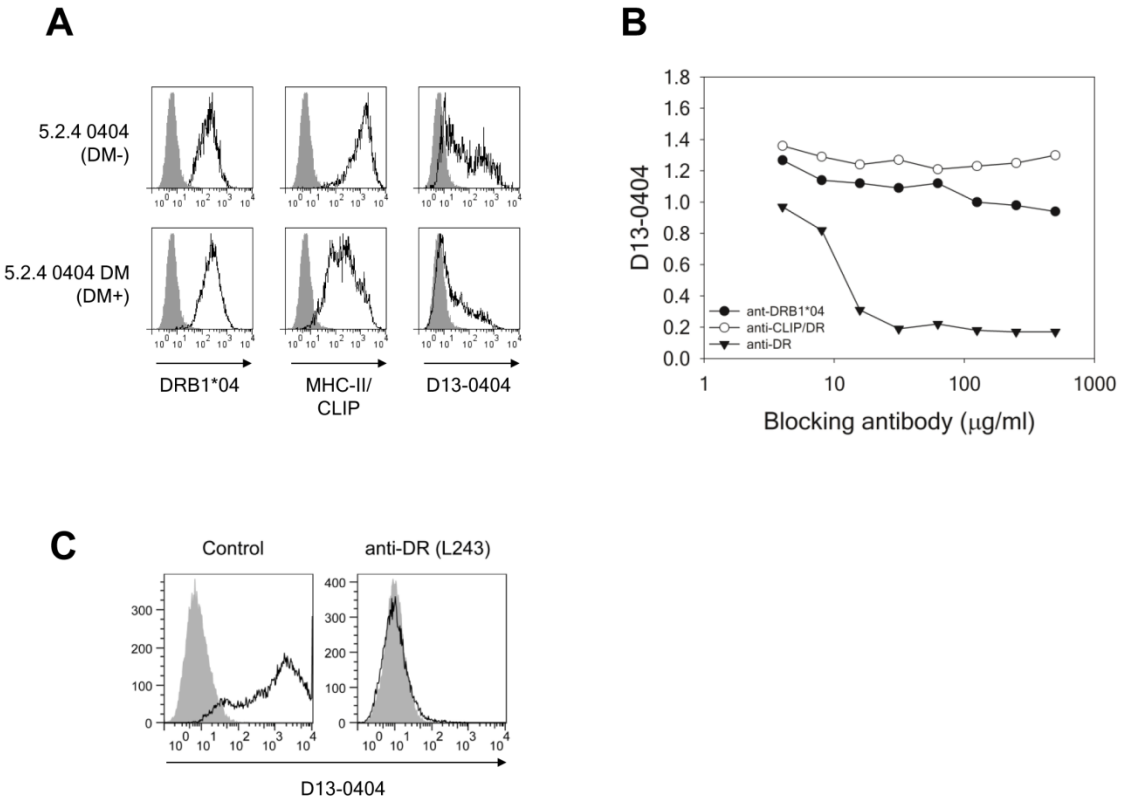
The cognate epitope D13-0404 recognized by the mAb NFLD.D13 was initially identified on DRB1*04:04 molecules and is restricted to pAPCs as well as DM- antigen presentation mutants transfected with DRB1*04:04 (283, unpublished). Flow cytometric analysis of D13-0404 expression demonstrated that this epitope is expressed by 5.2.4 0404 irrespective of DM expression, although at noticeably reduced levels in the DM+ cells (Figure 3.7A). Similar to the other DRB1*04 epitopes, D13-0404 is unlikely formed by DRB1*04:04/CLIP complexes as epitope blocking assays showed no inhibition of NFLD.D13 binding by the mAb cerCLIP (Figure 3.7B). However, successfully blocking by L243 and not with NFLD.D1 further suggests that the D13-0404 epitope is formed on mature pMHC-II complexes (Figure 3.7C).

3.2.6 Aspartyl and cysteinyl proteases are dispensable in formation of D13-0404

The contribution of peptides generated by endosomal and cytoplasmic antigen processing to the D13-0404 epitope was analyzed by CELISA on MT14B cells treated with pepstatin A, leupeptin, CBI II, or calpeptin. Expression of both total DR and DRB1*04:04, and the amounts of SDS-stable DR dimers were not appreciably affected by any of the inhibitors, although an accumulation of DRB monomers in leupeptin-treated cells was observed (Figures 3.8A, 3.8B). Whereas treatment with leupeptin, CBI II, or calpeptin noticeably altered the presentation of D11-0401 and D13-0401, expression of D13-0404 was unaffected (Figure 3.8A). Furthermore, additional experiments using lactacystin, wortmannin, bafilomycin, chloroquine, or the endosomal cysteine protease

Figure 3.7. The D13-0404 epitope is a DM-resistant epitope expressed on DRB1*04:04 cells.

(A) Surface expression of DRB1*04:01 (NFLD.D1), class-II-CLIP (cerCLIP), and D13-0404 was measured by flow cytometry in DO- 5.2.4 0404 cells with or without transfection with DM. Isotype controls are shown in grey. **(B)** The D13-0404 epitope is located near the peptide binding groove of DRB1*04 molecules. CELISA was used to determine binding of NFLD.D13 to the D13-0404 epitope on MT14B cells after incubation with the indicated concentrations of the blocking mAbs: anti-DRB1*04 (NFLD.D1), anti-MHC-II/CLIP (cerCLIP), anti-DR (L243), or an isotype control. D13-0404 expression is shown as OD values. **(C)** D13-0404 expression was measured by flow cytometry on MT14B cells previously incubated with the blocking mAb L243 (anti-DR) or with an isotype control mAb. Filled histograms represent isotype control staining. Unfilled histograms represent D13-0404 expression.


A
5.2.4 0404
(DM-)
5.2.4 0404 DM
(DM+)
DRB1*04
MHC-II/
CLIP
D13-0404
B
D13-0404
Blocking antibody (μg/ml)
ant-DRB1*04
anti-CLIP/DR
anti-DR
C
Control
anti-DR (L243)
D13-0404

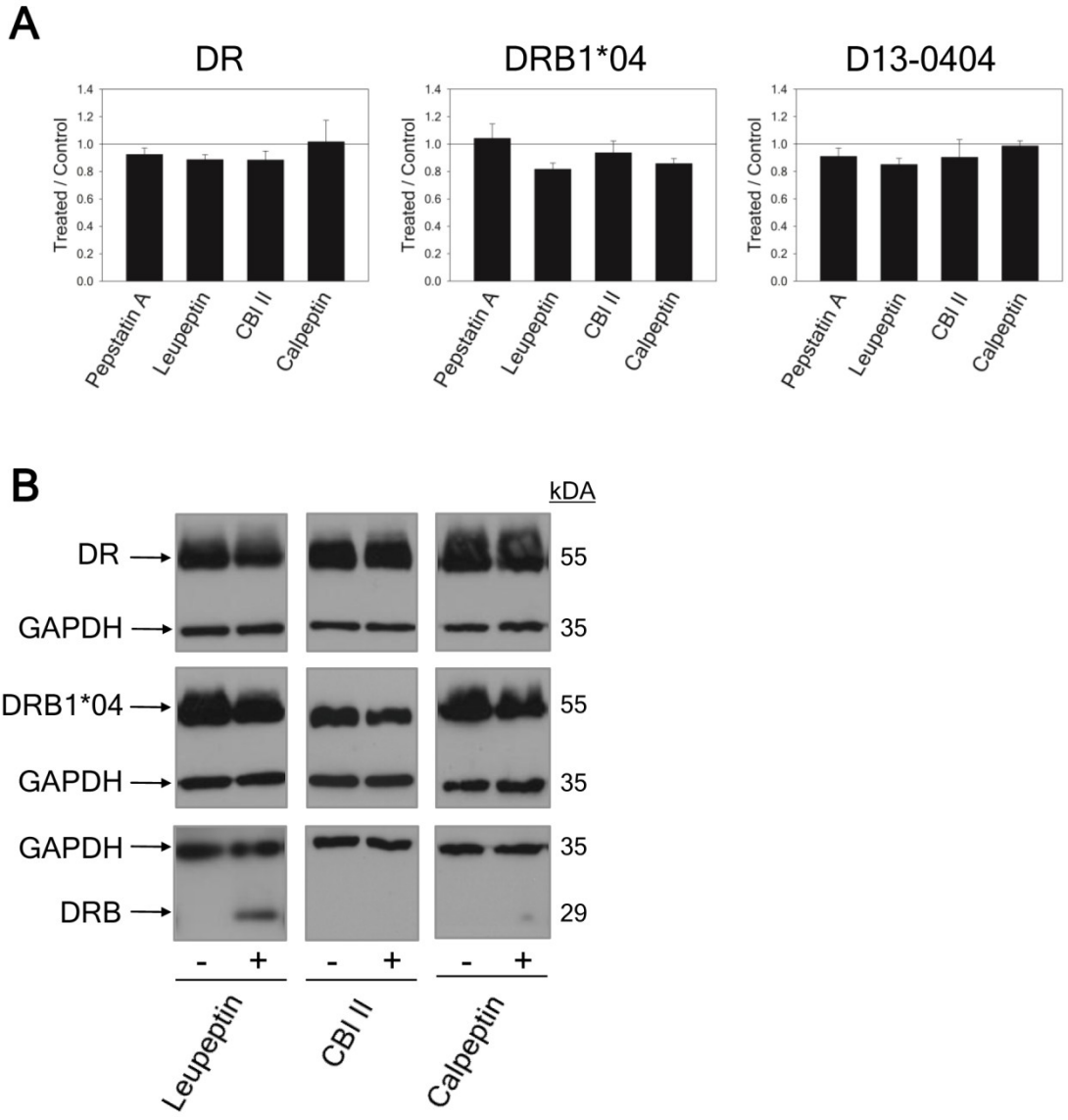
inhibitor E64d, did not significantly modify presentation of D13-0404 (Appendix A). These results demonstrate that endosomal and cytoplasmic cysteine proteases are dispensable in generating the peptides required for D13-0404 and that there is significant redundancy in the proteolytic processing of peptides necessary for formation this epitope.

3.2.7 Inhibition of Ii processing by protease inhibitors may modulate DRB1*04 peptide-dependent epitopes

For DM to facilitate efficient loading of endosomal peptides onto DR molecules, Ii must be cleaved by proteases into CLIP. Incomplete degradation of Ii with an accumulation of Ii degradation intermediates may occur in leupeptin-treated cells, resulting in unstable pMHC-II molecules and a decrease of CLIP depending on which MHC-II alleles are present (53, 78, 79, 117, 315, 316). Our studies on the DRB1*04 epitopes in DM+ cells using cysteine protease inhibitors similarly showed increased DRB monomers, although the amount of surface DR and SDS-stable dimers were still abundant (Figures 3.3, 3.6, 3.8). As an indicator of DM function, we determined the expression of DM and Ii intermediates in all four cell lines, as well as the surface expression of MHC-II/CLIP complexes. The amounts of DM were similar in inhibitor-treated and control cells, while an accumulation of the Ii degradation intermediate Ii-p10 was observed in cells treated with leupeptin, CBI II, or calpeptin, indicating some inhibition of Ii degradation to CLIP (Figure 3.9A). This is further supported by diminished cell surface expression of MHC-II/CLIP complexes in cells treated with cysteine protease inhibitors (Figure 3.9B). Notably, the largest decreases in MHC-II/CLIP complexes occurred on

Figure 3.8. Inhibition of aspartyl and cysteinyl proteases does not alter D13-0404 expression in MT14B cells.

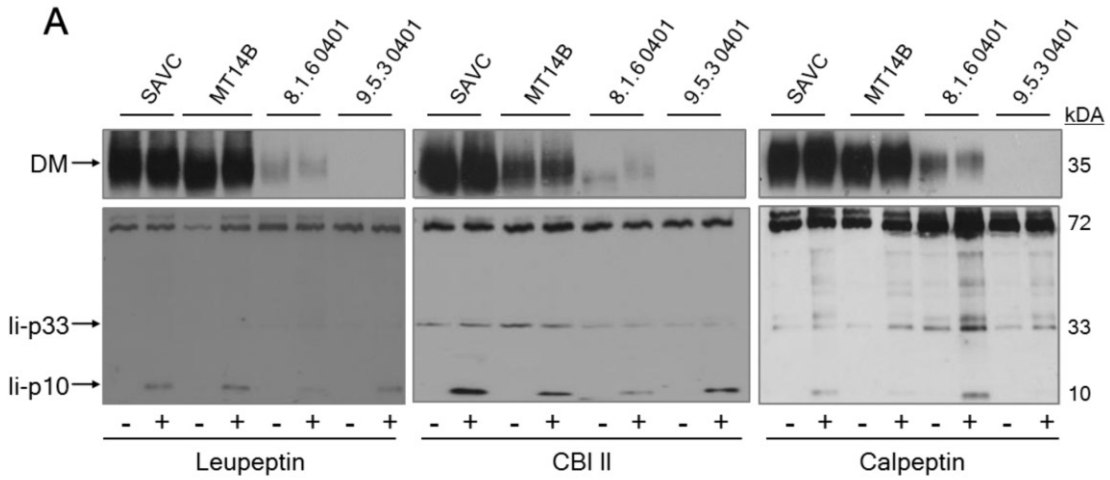
(A) MT14B cells treated with 25μM pepstatin A, 50μM calpeptin, 25μM leupeptin, 100μM CBI II, or with the appropriate diluent control were analyzed for total DR (L243), DRB1*04:01 (NFLD.D1), and D13-0404 by CELISA. The results are presented as the fold change in expression (treated/control), calculated by dividing the mean OD of inhibitor-treated cells by the mean OD of control cells, where a value equal to 1 (solid line) indicates no change in expression. Values are the mean of at the least three independent experiments with bars representing standard error ($n \geq 3$). Significant changes in expression due to protease inhibition were determined by comparing with total DR using a paired t-test, where an asterisk denotes $p < 0.05$. **(B)** The effect of cysteine protease inhibitors on the formation of SDS-stable DR dimers in MT14B cells. Whole cell lysates prepared from of MT14B cells treated with cysteine protease inhibitors or diluent as above were analyzed by SDS PAGE (20 μg/lane) under non-reducing conditions and western blotting to detect SDS-stable DR dimers (L243), DRB1*04 dimers (NFLD.D1), and DRB monomers (Tal 14.1). Detection of GAPDH served as a loading control. Results are representative of two experiments.

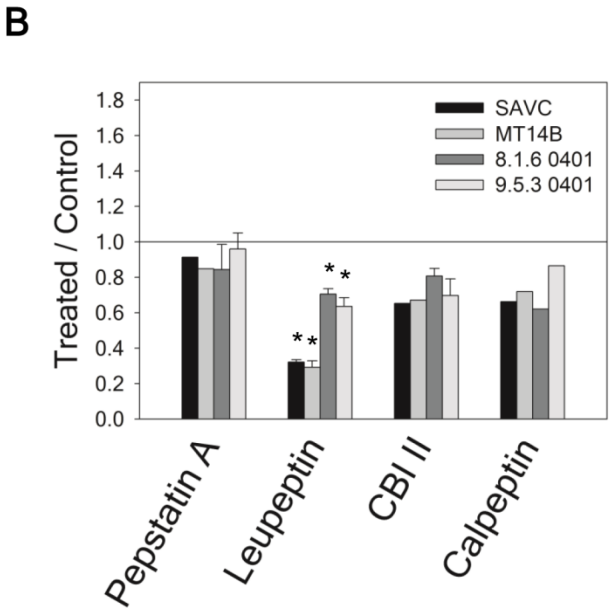

A
DR
DRB1*04
D13-0404
Treated / Control
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Pepstatin A
Leupeptin
CBI II
Calpeptin
B
kDA
DR
GAPDH
DRB1*04
GAPDH
GAPDH
DRB
55
35
55
35
35
29
-
+
-
+
-
+
Leupeptin
CBI II
Calpeptin

leupeptin treated SAVC and MT14B cells (Figure 3.9B). Taken together, these results suggest that impaired peptide loading of DRB1*04 molecules due to insufficient Ii cleavage may contribute to a portion of the observed protease inhibitor-induced reduction in expression of D11-0401 and D13-0401.

Figure 3.9. The effect of protease inhibitors on DM, Ii, and MHC-II/CLIP in DRB1*04 B cell lines.

(A) Whole cell lysates of SAVC , MT14B, 8.1.6 0401, and 9.5.3 0401 cells treated with the indicated cysteine protease inhibitors or diluent control were analyzed by SDS-PAGE (20 μg/lane) under non-reducing conditions and western blotting for expression of DMA (Tal 18.1) and Ii cleavage intermediates (PIN.1). **(B)** Inhibition of cysteine proteases reduces MHC-II/CLIP expression. The surface expression of MHC-II/CLIP (cerCLIP) was analyzed by flow cytometry on SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 cells treated with protease inhibitors or a diluent control. Results are displayed as the fold change in expression due to protease inhibitor treatment (treated/control), calculated as: (MFI of inhibitor-treated cells – MFI of isotype control) / (MFI diluent-control cells – MFI isotype control), where a value equal to 1 (solid line) indicates no change in expression. Displayed values are the mean of one to four independent experiments, with error bars indicating standard error (n = 1-4). Significant changes in expression due to protease inhibition were determined by comparing with total DR using a paired t-test, where an asterisk denotes $p < 0.05$.


A
SAVC
MT14B
8.1.6 0401
9.5.3 0401
kDA
DM
Ii-p33
Ii-p10
35
72
33
10
Leupeptin
CBI II
Calpeptin


B
Treated / Control
SAVC
MT14B
8.1.6 0401
9.5.3 0401
Pepstatin A
Leupeptin
CBI II
Calpeptin

3.3 Discussion

The results herein show that the cellular mechanisms underlying cell surface expression of antibody-defined allele-specific DRB1*04 epitopes are multifaceted. The epitopes, located on the peptide binding sites of DRB1*04 molecules, are formed by allele-specific residues with bound peptides contributing to their topology. While DM was previously known to be requisite for the D1l-0401 epitope and deleterious for the D13-0401 epitope, we show here that co-expression of its co-chaperone, DO, only marginally affected the epitopes in B-LCL and not in BL. Our results further suggest that both endosomal and cytoplasmic cysteine proteases are necessary for their expression; however, whether their role is to directly provide processed peptides or indirectly via inhibition of Ii degradation is currently unclear. By contrast, the D13-0404 epitope displayed much less dependence on DM or antigen processing pathways.

Preferential expression of these epitopes on EBV-transformed B-LCL suggests either cellular specific proteins, different antigen processing mechanisms, or both acting in concert to contribute to their presentation. The paucity of D11-0401 on BL 0401 transfectants cannot be fully explained by DM levels since DM expression in these cells, although reduced compared to SAVC, was at least equivalent to DM in the D11-0401+ 8.1.6 0401 cell line (Figure 3.1). We reasoned that since DM-mediated peptide loading is modulated by DO, expressed MHC-II alleles, and the peptide source, the absence of D11-0401 on BL cells may be due to their deficiency in DO expression (Figure 3.1C, 93, 314). However, no further enhancement of D11-0401 expression in BJAB DO 0401 (Figure 3.1E) nor in T2.Dw4DMDO cells (E. Mellins, unpublished) suggests otherwise. An

alternative explanation is that DO may indeed influence D11-0401 expression, but the peptide source for this epitope is not present in some cell types. For example, the peptidome in BL cell lines likely differs substantively from B-LCL as they are derived from germinal center B cells (317, 318), differentially express EBV genes and CD antigens (319), and are defective in some antigen processing mechanisms (320). Similarly, the T2 cell line, a T cell - B cell hybrid, is likely to have a different peptidome from EBV-transformed and normal B cells.

The potential role of DO in the DM-sensitive D13-0401 epitope was also explored. This epitope, like MHC-II/CLIP, is abundant on the DM- DO+ 9.5.3 0401 cell line, but is also amply expressed by DM- DO- mutant cell lines and abrogated by DM restoration (Figure 3.4C). However, low levels of D13-0401 on the T2.Dw4DMDO cells (E. Mellins, unpublished) as well as variable membrane and intracellular expression in SAVC cells, suggest that DO may sufficiently inhibit DM to allow low levels of D13-0401 expression. Such HLA-II-restricted epitopes including several endogenous minor histocompatibility antigens and CLIP have been previously described as DM-sensitive (102, 104). While MHC-II/CLIP was clearly upregulated in our DO+ transfected cells regardless of DM expression, there was no strict correlation between CLIP and D13-0401 expression. The absence of D13-0401 on the CLIP+ transfectants BJAB DO 0401 and 5.2.4 0401 combined with no inhibition by the MHC-II/CLIP-specific mAb cerCLIP (Figure 3.4), argues against CLIP contributing to the epitope.

Although we have not identified peptides contributing to the DRB1*04 epitopes, our data suggest that both D11-0401 and D13-0401 require endogenous and not exogenous proteins. We base this on our finding that calpeptin, an inhibitor of cytosolic

calpain, when combined with the endosomal cysteine inhibitors leupeptin or cathepsin B, severely reduced epitope expression in comparison to treatment with either single inhibitor. One interpretation is that cytoplasmic proteins first processed by calpain are translocated to MIIC to undergo further processing by cathepsin B and other leupeptin-sensitive proteases before binding to DRB1*04:01. Supporting this notion are several reports of MHC-II presentation of cytoplasmic antigens (30, 71, 139, 321-323) including a seminal study by Lich *et al.* (323) where they demonstrated that a DRB1*04:01-restricted endogenous glutamic decarboxylase immunodominant epitope required cytoplasmic processing with subsequent processing in endosomes or lysosomes. In addition to calpain, both the proteasome and autophagy have been described as mechanisms by which cytoplasmic antigens are degraded for presentation by the MHC-II pathway (71, 139, 322, 324-326). However, the proteasome inhibitor lactacystin and autophagy inhibitor wortmannin had little effect on their presentation, (Appendix A), suggesting a different mechanism is responsible for calpain-processed peptides gaining access to the MHC-II pathway.

In contrast to the D13-0401 loss in DM- 9.5.3 0401 cells, inhibition of calpain and cysteine proteases in DM+ SAVC and 8.1.6 0401cells resulted in an increase in the epitope (Figures 3.5, 3.6), suggesting that different peptides contribute to D13-0401 in DM- and DM+ cells. Among the repertoire of endogenously-derived presented peptides in DM+ cells are likely sets of peptides that form D13-0401 and D11-0401. However, DM-editing licenses transport and expression of D11-0401 molecules, but not D13-0401, for cell surface expression. In the absence of DM, DRB1*04:01/peptide complexes forming D13-0401 (or MHC-II/CLIP) are well-expressed on the cell surface. It is

tempting to speculate that similar peptides form DM-dependent D11-0401 and DM-sensitive D13-0401 since they are inhibited by the same protease inhibitors. However, the D13-0401 epitope formed in DM+ cells likely results from different peptides, which become more available by inhibition of cytoplasmic and endosomal proteases. These results suggest that it may be possible to induce the presentation of a MHC-II epitope normally destroyed by DM by inhibiting cellular proteases, similar to a study which demonstrated presentation of a MHC-I epitope after proteasome inhibition (327).

Current models of DM function propose that DM dissociates low-stability pMHC-II and stabilizes the unbound or open conformation of MHC-II thereby allowing binding of high affinity peptides, thus shaping the repertoire of epitopes expressed by pAPC (14). The differing DM requirements between D11-0401 and D13-0401 may provide insight into concepts of immunodominance and crypticity. Initial explanations for crypticity argued that differences in proteolytic processing of immunodominant and cryptic peptides could account for crypticity because these epitopes would be degraded during antigen processing. However, others have demonstrated that presentation of immunodominant epitopes are enhanced by DM while cryptic epitopes are antagonized by DM, suggesting that DM determines the immunodominant or cryptic fate of a given MHC-II epitope (328, 329). Our results support this view because even though both D11-0401 and D13-0401 epitopes exhibit similar protease requirements, they are differentially expressed depending on the presence or absence of DM. Furthermore, the intracellular levels of D13-0401 epitope in DM+ DO+ B-LCL suggest that this epitope forms within the endosomal pathway, but is antagonized by DM before it can reach the surface.

A major limitation of this study is peptide elution and mass spectrometry studies were not possible due to unsuitability of the NFLD.D11 and NFLD.D13 mAbs for immunoprecipitation. Although our results suggest cytoplasmic proteins are the likely source of peptides contributing to D11-0401 and D13-0401, we can only speculate on the actual protein source. Given the epitopes are largely restricted to EBV-transformed B cell lines, possible candidate antigens include B cell specific proteins such as immunoglobulin, or EBV viral proteins including nuclear antigen, or latent membrane proteins. Many of the latter are not expressed in BJAB and Ramos cells (330, 331), which were notably negative for D11-0401 or D13-0401 (Figures 3.1, 3.4). Peptides derived from HLA-I molecules are also strong candidates as they were previously shown to constitute an antibody-defined epitope in the context of DRB1*01 (255), and were abundant among peptides eluted from DRB1*04:01 molecules analyzed by mass spectroscopy (158). Incomplete reconstitution of D11-0401 on T2.Dw4DM, a line which poorly expresses HLA-I, combined with a lack of D11-0401 expression on HLA class-I-negative Daudi-Dw4 cells (280), initially suggested HLA-I molecules were the peptide source. However, failure to induce D11-0401 in Daudi-Dw4 by transfecting with B2M to reconstitute MHC-I expression, does not support this view (S. Drover, unpublished). A further argument against HLA-I as the peptide source is that BJAB and Ramos both express HLA-I, with the caveat that other defects in the HLA-I antigen processing pathway have been reported in BL cells (320).

Given the strong genetic relationship between both DRB1*04:01 and DRB1*04:04 and severe RA (284, 285, 332, 333), we previously tested and showed expression of D11-0401 and D13-0404 epitopes on DRB1*04+ B cells from RA patients

and healthy patients (310). In that study, DRB1*04 was identified using the DR4-specific mAb, NFLD.D1 and the designation of DRB1*04:01 and DRB1*04:04 was based on NFLD.D11 and/or NFLD.D13 binding, not allele-specific DRB1*04 typing. Since DM is critical to modulation of both D11-0401 and D13-0401 (potentially same topology as D13-0404) and given reports of decreased DM levels in B cells from RA patients (286), a further analysis of these epitopes combined with DM expression in RA is warranted. Such a study may provide further understanding of cross reactive HLA-DRB1*04 epitopes formed from self-peptides in the context of DM expression and DRB1*04-associated autoimmune disease.

Chapter 4

A study on the role of exosomes, lipid rafts, and tetraspanin microdomains in the formation of DRB1*04 epitopes

4.1 Preamble and objectives

In addition to presenting pMHC-II on their cell surface, pAPC constitutively secrete a significant amount of pMHC-II on exosomes that are able to directly and indirectly activate antigen-specific T cells both *in vitro* and *in vivo* (219, 220, 240, 241), as well as mediate the transfer of antigen between cells (244, 334). Exosomes have also been found to contain unique MHC molecules that are not expressed in the plasma membrane (245), suggesting that antigen presentation via exosomes may result in the presentation of unique epitopes that are not generated in the classical pathway of antigen presentation. Previous work by Spurrell D. R. identified expression of D11-0401 on exosome-like vesicles near the cell surface of B-LCL, as well as localization of D11-0401 and D13-0404 to sites of intracellular exosome biogenesis (283). Based on this, we hypothesized that the DRB1*04 epitopes are expressed on B-LCL-derived exosomes. Investigating the repertoire of pMHC-II expressed on exosomes has the potential to impact our understanding of how exosomes contribute to cellular communication between distant immune cells.

Recent studies suggest that pMHC-II are not uniformly distributed throughout the plasma membrane in pAPCs, but instead are concentrated and organized into supramolecular complexes such as lipid rafts or TEMs in a way which facilitates antigen presentation (145, 151-156, 163, 193). Preliminary work performed by Spurrell D.R. suggested that DR molecules bearing the D11-0401 and D13-0404 epitopes were associated with tetraspanins CD63 and CD82 and that disruption of TEMs caused a notable decrease in surface expression of these epitopes (283), suggesting that these

epitopes may be expressed on a distinct subset of DRB1 molecules that are associated with TEMs. However, these previous experiments did not include the analysis of appropriate control molecules. In addition, these experiments suggested no association of these epitopes with lipid rafts, but MBCD treatment to disrupt rafts was performed on cells fixed with paraformaldehyde, making it difficult to understand how this treatment could disrupt chemically cross-linked proteins. Using an updated methodology where MBCD treatment was performed prior to cell fixation and included analysis of additional control molecules, as well as preparation of DRMs containing rafts, we further investigated the hypothesis that the D11-0401, D13-0401, and D13-0404 epitopes are concentrated in TEMs and lipid rafts. The results from these experiments will hopefully help clarify the role of membrane microdomains in concentrating speicifc pMHC-II complexes on the cell surface. Since an effective immune response relies heavily upon the activation of T cells by APCs, this reaearch may further reveal the ways in wcih membrane organization of MHC-II enhances this process.

The specific objectives addressed in this chapter are:

1. To characterize the expression of the D11-0401, D13-0401, and D13-0404 epitopes on exosomes secreted from B-LCL to gain a further understanding of the formation of these epitopes within the antigen processing pathway.
2. To determine if lipid rafts and TEMs contain pMHC-II containing as specific repertoire of peptides by examining whether the D11-0401, D13-0401, and D13-0404 epitopes segregate into these membrane microdomains.

4.2 Results

4.2.1 DRB1*04-restricted epitopes are differentially expressed on B-LCL-derived exosomes.

MIICs, the late endocytic vesicles where most DM-mediated peptide loading of MHC-II occurs, are also important for exosome biogenesis (237). Co-localization of D11-0401 and D13-0404 with MIIC markers suggested these and possibly the D13-0401 epitope might be expressed on exosomes (283). Exosomes isolated from SAVC, MT14B, 8.1.6 0401 and 9.5.3 0401 cell cultures (Figure 4.1) were analyzed for DRB1*04 epitopes along with known exosomal markers using a modified flow cytometric assay in which exosomes were coupled to latex beads. The latex beads were easily detected by flow cytometry using forward and side scatter analysis, where single, double, and multiple clumps of beads were distinguishable (Figure 4.2A). Single beads typically represented 70% to 85% of the total bead population, denoted by the region R1 in Figure 4.2A, and were used for all subsequent analysis.

Adsorption of exosomes to the beads was confirmed by flow cytometric analysis using various markers (Figure 4.2B). CD82, CD86, and CD40 were present on exosomes from all cells while ICAM-1 was detected on all except MT14B. CD59, a GPI-anchored raft protein, was present on all except 9.5.3 0401 exosomes (Figure 4.2B), an unexpected finding since exosomes from the parental cell line 8.1.6 0401 contained CD59. Non-exosomal proteins CD71and LAMP-1 were not detected on exosomes (Figure 4.2B), even though they were abundant on all cell lines (Appendix B). Since the marker profile of

Figure 4.1. Whole-mount electron microscopy of purified exosomes from B-LCL.

Whole-mount electron microscopy of the 100 000 x *g* pellet from MT14B culture supernatants. The pellet consists of vesicles similar in size (< 200 nm) to that reported for exosomes. Arrows indicate exosomes. Similar results were observed for SAVC, 8.1.6 0401, and 9.5.3 0401. No vesicles were observed in the PBS control. Images are 40 000x. Bar = 200 nm. The image shown is representative of several fields of view for one experiment.

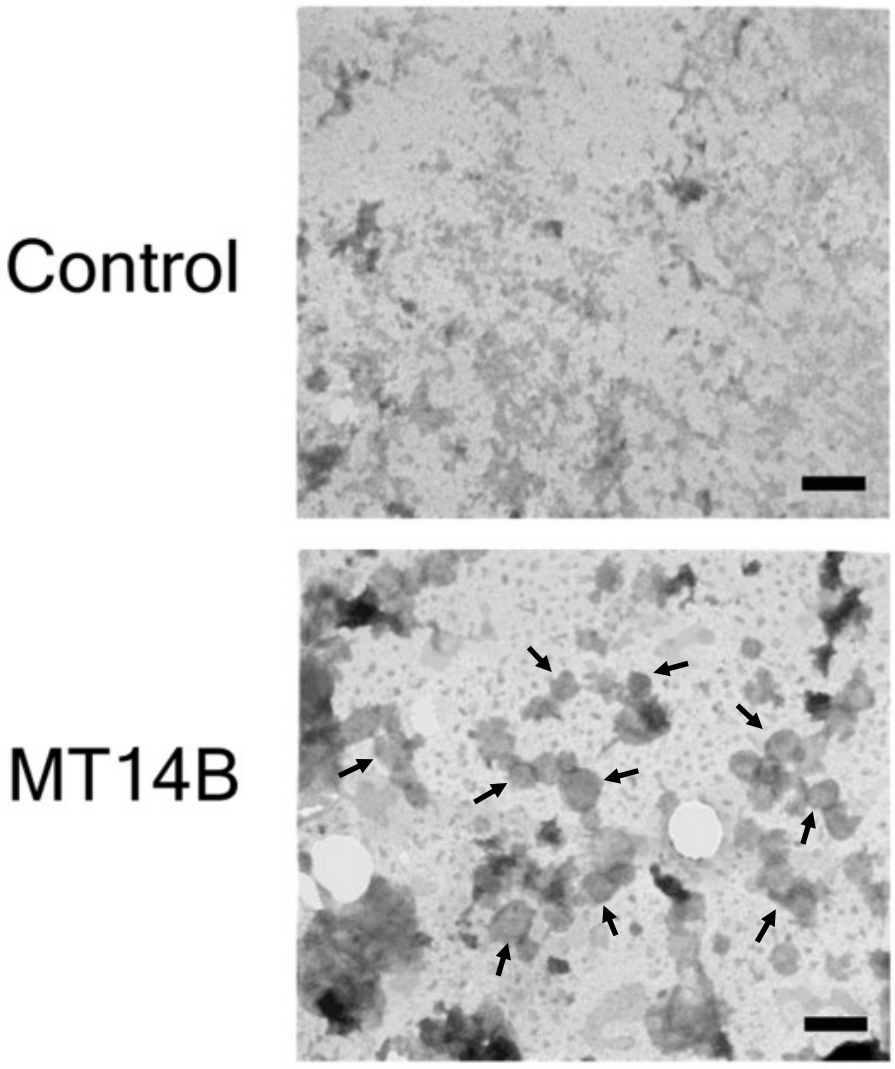
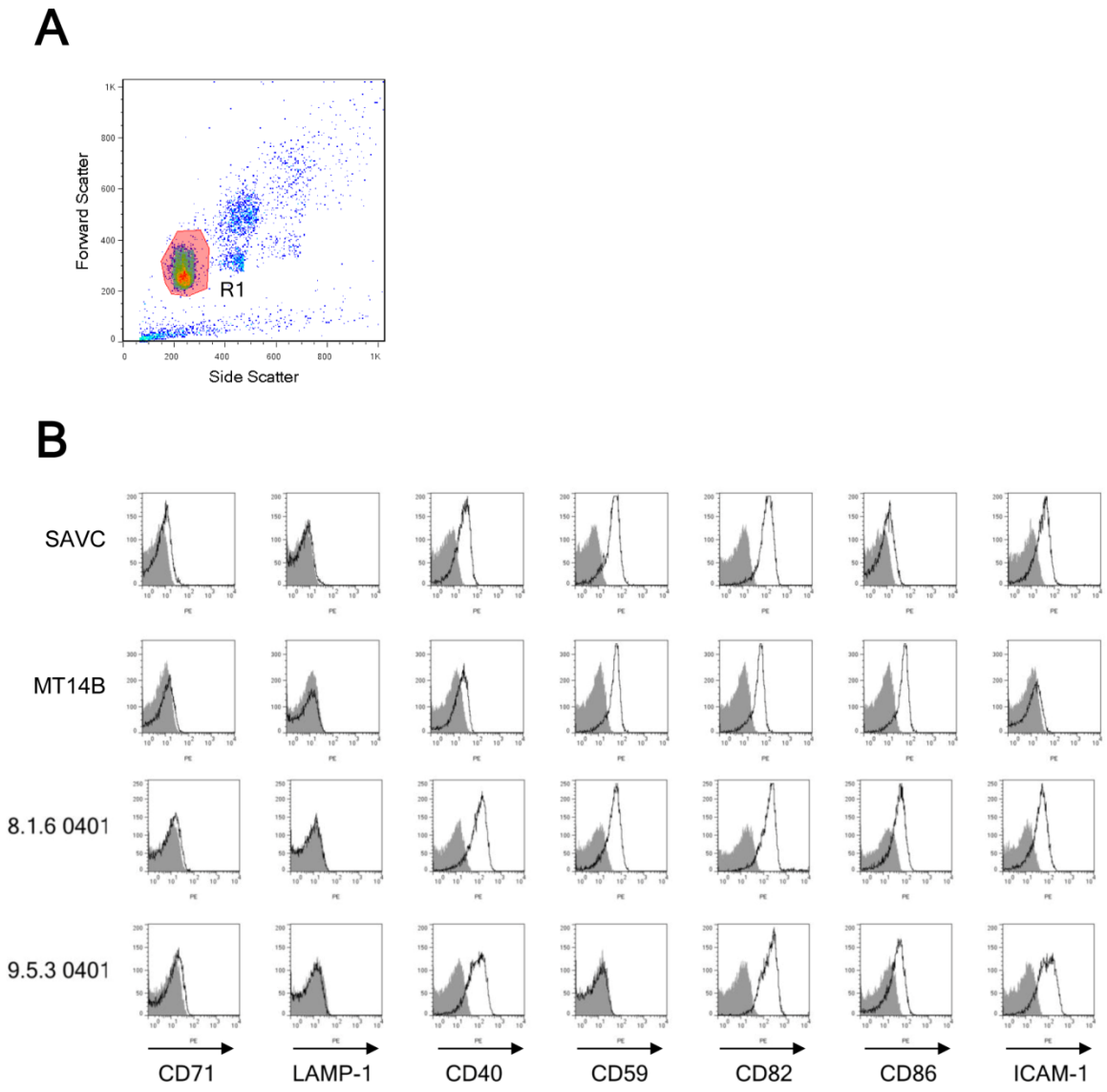

Control
MT14B

Figure 4.2. Characterization of exosomes isolated from B-LCL.

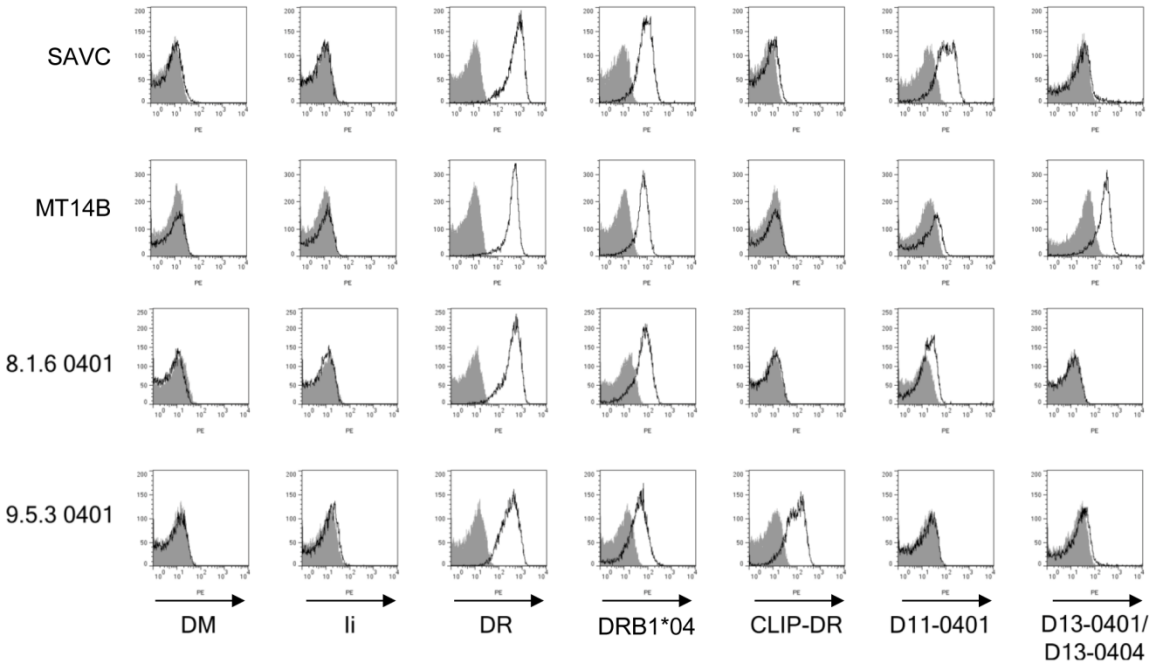
(A) Exosomes isolated from B cell culture supernatants as outlined in Figure 2.1 were coated onto 3.9 μm latex beads and analyzed by flow cytometry using forward versus side scatter analysis to distinguish single beads from clumps of multiple beads. Single beads, typically representing 70-85% of the total bead population and denoted by region R1, were selected for gating in all subsequent analysis. **(B)** Beads coated with exosomes from SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401were analyzed by flow cytometry to determine expression of relevant markers. Open histograms represent exosome-coated beads. Filled histograms represent unlabeled beads, which consistently had an MFI < 10. Beads incubated with exosome-depleted media and exosome-coated beads labelled with isotype control antibodies had comparable MFIs to unlabeled beads (data not shown). Results are representative of two independent experiments.


A
Forward Scatter
Side Scatter
R1
B
SAVC
MT14B
8.1.6 0401
9.5.3 0401
CD71
LAMP-1
CD40
CD59
CD82
CD86
ICAM-1

these vesicles was comparable to that of previously described exosomes (335, 336), we next analyzed them for antigen presentation molecules DM and Ii, and DR complexes including total DR, MHC-II/CLIP, and DRB1*04 epitopes. DM and Ii were not detected on exosomes from any of the cells (Figure 4.3). In addition, MHC-II/CLIP complexes were absent from exosomes isolated from DM+ cells (SAVC, MT14B, and 8.1.6 0401), but were abundant on DM- 9.5.3 0401 exosomes (Figure 4.3), likely due to the inability of CLIP to dissociate from MHC-II in the absence of DM. DR dimers and DRB1*04 molecules were present on exosomes from all four cell lines (Figure 4.3), consistent with previous reports of abundant MHC-II on APC-derived exosomes (335, 336). D11-0401 and D13-0404 were well expressed and restricted to SAVC and MT14B exosomes, respectively (Figure 4.3); however, D11-0401 expression was reduced on 8.1.6 0401 exosomes compared to SAVC exosomes, possibly due to reduced levels of exosomal DRB1*04:01 (Figure 4.3). As expected, D11-0401 and D13-0401 were not detected on 9.5.3 0401 and 8.1.6 0401 exosomes, respectively, since these epitopes are not naturally expressed on these cells. Thus, the exosomal profile of D11-0401 and D13-0404 is consistent with their cell surface expression. Surprisingly, D13-0401 was not detected on exosomes from 9.5.3 0401 despite abundant DRB1*04 molecules and total DR (Figure 4.3), suggesting that its expression may be dependent on different antigen processing or trafficking mechanisms. The intriguing finding that 9.5.3 0401 exosomes lacked the GPI-anchored protein CD59 led us to question whether GPI-anchored proteins influenced the expression of these DRB1*04 epitopes.

Figure 4.3. Epitopes D11-0401 and D13-0404, but not D13-0401, are present on exosomes from B-LCL.

Exosome-coated latex beads, as described for Figure 4.2A, were analyzed for expression of antigen presentation molecules and DRB1*04 epitopes by flow cytometry. Open histograms represent exosome-coated beads. Filled histograms represent unlabeled beads and consistently had an MFI < 10. Beads incubated with exosome-depleted media and exosome-coated beads labelled with isotype control antibodies had comparable MFIs to unlabeled beads (not shown). Results are representative of two independent experiments.


SAVC
MT14B
8.1.6 0401
9.5.3 0401
DM
Ii
DR
DRB1*04
CLIP-DR
D11-0401
D13-0401/
D13-0404

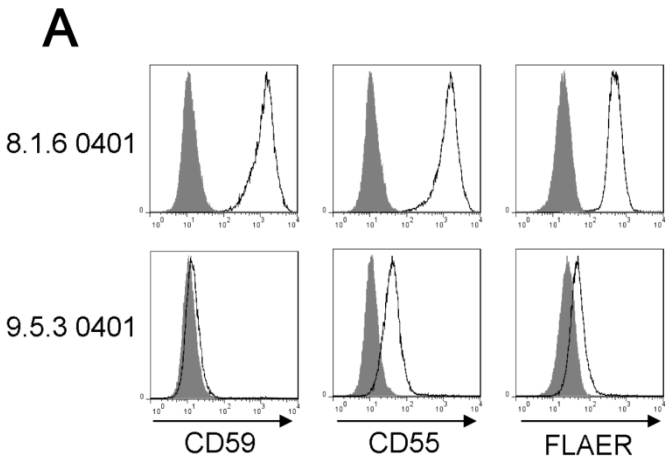
4.2.2 Analysis of the contribution of GPI-anchored proteins to expression of DRB1*04 epitopes

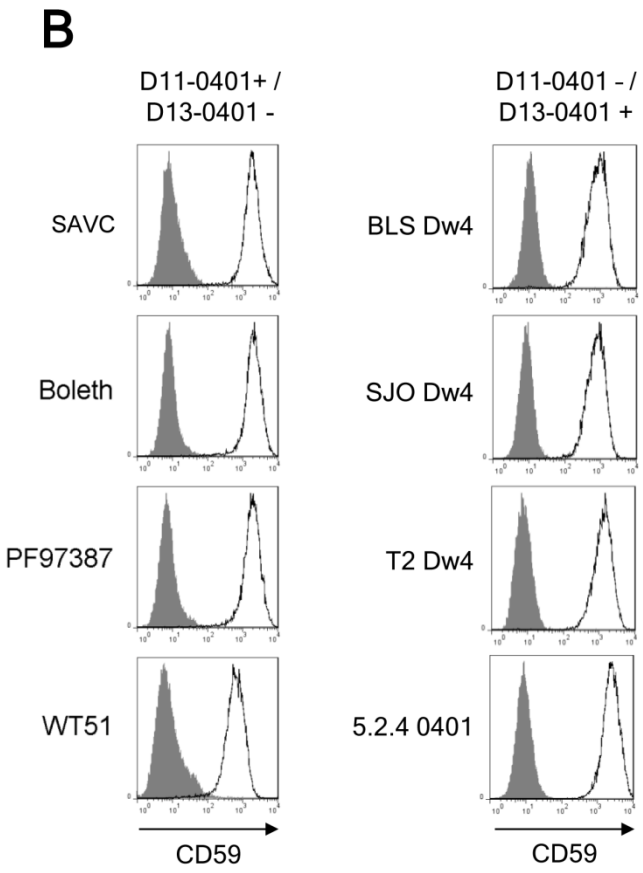
Consistent with the exosomal profiles, subsequent analysis using cell surface flow cytometry revealed abundant CD59 expression on 8.1.6 0401 but only a trivial amount on 9.5.3 0401 (Figure 4.4A). Similarly, CD55, another GPI-anchored protein was substantially reduced on 9.5.3 0401 compared to 8.1.6 0401 suggesting 9.5.3 is deficient in GPI-anchored proteins. Further testing using FLAER, a flow cytometric assay utilizing a fluorescently-labelled inactive variant of aerolysin which binds to GPI-linked proteins (299), revealed significantly reduced expression on 9.5.3 0401 compared to 8.1.6 0401 (Figure 4.4A). Since 9.5.3 0401 also does not express DM and D11-0401, but is strongly positive for D13-0401, and the converse is true for 8.1.6 0401, we hypothesized that lipid rafts or lipid raft-associated GPI-anchored proteins may influence expression of these epitopes.

To address this, CD59 was analyzed by flow cytometry on a panel of DM+ and DM- DRB1*04:01 cell lines. All DM+ / D11-0401+ / D13-0401- cell lines including SAVC, Boleth, PF97387, WT51, and 8.1.6 0401 had comparable amounts of CD59 (Figure 4.4B). Similarly, DM- / D11-0401- / D13-0401+ cells such as SJO Dw4, T2 Dw4, BLS Dw4, and 5.2.4 0401 were also positive for CD59 (Figure 4.4B). These results suggest 1) expression of GPI-linked proteins are not required for D11-0401 expression, or if so, it is only in the presence of DM; and 2) GPI-linked proteins do not contribute to the D13-0401 epitope since CD59 is abundant on other D13-0401+ cell lines (Figure 4.4B). Thus, while GPI-anchored proteins or lipid rafts cannot be eliminated as contributing

Figure 4.4. GPI-anchored proteins are severely reduced in 9.5.3 0401 cells, but do not associate with expression of D11-0401 or D13-0401 epitopes.

(A) Surface expression of GPI-anchored proteins CD59 (MEM-43) and CD55 (IA10), and total GPI-anchored proteins in 9.5.3 0401 and 8.1.6 0401 were compared using flow cytometry and the FLAER assay as described in Materials and Methods. FLAER, a non-lysing form of proaerolysin conjugated with Alexa Fluor 488, specifically binds to the GPI moiety of GPI-linked proteins. CD59, CD55, and FLAER expression are represented by the open histograms. Filled histograms indicate isotype controls. **(B)** Surface expression of CD59 was measured on D11-0401+/D13-0401– and D11-0401– /D13-0401+ B-LCL by flow cytometry. Open histograms represent CD59 expression. Filled histograms indicate isotype controls. Results are representative of two independent experiments.


A
8.1.6 0401
9.5.3 0401
CD59
CD55
FLAER


B
D11-0401+ /
D13-0401 -
D11-0401 - /
D13-0401 +
SAVC
Boleth
PF97387
WT51
BLS Dw4
SJO Dw4
T2 Dw4
5.2.4 0401
CD59
CD59

factors to presentation of the DM-dependent D11-0401 epitope, the results suggest they do not influence D13-0401 expression.

4.2.3 Association of DRB1*04 epitopes with membrane microdomains

MHC-II molecules are not uniformly distributed throughout the plasma membrane, but are organized into smaller patches or clusters on the surface of pAPC, which facilitate efficient antigen presentation to CD4+ T cells especially in circumstances of limited antigen (152, 177, 337). Two types of membrane microdomains, lipid rafts and tetraspanin enriched microdomains (TEMs), have been implicated in clustering MHC-II on the surface of APC prior to interaction with T cells (153, 163). Since lipid rafts are enriched in GPI-anchored proteins such as CD59 (338) and given the differential expression of GPI-anchored proteins between 8.1.6 0401 and 9.5.3 0401 as previously shown, we queried whether differences in lipid raft dynamics or other types of membrane microdomains might underlie the expression of the DRB1*04 epitopes. This idea was also supported by our finding that both D11-0401 and D13-0404 colocalize with tetraspanins CD63 and CD82 and preliminary experiments showing that chemical disruption of TEMs in B -LCL resulted in some loss of D11-0401 and D13-0404 (283). As described below, we investigated whether D11-0401, D13-0401, and D13-0404 epitopes are modulated by clustering into membrane microdomains.

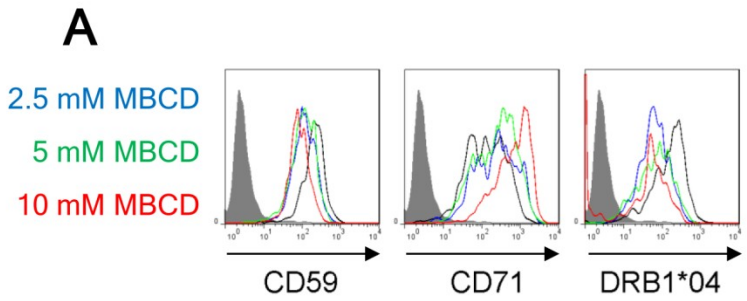
4.2.3.1 DRB1*04 epitopes are differentially susceptible to lipid raft disruption

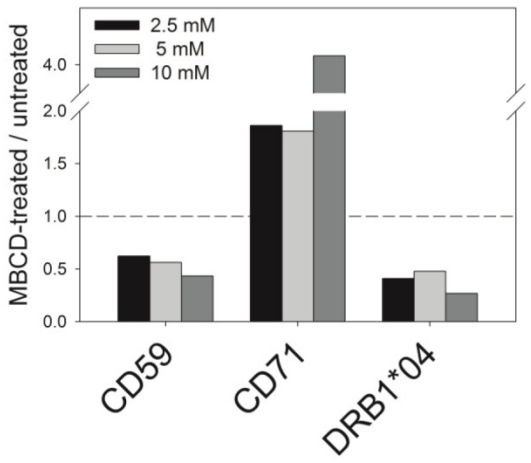
To evaluate whether the DRB1*04 epitopes associate with lipid raft membrane microdomains, raft integrity was disrupted using MBCD, which extracts cholesterol from the plasma membrane and disturbs protein association with cholesterol-enriched rafts (304-306). Preliminary experiments were performed with MT14B cells to determine the optimal concentration of MBCD that would selectively disrupt surface expression of lipid rafts in cells treated with 2.5 mM, 5 mM, and 10 mM MBCD. MBCD treatment resulted in a 30% or more decrease in CD59 and DRB1*04, while CD71 was not inhibited but actually increased, suggesting that a proportion of cell surface DRB1*04 molecules are associated with lipid rafts (Figures 4.5A, 4.5B). Additional analysis by confocal microscopy confirmed the MBCD-induced disruption of lipid raft protein expression, as evidenced by decreased CD59 and HLA-DR in MBCD-treated MT14B cells, whereas no obvious change in CD71 expression was observed (Figure 4.5C). Furthermore, MBCD treatment resulted in a reduction of GM1 ganglioside (Figure 4.5C), a well-known marker of membrane rafts (174, 339, 340). Subsequent experiments were performed with 5 mM MBCD to minimize any toxic effect of MBCD while still mainaining adequate lipid reaft disruption.

MBCD- and control-treated SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 cells were analyzed for surface expression of membrane proteins and DRB1*04 epitopes by flow cytometry (Figure 4.6). Compared to control, MBCD-treatment significantly reduced CD59 on all B-LCL except the GPI-linked protein deficient 9.5.3 0401 (Figure 4.6A), significantly reduced CD45 on MT14B, and markedly reduced CD82 and MHC-I on all B-LCL. In contrast, CD71 expression was increased on all MBCD-treated cells

Figure 4.5. Surface expression of lipid-raft associated molecules and HLA-DR is adversely affected by MBCD treatment.

(A) Titration of MBCD was performed in a single experiment to determine the optimal concentration to disrupt lipid rafts. MT14B cells were treated with 2.5 (blue), 5 (green), and 10 mM (red) MBCD as described in the Materials and Methods. Surface expression of CD59 (MEM-43), CD71 (M-A712), and DRB1*04 (NFLD.D1) was analyzed by flow cytometry and compared to untreated control cells (grey histograms). Filled histograms represent isotype controls. **(B)** The bar chart shows the degree of MBCD disruption of lipid rafts. MFI values of CD59, CD71, and DRB1*04 for MBCD-treated cells were divided by the MFI expression values of untreated control cells. The broken line represents no change in expression mediated by MBCD. Results are representative of one experiment. **(C)** MT14B cells were treated with 5 mM MBCD and surface expression of CD59, CD71, total HLA-DR (L243), and GM1-ganglioside (Vybrant Lipid Raft Labeling Kit) was compared to untreated control cells by immunofluorescence and confocal microscopy. Each field of view contains approximately equal number of cells. Images were acquired at 20x objective magnification and are representative of several fields of view from at least two independent experiments.


A
2.5 mM MBCD
5 mM MBCD
10 mM MBCD
CD59
CD71
DRB1*04


B
2.5 mM
5 mM
10 mM
MBCD-treated / untreated
4.0
2.0
1.5
1.0
0.5
0.0
CD59
CD71
DRB1*04

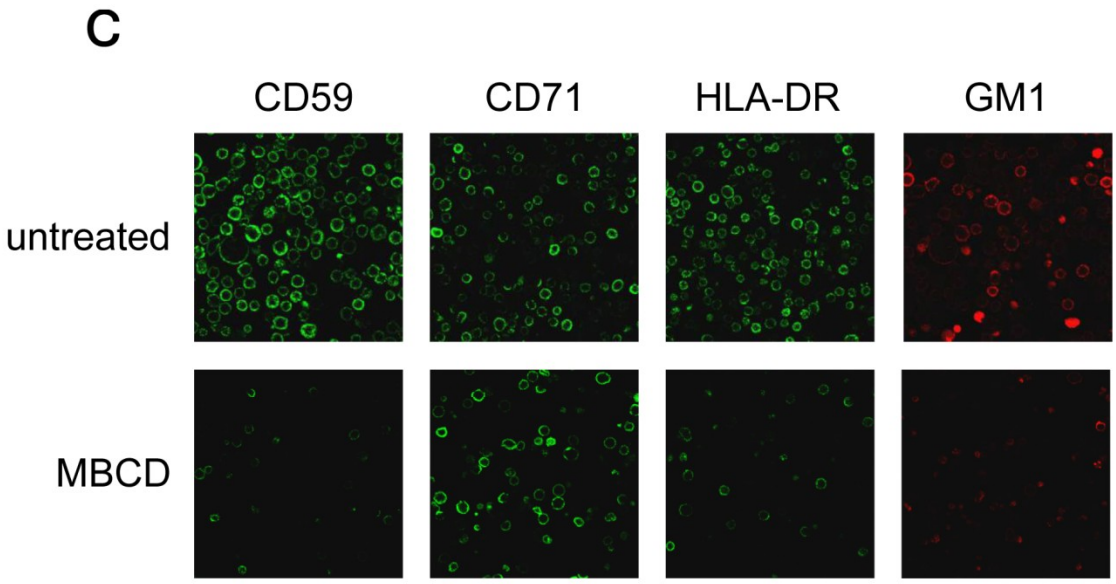
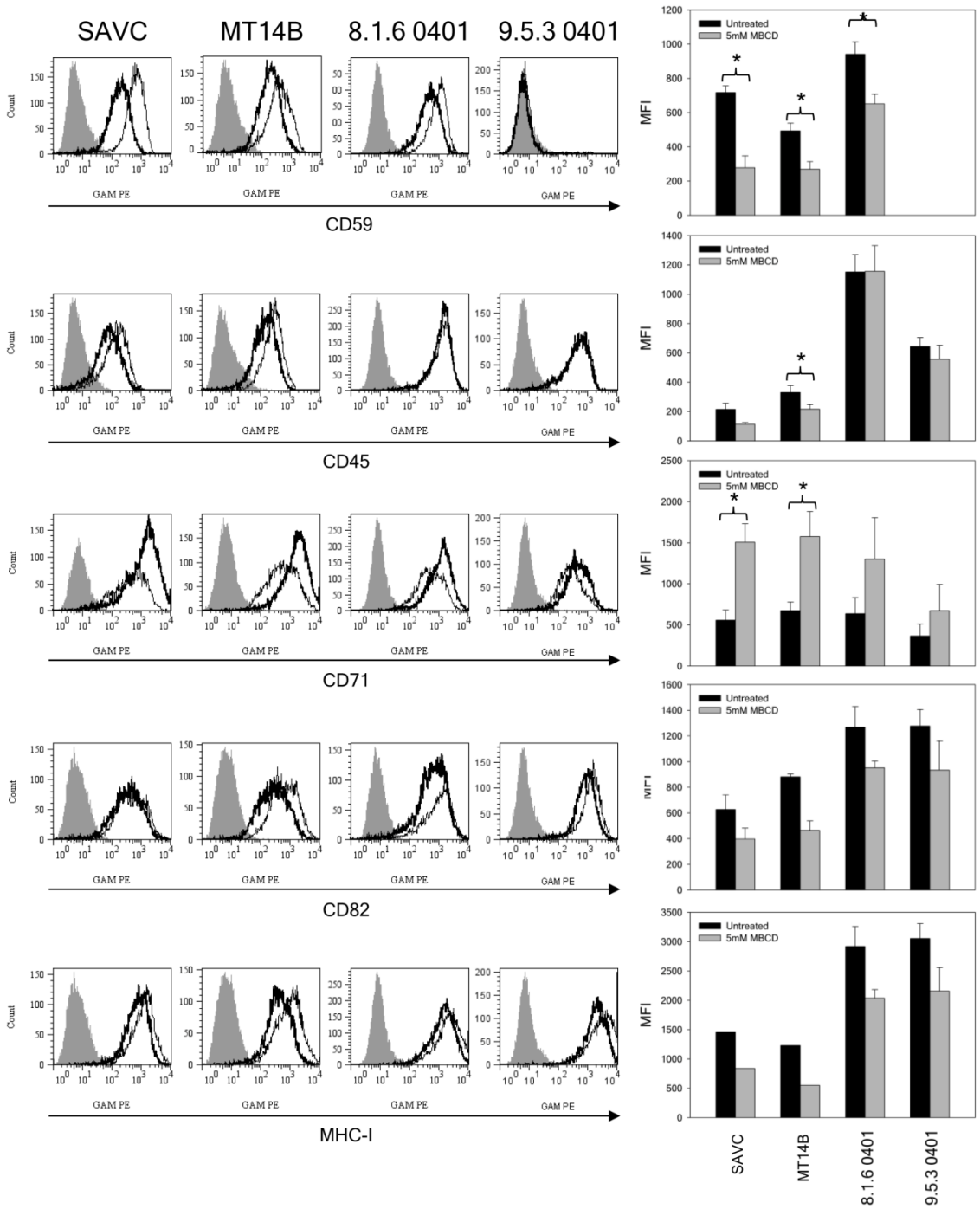

C
CD59
CD71
HLA-DR
GM1
untreated
MBCD

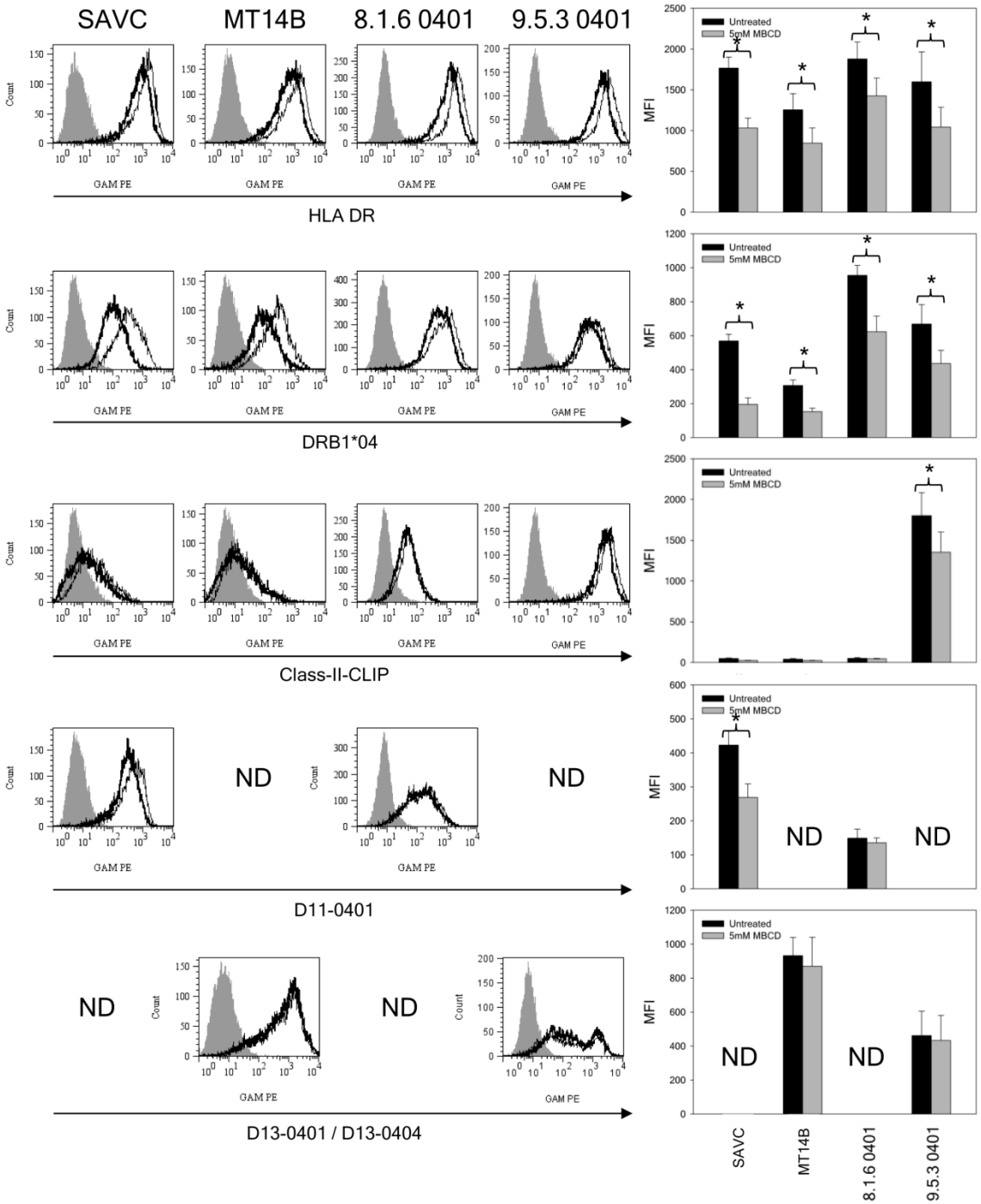
Figure 4.6. The effect of lipid raft disruption on the surface expression of transmembrane proteins and DRB1*04 epitopes.

Lipid rafts were disrupted using MBCD to treat SAVC, MT14B, 8.1.6 0401, or 9.5.3 0401 cells. Surface expression of **(A)** CD59 (MEM-43), CD45 (HI30), CD71 (M-A712), CD82 (TS82b), MHC-I (W6/32), and **(B)** total DR (L243), DRB1*04 (NFLD.D1), MHC-II/CLIP (cerCLIP), D11-0401, and D13-0401/D13-0404 was determined following MBCD treatment (thick lines) and compared to untreated control cells (thin lines). Filled histograms represent isotype controls. Representative data from one experiment are shown in the histogram plots. Cumulative data from one to five independent experiments are shown in the bar graphs (n = 1-5), where the mean MFI was compared between MBCD-treated (grey bars) and untreated control (black bars) cells. A paired t-test was performed to compare expression between MBCD-treated and untreated cells and significant differences are indicated by an asterisk ($p < 0.05$). Bars represent standard error. ND = not determined. Data from individual experiments for (A) and (B) are shown in Appendix D and E, respectively.



Continued on the next page.

B


SAVC
MT14B
8.1.6 0401
9.5.3 0401
Count
GAM PE
HLA DR
DRB1*04
Class-II-CLIP
ND
D11-0401
D13-0401 / D13-0404
MFI
Untreated
5mM MBCD
*

(Figure 4.6A). Total DR and DRB1*04 were also significantly reduced in all four cell lines (Figure 4.6B), suggesting that a large portion of DR molecules are located in lipid raft microdomains. MBCD treatment had no effect on MHC-II/CLIP expression on DM+ SAVC, MT14B, and 8.1.6 0401 cells, but significantly decreased its normally strong expression in DM- 9.5.3 0401 cells (Figure 4.6B). Similar to total DR and DRB1*04, D11-0401 was significantly reduced on MBCD-treated SAVC cells, but unchanged on 8.1.6 0401 cells (Figure 4.6B). Both D13-0404 and D13-0401 epitopes in MT14B and 9.5.3 0401, respectively, were unaffected by MBCD-treatment (Figure 4.6B). Taken together, the results suggest that only the D11-0401 epitope is associated with lipid rafts in the plasma membrane.

Since D11-0401 expression was susceptible to lipid raft disruption in SAVC, but not in 8.1.6 0401 cells, we investigated this in additional D11-0401+ B-LCL, Boleth and PF97387. Similar to SAVC and 8.1.6 0401, MBCD treatment resulted in lipid raft disruption in both cell lines as demonstrated by substantially reduced CD59, total DR, and DRB1*04, with no change in CD71 (Appendix C). More importantly, D11-0401 expression was diminished in both Boleth and PF97387 comparable to that observed in SAVC cells (Appendix C). Therefore, these results demonstrate that D11-0401 is partially associated with membrane rafts in cells that naturally express DRB1*04:01.

To confirm that the D13-0404 epitope does not associate with lipid-rafts as was observed on MT14B (Figure 4.6B), we performed similar experiments using another D13-0404+ B-LCL, BM92. In contrast to MT14B, the D13-0404 was markedly decreased in MBCD-treated BM92 (Appendix C) while changes in CD59, total HLA-DR, and DRB1*04 and CD71 were consistent with those observed in MT14. Thus, the incongruent

results are not due to technical issues with MBCD, but suggest D13-0404 association with rafts could be cell type-dependent.

4.2.3.2 The D13-0404 epitope is not associated with detergent resistant membranes extracted from MT14B

Detergent resistant membranes (DRMs) extracted from cells are closely related to lipid rafts because both are enriched in similar lipids and proteins including cholesterol, GPI-anchored proteins, and specific transmembrane proteins (341). Similar to DRMs, lipid rafts are resistant to nonionic detergents, such as Triton X-100, due to extensive, strong interactions between their constituents (342). Therefore, the association of a protein with DRMs provides a good measure of its affinity to lipid rafts *in vivo*. To determine the affinity of DRB1*04 epitopes for lipid rafts, DRMs were isolated from MT14B, 8.1.6 0401, and 9.5.3 0401 cells using sucrose density gradient ultracentrifugation followed by western blot analysis of gradient fractions. Although the D11-0401 and D13-0401 epitopes are either not or poorly detected by western blotting of total cell lysates, the possibility that DRM isolation would result in DRB1*04 molecules with these epitopes concentrating in DRMs or detergent-soluble fractions warranted these experiments.

As controls, the DRM-association of the GPI-anchored protein CD59 and the non-DRM protein CD71 were analyzed. CD59 was mostly located in fractions 2 to 4 in MT14B and 8.1.6 0401, corresponding to the expected location of isolated DRMs (Figures 4.7, 4.8A). Consistent with exosomal and surface expression, no CD59 was

Figure 4.7. The D13-0404 epitope is located on detergent soluble DRB1*04:04 molecules.

Detergent resistant membranes were isolated by sucrose density ultracentrifugation from MT14B total cell lysate as described in the Materials and Methods. Fractions were collected from the top of the sucrose gradient and subject to SDS-PAGE under reducing conditions followed by western blotting for molecules CD59 (MEM-43), CD71 (M-A712), DR dimer (L243; Tal 14.1), DRB1*04 (NFLD.D1), DM (Tal 18.1), Ii (LN2), and D13-0404. DRMs migrated to the 5/35% sucrose interface of the sucrose gradient, which visually corresponded to fractions 2 to 4. The approximate size (kDA) of the identified protein bands is indicated.

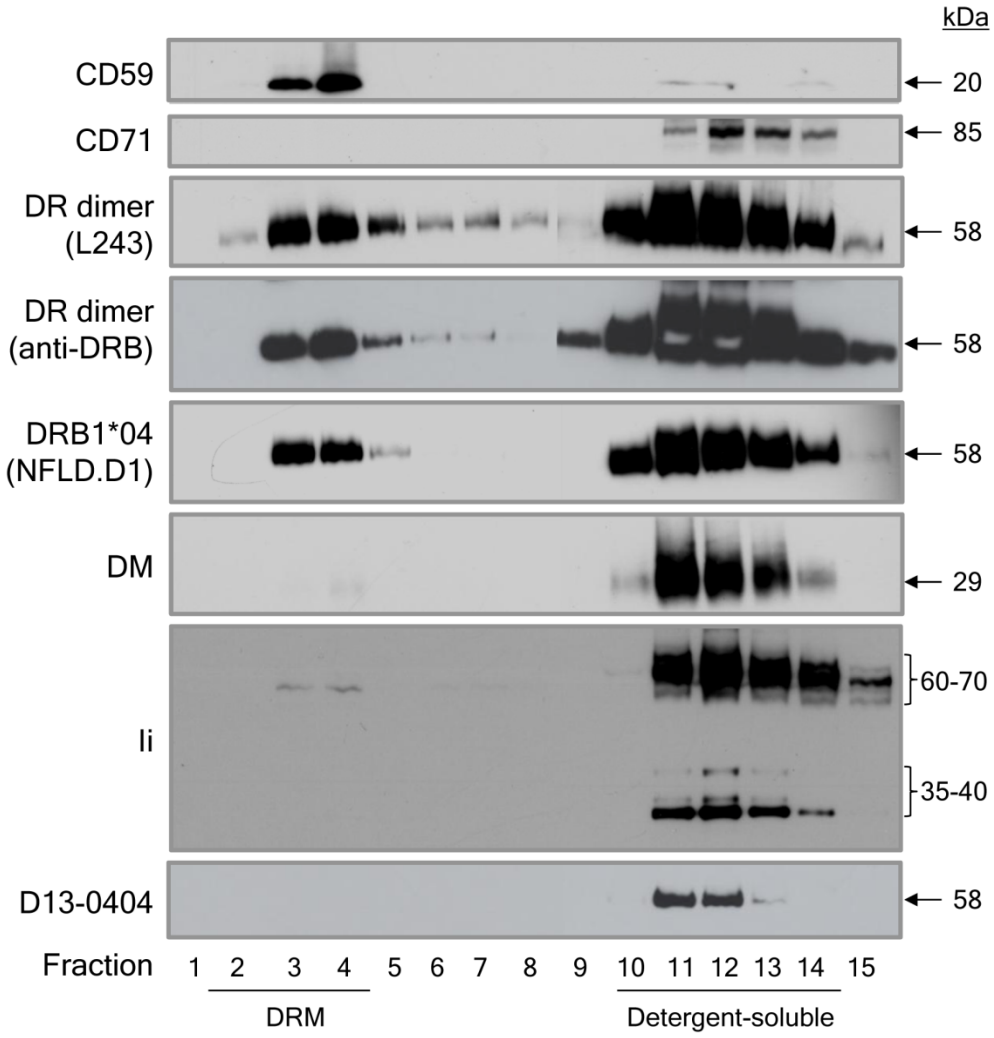
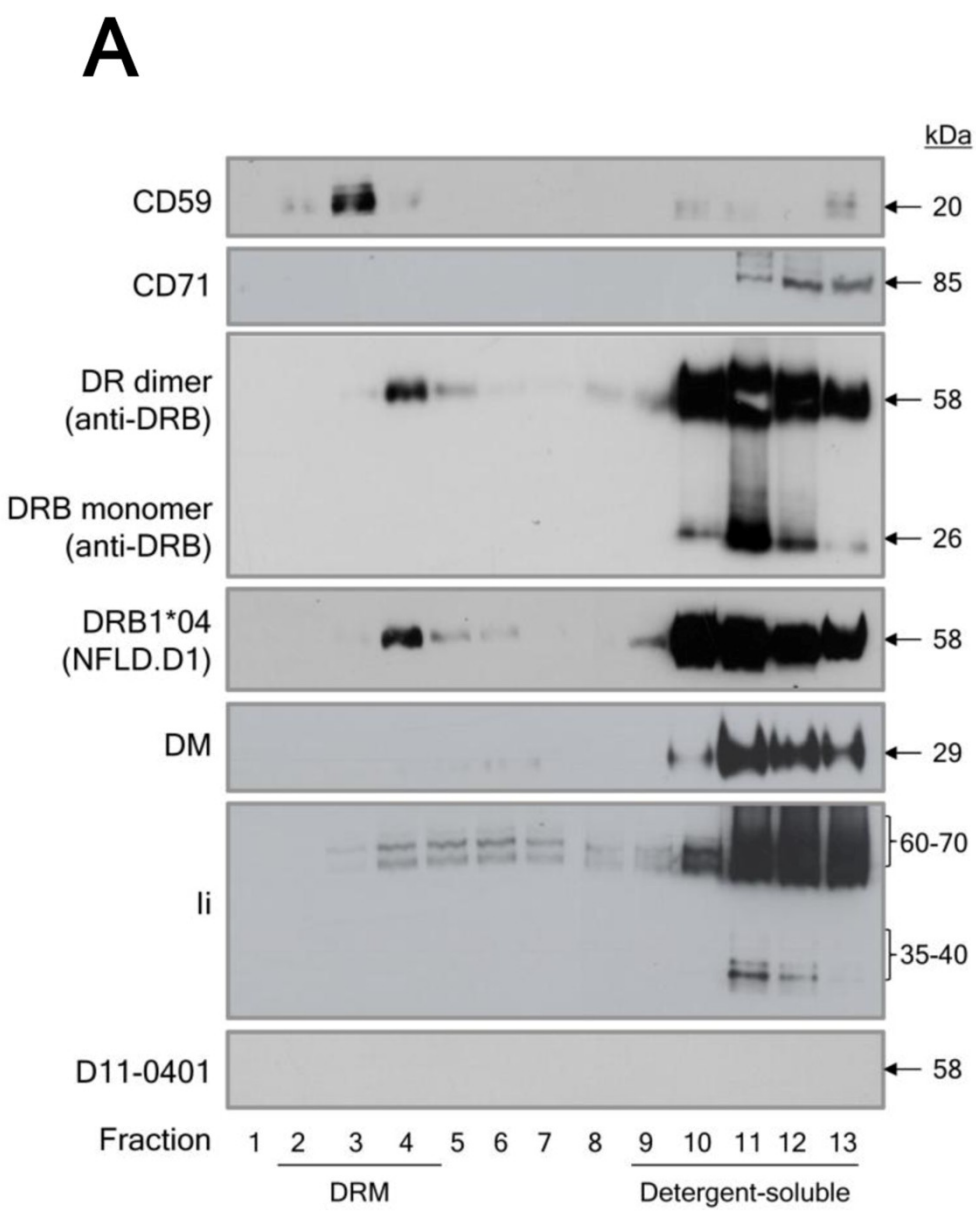

kDa
CD59
CD71
DR dimer
(L243)
DR dimer
(anti-DRB)
DRB1*04
(NFLD.D1)
DM
Ii
D13-0404
20
85
58
58
58
29
60-70
35-40
58
Fraction
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
DRM
Detergent-soluble

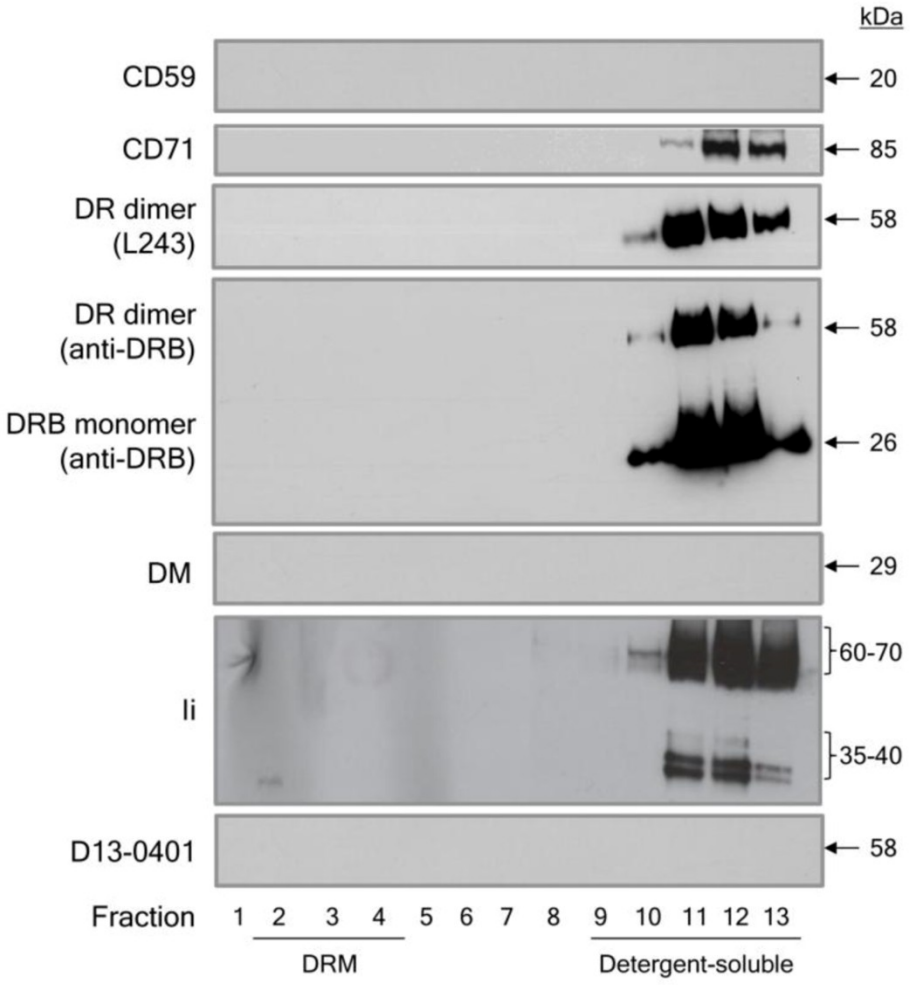
Figure 4.8. 9.5.3 0401 exhibits reduced DRM-associated proteins compared to 8.1.6 0401.

Detergent resistant membranes were isolated by sucrose density ultracentrifugation from 8.1.6 0401 **(A)** and 9.5.3 0401 **(B)** total cell lysates as described in the Materials and Methods. Fractions were collected from the top of the sucrose gradient and subject to SDS-PAGE under reducing conditions followed by western blotting for molecules CD59 (MEM-43), CD71 (M-A712), DR dimer (L243; Tal 14.1), DRB monomer (Tal 14.1), DRB1*04 (NFLD.D1), DM (Tal 18.1), Ii (LN2), D11-0401, and D13-0404. DRMs migrated to the 5/35% sucrose interface of the sucrose gradient, which visually corresponded to fractions 2 to 4. The approximate size (kDA) of the identified protein bands is indicated.



Continued on the next page.

B


kDa
CD59
20
CD71
85
DR dimer
(L243)
58
DR dimer
(anti-DRB)
58
DRB monomer
(anti-DRB)
26
DM
29
Ii
60-70
35-40
D13-0401
58
Fraction
1 2 3 4 5 6 7 8 9 10 11 12 13
DRM
Detergent-soluble

detected in the gradient fractions of 9.5.3 0401 cell lysates. CD71 was exclusively detected in fractions 11 to 14 from MT14B (Figure 4.7), 8.1.6 0401 (Figure 4.8A), and 9.5.3 0401 (Figure 4.8B), corresponding to the detergent-soluble fractions. Taken together, these results confirm that DRM-associated proteins localize to fractions 2 to 4, while non-DRM proteins remain in the detergent-soluble lysate fractions 11 to 15.

Analysis of DRM-association of DR in MT14B showed the majority DR dimers localized to the detergent-soluble fractions, as shown by three different anti-DR antibodies (Figure 4.7). However, a subset of DR dimers was detected in the DRMs, indicating a minor pool of lipid-raft associated DR molecules. Both DM and Ii were predominantly found in detergent-soluble fractions (Figure 4.7), with only a tiny fraction of Ii-complexes (60-70 kDa) and DM detected in DRMs. DRB1*04:04 molecules containing the D13-0404 epitope were only detected in the detergent soluble fractions in MT14B, indicating that DR molecules with this epitope do not have an affinity for lipid rafts (Figure 4.7). These results are consistent with the previous findings where the disruption of lipid rafts with MBCD did not affect D13-0404 expression in MT14B cells (Figure 4.6B).

Similar to MT14B, SDS-stable DR dimers in 8.1.6 0401 were primarily in detergent-soluble fractions, with only small amounts present in DRM fractions (Figure 4.8A). DRB monomers were solely detected in the detergent-soluble fractions (Figure 4.8A). Both DM and Ii molecules were confined to the detergent-soluble fractions in 8.1.6 0401, except for a small proportion of Ii-complexes found in DRMs (Figure 4.8A). As shown, no D11-0401 epitope was detected in any fractions, even though a portion of DRB1*04 was detected in the DRM fractions. Most likely this is due to the instability of this epitope to detergents, as it is also poorly detected in lysates prepared from its cognate

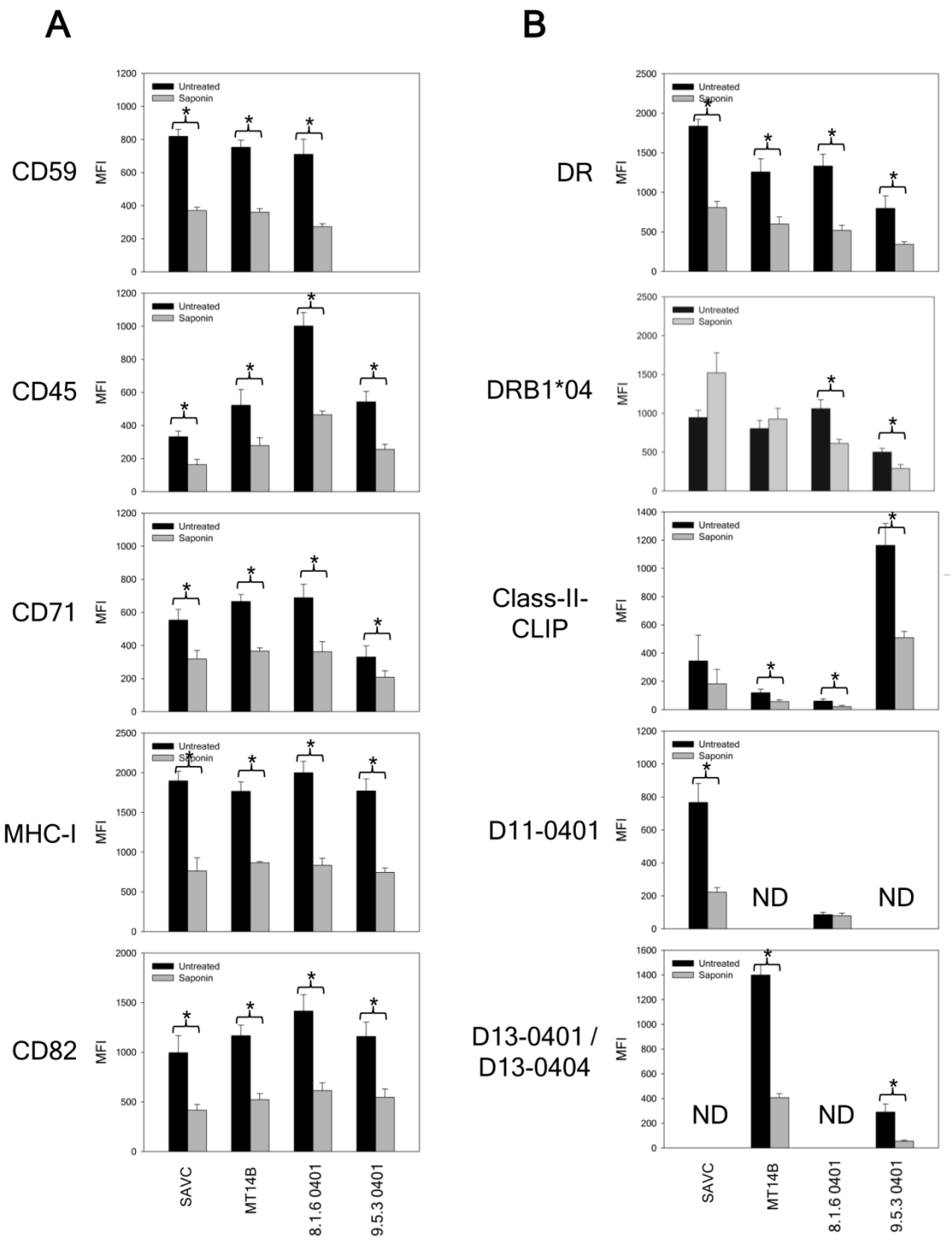
cell line SAVC (data not shown). No SDS-stable DR dimers or DRB monomers were detected in the DRM fractions in 9.5.3 0401 (Figure 4.8B), which was not surprising given that it does not express DM (Figure 4.8B). Ii and Ii-complexes were exclusively detected in detergent-soluble fractions in 9.5.3 0401 (Figure 4.8B). Although the NFLD.D13 mAb detected its cognate D13-0404 epitope in the detergent soluble fractions of MT14B (Figure 4.8A), it does not detect the cross-reactive epitope D13-0401 in 9.5.3 0401 fractions (Figure 4.8B).

4.2.3.3 Saponin treatment of cells resulted in an overall decrease of membrane proteins

Saponin treatment of B cells was previously shown to interfere with formation of TEMs containing MHC-II molecules carrying a select set of peptide antigens defined by a specific epitope, CDw78 (153). To determine whether D11-0401, D13-0401, or D13-0404 associate with TEMs, their expression was measured on B-LCL after treatment with saponin and compared to untreated control cells. To ensure saponin treatment selectively disrupted TEMs, TEM-associated and non-TEM proteins were analyzed by flow cytometry. Although the tetraspanin marker CD82 was significantly decreased on saponin-treated cells, non-tetraspanin markers CD59, CD45, and CD71 were also significantly reduced (Figure 4.9A). In addition, MHC-I, total HLA-DR, and MHC-II/CLIP complexes were dramatically reduced in all cell lines (Figures 4.9A, 4.9B). Interestingly, DRB1*04 expression was unaffected on saponin-treated SAVC and MT14B cells despite significantly reduced total DR (Figure 4.9B). As for the DRB1*04 epitopes,

Figure 4.9. Saponin treatment of B-LCL results in a non-specific decrease in cell surface protein expression.

SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 cells were treated with saponin to preferentially disrupt tetraspanin microdomains and surface expression of **(A)** CD59 (MEM-43), CD45 (HI30), CD71 (M-A712), MHC-I (W6/32), CD82 (TS82b) and **(B)** total DR (L243), DRB1*04 (NFLD.D1), MHC-II/CLIP (cerCLIP), D11-0401, and D13-0401/D13-0404 was compared to control cells. Cumulative data from one to five independent experiments are displayed (n = 1-5). A paired t-test was performed to compare expression between saponin-treated and untreated cells and significant differences are indicated by an asterisk ($p < 0.05$). Bars represent standard error. ND, not determined.


A
B
CD59
CD45
CD71
MHC-I
CD82
DR
DRB1*04
Class-II-CLIP
D11-0401
D13-0401 / D13-0404
MFI
Untreated
Saponin
ND
SAVC
MT14B
8.1.6 0401
9.5.3 0401

D11-0401 was significantly reduced by saponin treatment in SAVC, but not on 8.1.6 0401-treated cells (Figure 4.9B). Presentation of both D13-0404 and D13-0401 epitopes was decreased in saponin-treated MT14B and 9.5.3 0401 cells, respectively (Figure 4.9B). Taken together, these results suggest that the effects of saponin are not restricted to TEMs or TEM-associated proteins, making this method impractical for evaluating association of pMHC-II with TEMs on intact cells.

4.3 Discussion

Our previous studies on the DM-dependent D11-0401 and the DM-resistant D13-0404 epitopes indicated they require nascent DRB1*04 molecules and are formed in DM+ CD63+ CD82+ intracellular compartments resembling late endosomes and MVBs from which exosomes are secreted (283). In this study, we show strong expression of D11-0401 and D13-0404 epitopes on exosomes prepared from SAVC and MT14B cells, respectively, thus supporting their generation in late endocytic vesicles. However, the DM-sensitive D13-0401 epitope was not present on 9.5.3 exosomes while MHC-II/CLIP complexes, also DM-sensitive, were abundantly expressed. Since MHC-II/CLIP is generated in late endocytic vesicles and amply expressed on DM- cells, the absence of D13-0401 on exosomes suggests it is not formed in late endosomal compartments.

Exosomes are only one of the many types of extracellular membrane vesicles, all of which differ in origin, function, size, sedimentation, and protein composition (229). Based on appearance and size by electron microscopy, < 200 nm in diameter, and protein marker profile (ICAM-1+, CD59+, CD82+, CD86+, HLA-DR+, DRB1*04+, and CD71-

or LAMP-1-), the preparations used in this study are largely consistent with previously described exosomes (335, 343, 344). However, the presence of CD40 on exosomes from all four B-LCL somewhat contrasts with other reports that did not detect CD40 on B cell-derived exosomes (335, 343, 345). Others have shown that signaling via CD40 on B cells can lead to B cell activation and increased exosome release from the activated cells (346-348). Since the EBV-antigen, LMP-1, a known CD40 mimic and activator of B cells (349, 350) is expressed on most B-LCL, it is possible that this led to CD40 packaging in the exosomes described herein.

Although expression of pMHC-II complexes on exosomes from various cell types is well documented, evaluation of DM-modulated allele-specific epitopes on exosomes is lacking (219, 335, 343). Here we showed DRB1*04 molecules bearing either the DM-dependent D11-0401 or the DM-resistant D13-0404 epitope were expressed on the relevant exosomes, whereas the DM-sensitive D13-0401 epitope was completely excluded from 9.5.3 0401 exosomes despite strong expression of DRB1*04:01 (Figure 4.3). To our knowledge, this is one of the first described instances of differential pMHC-II presentation on exosomes compared to the cell membrane within a given cell type. A previous report found that exosomes derived from human monocyte-derived DCs, various cell lines, and human plasma contained novel MHC-I structures as detected by conformational-dependent antibodies, suggesting that exosomes may contain unique MHC-I complexes or epitopes for recognition by T cells that are not present on the surface of APCs (245). Our results suggest that D13-0401 is not present in MVBs, which is consistent with the hypothesis that this epitope may form in early or recycling

endosomes similar to previously described DM-sensitive epitopes (102) or 'type B' conformers (258, 274, 351).

An alternative explanation is D13-0401+ molecules are actively removed from ILVs prior to secretion or excluded from packaging onto exosomes entirely. Mechanisms governing protein packaging onto ILVs for extracellular release are incompletely understood. For example, some studies show entry into ILVs required ubiquitination and involves the ESCRT protein machinery (352, 353), while others show ubiquitination is not required for DR entry onto exosomes (354-357). Other proposed mechanisms include a ceramide-dependent mechanism (358) and a lipid raft domain-mediated mechanism (194, 359), but neither are well characterized. Although 9.5.3 0401 exosomes were negative for D13-0401, the presence of ample DR and specifically DRB1*04:01 show this cell line is capable of packaging DR complexes onto exosomes. While our results suggest D13-0401 is not generated in the late endosomal compartments, unfortunately, its susceptibility to fixation and permeabilization makes it difficult to evaluate by immunocytochemistry and confocal microscopy. Given the ability of exosomes to function as APCs with the capacity to activate antigen-specific or autoimmune T cell responses, the finding that exosomes and the plasma membrane may contain a different repertoire of peptide/DRB1*04:01 complexes emphasizes the importance of determining the mechanisms responsible for recruitment of pMHC-II onto exosomes.

Our finding that DM and Ii were also excluded from B-LCL-derived exosomes is consistent with previous reports (343, 344). During transport to multivesicular antigen processing compartments, some MHC-II complexes are sorted into the ILVs of MVBs, where proteolysis within this acidic protease rich environment leads to the degradation of

Ii to CLIP. DM, which is present in the MVB internal and limiting membranes, facilitates CLIP removal and peptide binding (13, 42). Thus, both Ii degradation and CLIP dissociation are likely completed prior to exosome release. This is further supported by the absence of MHC-II/CLIP complexes on exosomes from the DM+ cells SAVC, MT14B, and 8.16 0401, and abundant expression on DM- 9.5.3 0401 exosomes.

An unexpected finding regarding 9.5.3 0401 was the absence of exosomal CD59, which was subsequently shown to be due to a general deficiency of GPI-linked proteins on 9.5.3 despite normal levels on 8.1.6. Since 9.5.3 was derived from 8.1.6 by ethyl methane sulfonate (EMS) mutagenesis and immunoselection with the 16.23 mAb specific for a DM-dependent DRB1*03 epitope followed by complement lysis (271), it is possible that EMS introduced random mutations not only in the *HLA-DMB*, but also in *PIGA* gene which is required for biosynthesis of GPI-anchors (270, 360). Indeed, EMS has been previously shown to cause preferential mutations in the *CD59* gene or generate GPI-deficient cells (361, 362).

Abundant expression of the D13-0401 epitope coinciding with absence of GPI-anchored membrane proteins on exosomes and the cell membrane of 9.5.3 0401, raised the possibility that D13-0401 and other DRB1*04 epitopes were modulated by CD59 or by assembly into lipid rafts. GPI-anchored proteins are a defining component of lipid rafts, with which they associate through their distinctive lipid anchors (363). Furthermore, GPI-anchored proteins promote the incorporation of additional membrane proteins into larger functional clusters that may interact with rafts (364), suggesting a deficit of GPI-anchored proteins in a cell may significantly affect the protein composition of rafts. Although further analysis of several normal and mutant DRB1*04:01 B-LCLs, either

D11-0401+ / D13-0401- or D11-0401- / D13-0401+, revealed no correlation between CD59 and their expression (Figure 4.4), it remains possible that DM-dependent epitopes such as D11-0401 requires the combination of DM and CD59. Interestingly, BL cells which poorly express the D11-0401 epitope (Figure 3.1), are reported to have reduced levels of GPI-linked proteins (365, 366), a finding that we subsequently confirmed (S. Drover, unpublished). A more direct test of this hypothesis would involve monitoring D11-0401 expression after knockdown or transfection of CD59 in DM+ / D11-0401+ or DM+ / D11-0401- cells, respectively. These findings would provide some insight into the possible importance and role of CD59, or other GPI-linked proteins, to the presentation of DM-modulated eptiopes.

A considerable proportion of pMHC-II at the cell surface and in internal membranes is associated with lipid rafts. This association occurs early in MHC-II biosynthesis during transit through the Golgi apparatus, resulting in more than half of newly synthesized MHC-II complexes being lipid raft-associated after peptide loading for final transport to the plasma membrane (184, 193). The presence of pMHC-II in lipid rafts is particularly important for T cell activation by APCs as rafts concentrate the normally low levels of a specific pMHC-II on their surface (154-156). For example, Bosch *et al.* demonstrated that newly formed pMHC-II generated in intracellular antigen processing compartments in DCs arrive at the plasma membrane in microclusters which could be dispersed by MBCD treatment (156). In this study, we found a substantial proportion of global surface DR species and DRB*04:01 molecules reside in lipid rafts in several B-LCL (Figure 4.6B, 4.7, 4.8). Significantly reduced amounts of D11-0401 on MBCD-treated SAVC and other DRB1*04:01 B-LCL (Figure 4.6B, Appendix C)

indicated that optimal expression of this DM-dependent epitope requires intact lipid rafts. The exception to this was the meagre reduction of D11-0401 on 8.1.6 0401 (Figure 4.6B), which may be related to reduced levels of DM and DO in 8.1.6 due to it being hemizygous for these loci (112). Both DM and DO have a role in the localization of proteins within MVB subdomains and are associated intracellularly with lipid rafts (367, 368).

Similar amounts of the DM-sensitive D13-0401 was found on untreated control and MBCD-treated 9.5.3-0401 cells, suggesting this epitope segregates with DRB1*04:01 molecules that are not partitioned in lipid raft microdomains. It is possible the absence of GPI-anchored proteins in 9.5.3 0401 may have significantly altered raft protein composition based on the lack of DRMs in lysate fractions prepared from 9.5.3 0401 as compared to 8.1.6 0401 (Figure 4.8). However, its resistance to MBCD is not entirely surprising given its sensitivity to DM, its absence on exosomes, and considerable evidence that both exosomes and lipid raft-associated MHC-II molecules arise from DM+ late endosomes and IVLs (145, 195-197). Intriguingly, the DM-resistant D13-0404 epitope was also unaffected by MBCD-treatment of MT14B cells, although its expression was reduced in another DRB1*04:04 cell line, BM92 (Appendix C). Aside from cell context differences, we have no explanation for this incongruity; however, the finding that the D13-0404 epitope segregated in the detergent-soluble and not in the DRM fractions of lysates prepared from MT14B (Figure 4.7) is consistent with its resistance to lipid raft disruption on MBCD-treated MT14B cells. Thus, although this epitope localizes to MIIC and is found on exosomes, it is primarily DM resistant and protease redundant,

suggesting this epitope may be formed in other intracellular vesicles that contain a smaller proportion of rafts.

In comparison to lipid rafts, tetraspanin protein family members contribute to the formation of membrane microdomains termed TEMs, which are responsible for clustering of some pMHC-II (153). Tetraspanins form homodimers and heterodimers resulting in the formation of larger membrane microdomain complexes which promote the recruitment of several other membrane proteins, lipids, and signaling molecules (151). In B cells, CD63 and CD82 form complexes with HLA-DR, DM, and DO within endosomal compartments (182), suggesting that tetraspanins and TEMs are important in clustering and transporting pMHC complexes to the plasma membrane. Indeed some studies show that newly loaded pMHC-II molecules arriving from MIIC segregate into TEMs on the plasma membrane (145, 153, 343). Since the FN1 mAb was shown to specifically recognize oligomerized pMHC-II in tetraspanin-enriched clusters that contain a select set of peptides (213, 369), we hypothesized that the DRB1*04 epitopes may also associate with TEMs. However, our efforts to prove this hypothesis were problematic as treatment with saponin, which has been reported to disrupt tetraspanin-tetraspanin interactions in a cholesterol dependent manner but without disturbing lipid rafts or affecting total surface DR expression (153, 210), had a negative impact on TEM- and non-TEM-associated markers. For example, not only did saponin treatment decrease the tetraspanin CD82, it also significantly decreased lipid raft-associated CD59, non-TEM-associated proteins CD45 and CD71, as well as MHC-I, peptide/DR, and MHC-II/CLIP complexes (Figure 4.9).

Intriguingly, DRB1*04 molecules, determined by the peptide-independent and DM-resistant pan-DRB1*04-specific NFLD.D1 mAb, were the only molecules that were

increased by saponin treatment (Figure 4.9). Thus, significantly decreased amounts of D11-0401, D13-0401, and D13-0404 in saponin treated cells cannot be easily discounted given that the D13-0401 and D13-0404 epitopes were not altered by lipid raft disruption. It is possible that the global effect of saponin-mediated changes in TEM and non-TEM expression may result from a disruption of several types of microdomains, especially since the distinction between TEMs, lipid rafts, and other specializations of the plasma membrane may not be clearly defined as once thought (370). The incorporation of cholesterol in both TEMs and lipid rafts is a defining feature for both microdomains in terms of their formation and biological function (210, 371-373). Thus, it is probable that the cholesterol-dependent action of saponin for disruption of TEMs was also responsible for the alteration of additional membrane microdomains including lipid rafts.

The exosomal expression and raft-association of the DRB1*04 epitopes in this study may give some insight into the presentation of unique pMHC-II epitopes. The D11-0401 epitope is reminiscent of the previously described Ia.2 epitope, which is recognized by the 11-5.2 mAb and defines a subset of cell surface I-A^k molecules predominantly found within membrane lipid rafts (186). Thus, D11-0401 may represent an ideal epitope to study the model of lipid raft-mediated coordination of MHC-II peptide loading and transport to the plasma membrane proposed by Roche and colleagues (152). In addition, the findings in this study support further investigation into the functional or therapeutic role of D11-0401+ exosomes, especially given the strong linkage of DRB1*04:01 to autoimmune diseases such as RA. Conversely, the discordant expression of D13-0401 between the plasma membrane and exosomes suggests that this epitope might be actively

excluded from presentation, which may provide a basis for further studies into antigen presentation mechanisms to limit the presentation of DM-sensitive self-epitopes.

Chapter 5

Summary and Future Directions

5.1 Overview

The focus of the thesis was to further characterize the factors which influence the formation and expression of DRB1*04 epitopes defined by the mAbs NFLD.D11 and NFLD.D13. These antibodies, along with other anti-DRB1*04 mAbs, were originally developed and characterized by Dr. S. Drover and Dr. W.H. Marshall (78, 276-280, 283, 310). The study of both NFLD.D11 and NFLD.D13 mAbs was initially undertaken given that they recognize epitopes on DRB1*04 molecules that contain the "shared epitope" (SE), a five amino acid sequence motif in residues 70-74 of the DRB chain (QKRAA / QRRAA), which is strongly associated with the development of severe rheumatoid arthritis (284, 285, 332). The mechanisms which account for the association of the SE with rheumatoid arthritis are unclear and inconclusive, with some attributing it to the selection of the T cell repertoire (374) or the presentation of arthritis-related autoantigens (375). Previous studies in our laboratory suggested that a specific set of peptides contribute to formation of the D11-0401, D13-0401, and D13-0404 epitopes (278-280, 283). Furthermore, the presentation of these epitopes depends on a delicate balance of factors including DM expression, peptide availability, and other unknown cellular elements (78, 280, 283). In order to identify these unknown factors, one of the major goals of this work was to ascertain under what conditions these DM-modulated epitopes are optimally expressed, with a particular focus on the effect of HLA-DO, association with membrane microdomains, and exosomal expression. Given the relationship between DM-sensitivity of MHC-II epitopes and autoreactive T cell development in autoimmune

disease, further study into these contributing elements may provide insight into a mechanism for the development of SE-associated autoimmunity.

5.2 Summary and potential implications of major findings

The research presented in this thesis examined the mechanisms involved in the presentation of antibody-defined DRB1*04 epitopes making use of B-LCL and antigen presentation defective cell lines which naturally express these epitopes. The D11-0401, D13-0401, and D13-0404 epitopes have been previously studied in some detail (78, 280, 283). Nevertheless, there remains an incomplete understanding of the conditions under which these epitopes are optimally expressed.

Previous studies have categorized peptide antigens or epitopes based on the effect of DM on their presentation or expression (102-104). DM-resistant peptides or epitopes are unaffected by DM expression and can be expressed on any MHC-II-expressing cells irrespective of DM or DO co-expression. Conversely, DM-sensitive peptides or epitopes are abolished by the presence of DM, but can be restored by the expression of DO which is typically restricted to pAPC (102). Finally, DM-dependent peptides or epitopes require the expression of DM for presentation (103), suggesting that these epitopes would be abrogated by DO. The findings presented in this thesis, particularly Chapter 3, help classify the D11-0401, D13-0401, and D13-0404 epitopes into the above defined categories.

The most important observations from previous experiments focused on D11-0401 were that (a) D11-0401 requires DM for expression in B-LCL, and (b) a subset of

endolysosomal and/or cytoplasmic cysteine proteases are required for epitope expression (78, 283), thus suggesting that the peptide(s) forming this epitope is DM-dependent. However, analysis of D11-0401 expression in this study on several DM+ cell lines such as BL cell lines Daudi, BJAB, and Ramos, and in DM-restored 5.2.4 0401 cells indicated that additional factors, possibly DO are required. Transfection of DO into BJAB did not induce D11-0401 formation, indicating that DO is not the missing factor for generating this epitope in BL cell lines. Without knowing the exact peptide(s) that contribute to this epitope, it is difficult to definitively rule out DO as a contributing factor, since a possible explanation for D11-0401 deficient expression on these DM-positive cell lines is they simply do not contain the proper antigens for proteases to generate the peptide(s) required for epitope expression. It can only be concluded from these experiments that DO may still be required along with DM, but other factors such as an appropriate antigenic peptide source may be missing in BL cell lines and other DM+ D11-0401- cells.

In contrast to D11-0401, the D13-0401 epitope was previously found to be only expressed in the absence of DM expression, indicating that the peptide(s) forming this epitope is DM-sensitive (78, 280). Additional expression analysis of D13-0401 on DM- and DM+ cell lines in Chapter 3.2.3 confirmed that expression of this epitope is confined to DM- cells, but also suggested that other cellular factors such as a specific antigen or peptide are required for epitope formation. Whereas the role of DO in D11-0401 expression was intriguing to examine given its DM-dependence, the contribution of DO to D13-0401 formation in the absence of DM is more difficult to envision given that DO performs its function by directly binding DM. For this reason, the involvement of DO in D13-0401 formation was not investigated in detail in this study. However, it is possible

that under circumstances of abnormally high DO expression, where the majority of DM remains bound to DO, this inhibition of DM may lead to the expression of D13-0401, as previously described for DM-sensitive epitopes (102, 104).

Regarding the DM-sensitivity and dependency of D13-0401 on specific proteases, it is reminiscent of another previously described epitope consisting of a peptide from type II collagen (aa261-273) bound to HLA-DRB1*04 (376). Type II collagen is a candidate autoantigen for rheumatoid arthritis and the peptide aa261-273 forms an immunodominant pathogenic epitope with HLA-DRB1*04 which can activate CD4+ T cells. Interestingly, this epitope is expressed by APC lacking DM and its presentation is inhibited by DM in the recycling pathway, resulting in decreased presentation and T cell activation (376).

Concerning the D13-0404 epitope, prior experiments demonstrated that DM expression and specific proteases were not necessary for expression (283). This is further demonstrated in Chapter 3.2.5 where the DM- 5.2.4 0404 cell line was found to abundantly express D13-0404. Furthermore, transfection of DM into 5.2.4 0404 only minimally affected D13-0404 expression and only in cells exhibiting the greatest expression. Therefore, this epitope is similar to that described above for DM-resistant epitopes (103).

Consistent with previous findings by Spurrell, D.R. (283), endolysosomal and cytoplasmic cysteine proteases were necessary for optimal expression of DM-dependent D11-0401 and DM-sensitive D13-0401, while being dispensable for DM- resistant D13-0404. Contribution of calpains to D11-0401 and D13-0401formation, suggests cytoplasmically-derived proteins could be a source of the putative peptide(s) involved in

epitope formation. This would not be surprising since as about 30% of the peptides bound to MHC-II are derived from nuclear and cytoplasmic proteins (136), which may access the endosomal pathway by autophagy (137). Autophagy in APCs can result in the generation of citrullinated peptides which are presented in the context of MHC-II (142), and the presence of anti-citrullinated protein/peptide antibodies is a defining characteristic of rheumatoid arthritis (377). The presentation of citrullinated peptides by MHC-II have been linked to the development of rheumatoid arthritis, and interestingly, the production of these autoantibodies is associated with the HLA-DRB1 shared epitope (143, 377, 378). Since both D11-0401 and D13-0401 may consist of cytoplasmic-derived peptide(s) bound to DRB1*04:01 and because D11-0401 was detected on PBMC from rheumatoid arthritis patients (310), it is speculative that these epitopes consist of citrullinated peptides processed in the cytoplasm of APCs and transported to the endosomal pathway via autophagy. Although briefly mentioned in Chapter 3.3 and Spurrell, D.R. (283), the role of autophagy in D11-040, D13-0401, and D13-0404 formation was investigated using the inhibitor wortmannin, with inconclusive results. For this reason, we believe that the role of autophagy requires further study, and that the use of more specific synthetic protease and autophagy inhibitors available today will provide a more precise definition concerning the peptide species bound to DRB1*04 that forms these epitopes.

A significant question that remains from this study concerns the structural basis of the D13-0401 epitope. The results presented in Chapter 3.2.4 indicate that specific proteolytic processing events affect D13-0401 expression, suggesting that this epitope defines a particular peptide or subset of peptides in combination with DRB1*04:01. Alternatively, since inhibition of the same proteases caused a strikingly similar decrease

of D13-0401 in 9.5.3 0401 as with D11-0401 in SAVC and 8.1.6 0401, one could argue that these epitopes define conformational isomers of DRB1*04:01 independent of the bound peptide like that reported for type A and type B pMHC-II complexes, described by Unanue (258). Although the exact structural differences between types A and B pMHC-II are unknown, it is well established that type A molecules require DM, whereas type B complexes are destroyed by interaction with DM. Further support of the idea that D13-0401 corresponds to type B complexes is that this epitope is expressed intracellularly in SAVC cells, but only minimally and sometimes undetectable at the cell surface. If this epitope is directly destroyed by DM binding in SAVC independent of bound peptide, then DRB1*04:01 molecules on route to MIIC which have not yet encountered DM would contain the D13-0401 epitope and only after arrival in the DM-rich MIIC environment would this epitope be destroyed. Further support for this hypothesis is the observation that exosomes released by SAVC cells do not express D13-0401 as demonstrated in Chapter 4.2.1. Since exosomes originate from MIIC and contain very little MHC-II-CLIP, exosomal pMHC-II have likely come into contact with DM, thus inducing a conformational change which destroys D13-0401. Moreover, the absence of D13-0401 on 9.5.3 0401 exosomes may indicate that this epitope is not present in MIIC, which is also comparable to type B complexes (379).

Recent studies suggest that for successful stimulation of T cells, pMHC-II complexes require concentration in distinct microdomains including lipid rafts and TEMs within the plasma membrane (150-154). The relevance and mechanisms of pMHC-II association with lipid rafts TEMs is not fully understood; however, it has been previously hypothesized that membrane microdomains contain MHC-II that bind and display

specific sets of peptides (153, 163). Since prior experiments in the lab indicated possible association of the DRB1*04 epitopes with membrane microdomains (283), we questioned whether the DM-sensitivity of these epitopes affected their ability to segregate in different microdomains. By examining surface expression on B-LCL using flow cytometry prior and subsequent to chemical disruption of lipid rafts and TEMs, we found that a fraction of DM-dependent D11-0401 is associated with rafts in the plasma membrane, while presentation of both DM-sensitive D13-0401 and DM-resistant D13-0404 was not affected (Chapter 4.2.3). In support of this later observation, D13-0404+ DRB1*04:04 molecules were not observed in DRM fractions isolated from MT14B cells. Previous experiments by Spurrell, D.R. indicated that the D11-0401 and D13-0404 epitopes were not affected by MBCD-disruption of lipid rafts (283); however, these previous experiments involved MBCD treatment on PFA-fixed cells whereas the current procedure used live cells. This discrepancy between findings is likely due to the difference in methodologies; however the current method appears to be appropriate given the specific disruption of raft-associated membrane proteins but not non-raft proteins.

A previously described MHC-II epitope on I-A^k molecules termed the Ia.2 epitope, which is recognized by the 11-5.2 mAb and defines a subset of cell surface I-A^k predominantly found within lipid rafts (186). This study also demonstrated that the Ia.2-positive MHC-II molecules are critically necessary for a successful interaction between B and T lymphocytes, especially under circumstances of limited antigen. This is consistent with previous reports detailing the importance of raft-resident pMHC-II in antigen presentation (154, 186). Unlike the Ia.2 epitope, it is not clear from our studies whether D11-0401 defines a unique subset of pMHC-II that are sequestered in lipid raft domains.

Since complete abrogation of D11-0401 was not observed after lipid raft disruption in SAVC, Boleth, and PF97387 cells, and no disruption of epitope expression occurred on 8.1.6 0401 cells, it is unlikely that this epitope is created from a pMHC-II association with lipid rafts, but that D11-0401 instead defines a subset of pMHC-II that dynamically passage in and out of rafts at the cell surface. This is supported by our previous findings that the main determining factors governing D11-0401 expression are DM and peptide availability, and that D11-0401-positive pMHC-II are present both in the presence and absence of the lipid raft marker CD59.

Preliminary experiments performed by Spurrell, D.R. (283) found that cell surface D11-0401 expression was disrupted by low concentrations of the detergent saponin, suggesting that this epitope is concentrated in TEMs similar to that described for the CDw78 epitope (153, 210). We performed the same protocol in this study and also observed a significant decrease in the DRB1*04 epitopes as shown in Chapter 4.2.3; however, saponin treatment also caused a general decrease in all surface molecules investigated, including proteins not known to localize within TEMs. These non-specific results, combined with a criticism of this method by Poloso *et al.* (212) who questioned the use saponin treatment on cells that have been previously fixed with PFA to disrupt membrane protein interactions, makes it difficult to arrive at a conclusion concerning the association of D11-0401, D13-0401, or D13-0404 with TEMs. An attempt was made in this study to visualize D11-0401 and D13-0404 with TEMs simultaneously using immunofluorescent staining confocal microscopy, but this proved to be technically difficult. The concept that TEMs contain unique MHC-II epitopes such as CDw78 was contested by Poloso et al. (212), who found that CDw78 does not uniquely identify

tetraspanin-associated MHC-II, thus calling into question the use of this epitope as an indicator of MHC-II-tetraspanin complexes, and suggests that this epitope defines a subset of pMHC-II associated with tetraspanins that reside in lipid rafts.

An additional aim of this study was to characterize the expression of the D11-0401, D13-0401, and D13-0404 epitopes on B-LCL-derived exosomes. Purified exosomes expressed typical exosomal proteins reported in the literature, including DR. However, the expression of the DRB1*04 epitopes was cell specific and independent of the epitope expression at the cell surface. While both DM-dependent D11-0401 and DM-resistant D13-0404 epitopes were abundantly expressed on exosomes from DM-expressing B-LCL, exosomes from DM-deficient cells were devoid of DM-resistant D13-0401 expression. Taken together, these results suggest a role for DM in the recruitment of specific pMHC-II to exosomes during their biogenesis.

The finding that the D11-0401 and D13-0404 epitopes are contained on exosomes has several important implications. It reaffirms that these epitopes are formed on nascent DR molecules trafficking through the classical MHC-II antigen processing pathway to peptide-loading late endocytic vesicles, since exosomes originate from ILVs within MIIC. It also supports the assertion that D11-0401+ and D13-0404+ pMHC-II consist of mature molecules containing bound peptide because MHC-II-CLIP complexes are sorted onto ILVs during the process of DM-mediated peptide loading (13). Finally, exosomal expression also supports the observation that D11-0401 associates with lipid rafts, since ILVs are known to contain several raft-associated molecules (195-197) and the majority of exosomal pMHC-II can reside within lipid rafts (195). However, these assertions are complicated by the observations that D13-0404 does not appear to be associated with

lipid rafts. Nonetheless, the D11-0401 epitope may represent an ideal epitope to study the hypothetical model of lipid raft-mediated coordination of MHC-II peptide loading and transport to the plasma membrane proposed by Roche and colleagues (152). However, additional research is required to clarify the relevance of the exosomal expression of these epitopes to the factors governing their expression.

The lack of D13-0401 despite abundant pMHC-II complexes on exosomes from 9.5.3 0401 is intriguing for several reasons. First, it provides additional support that the D13-0401 epitope is not comprised of MHC-II-CLIP molecules, since substantial MHC-II-CLIP was observed on the same exosomes. Secondly, assuming that this epitope is found within MIIC given its DM-sensitive classification, the lack of D13-0401 on exosomes from SAVC, 8.1.6 0401, and 9.5.3 0401 suggests that pMHC-II bearing this epitope are actively prohibited from trafficking onto exosomes. Although it cannot be determined from this study what mechanisms are responsible for this exclusion, a potential explanation may involve the resistance of this epitope to MBCD-induced lipid raft disruption. Previous studies have proposed a lipid raft-mediated mechanism involvement in the recruitment of pMHC-II to exosomes (194, 359). Alternatively, the lack of GPI-anchored proteins or MHC-II accessory molecules such as DM in 9.5.3 0401 may point to a direct role for these molecules in loading pMHC-II onto exosomes. Regardless, the idea that there is an active mechanism in pAPC to ensure that only DM-edited pMHC-II complexes are presented on exosomes to prevent exosomal presentation of low-affinity potentially autoantigenic epitopes leading to undesired autoimmune activation is an attractive hypothesis to consider. In line with this thinking, the idea that non-APCs which upregulate MHC-II but not DM or DO can present DM-unedited or

DM-sensitive low affinity pMHC-II epitopes on exosomes activation of auto-reactive T cells is also intriguing. Finally, the idea that APC have the ability to regulate which particular pMHC-II epitopes are released to the external environment via exosomes raises many questions concerning their significance in immune activation and regulation. Whether particular antigens or peptides are preferentially presented on exosomal MHC-II and under what circumstances can dysregulation of this process lead to the presentation of autoantigenic MHC-II epitopes and subsequent activation of autoimmune responses would be an avenue worth exploring. The D13-0401 epitope may represent a unique tool, and in combination with D11-0401 and their associated cell lines, a potential model to investigate mechanisms of MHC-II incorporation onto exosomes.

5.3 Study limitations and future directions

The work described in this thesis has furthered our understanding of the factors influencing expression of unique antibody-defined DRB1*04 epitopes. However, there are several limitations to consider and unanswered questions for future studies, as well as new research avenues that have the potential for investigation.

1) Many of the same proteases required for antigen processing and MHC-II presentation also degrade the Ii chain, an integral step preceding peptide loading. Thus, inhibition of these same proteases can have several adversely affect the MHC-II presentation pathway, including blocking transport of MHC-II to antigen loading compartments and the cell surface (117, 380, 381). A limitation of this study is that

incomplete invariant chain degradation was detected in protease inhibitor-treated B-LCL as shown in Chapter 3.2.7, suggesting that a general impairment of peptide loading and MHC-II transport instead of a decrease in a specific peptide species may be responsible for the observed reduction in D11-0401, D13-0401, and D13-0404 in these experiments. This does not fully discount the conclusion that these epitopes represent a specific peptide or group of peptides bound to DRB1*04, but instead suggests that at some point during their synthesis, these epitopes transit through the classical pathway of MHC-II presentation.

2) Since undertaking this study, the precise function and effect of DO on the repertoire of peptides presented by MHC-II has been further clarified in the literature (81, 382). Because DO is a MHC-II substrate mimic for DM resulting in reduced DM-function, the overall pMHC-II expression at the cell surface is ultimately controlled by the ratio of active DM to inactive DM-DO complexes (383). Therefore, perhaps a more relevant set of experiments to determine the contribution of DO to expression of both D11-0401 and D13-0401 would be to quantify the ratio of DO to DM by using western blot or flow cytometry analysis of expression in cell lines constitutively expressing each epitope. Given this new understanding of DO function, it is likely that optimal D11-0401 expression requires high DO levels since the epitope likely consists of low stability self-peptide(s) bound to DRB1*04:01 molecules. Thus, optimal D11-0401 expression is conceivably dependent on (1) the presence of the appropriate protein(s) or peptide(s) and (2) the ratio of functional DM to inactive DO-bound DM. Alternatively, a more direct approach to measure the effect of DO on D11-0401 or D13-0401 would be to knock

down DO expression in D11-0401+ or D13-0401+ cells, respectively, utilizing RNA interference knockdown or CRISPR/Cas9 gene editing technology. However, interpreting the results of these experiments would be complicated by the fact that knockdown of DO would also affect the level of functional DM. Regardless, a more complete understanding of how DM and DO simultaneously shape the presentation of specific epitopes could provide insight to mechanisms of immune tolerance and initiation of autoimmunity.

3) The current study is limited in its conclusions regarding the specificity of the cellular proteases contributing to D11-0401, D13-0401, and D13-0404 formation and future experiments should make use of more specific protease and cellular pathway inhibitors to clarify the nature of the antigen or peptides which form these epitopes. Cysteine cathepsins, which constitute a major portion of the endolysosomal proteolytic activity, are responsible for the generation of several MHC-II epitopes (61, 384, 385). In addition, both GILT and AEP have been shown to contribute to the generation of MHC-II epitopes (125, 126, 128, 386). Therefore, particular attention should be devoted to specific lysosomal cysteine proteases such as cathepsins L and S, GILT, and AEP. Furthermore, additional focus should be made to the contribution of autophagy to the formation of these epitopes, especially since cytoplasmic-derived citrullinated peptides presented by DRB1*04:01 may represent these epitopes as previously discussed. An alternative method to determine whether citrullinated proteins or peptides contribute to epitope formation would be to (1) test whether anti-citrullinated protein abs block NFLD.D11 and NFLD.D13 binding, or (2) stain D11-0401+, D13-0401+, and D13-0404+ cells with anti-citrullinated proteins/peptide antibodies and visualize by confocal

microscopy for cellular localization. The identity of the specific peptide or peptides that are bound by DRB1*04 molecules forming these epitopes would be valuable given the potential biologically relevance of this epitope to DRB1*04:01and *04:04 associated autoimmunity.

4) The role of one or more tetraspanin proteins in expression of D11-0401, D13-0401, and D13-0404 remains unanswered. A more appropriate approach to examine the importance of D11-0401-tetraspanin interactions in the expression of this epitope might be to make use of RNA interference or CRISPR/Cas9 gene editing techniques to knockdown individual tetraspanin expression. However, the question of whether D11-0401 associates with larger tetraspanin-enriched microclusters distinct from lipid rafts might be difficult to ascertain, especially given that both microdomains may be more closely related than what was initially postulated (152).

5) Due to the aforementioned difficulty of using the NFLD.D11 and NFLD.D13 antibodies in several assays, a complete analysis of the association of these epitopes with DRMs could not be determined, as well as immunofluorescence colocalization experiments were inconclusive, both of which would have provided additional insight into epitope raft association. As an alternative to the sucrose density gradient ultracentrifugation technique, a unique flow cytometric assay of differential detergent resistance (FCDR) was employed, which is based on the resistance of lipid rafts and associated proteins to be solubilized in nonionic detergents such as Triton X-100 (387-389). Contrary to the previous experiments utilizing MBCD to disrupt lipid raft integrity,

the FCDR results indicated that D11-0401 was detergent soluble in SAVC and 8.1.6 0401 cells, suggesting limited association of this epitope with cell surface lipid rafts (Appendix G). However, the FCDR results are less reliable since detergent resistance or solubility can be affected by several other factors independent of raft-association (390). We propose additional experiments are performed which involve the biotinylation of the NFLD.D11 and NFLD.D13 mAbs prior to incubation with D11-0401-positive cells, followed by streptavidin-HRP labelling and lipid raft isolation by sucrose density gradient ultracentrifugation. This would allow examination of the distribution of the HRP-tagged mAbs in raft and non-raft fractions, thus allowing calculation of the proportion of surface pMHC-II bearing these epitopes that is raft-associated.

6) Given the similarity of the D13-0401 epitope to another DRB1*04-restricted epitope consisting of a peptide from type II collagen as described above (376), it might be worthwhile to examine the functional relevance of D13-0401, with a particular focus on the *in vivo* expression profile in the context of DRB1*04-associated autoimmune disease such as RA or the ability of this mAb to block autoreactive T cell activation. For example, expression of D13-0401, as well as D11-0401 and D13-0404, could be investigated in arthritic tissue sections or primary cells from inflamed synovium by immunohistochemistry and immunocytochemistry, respectively. Alternatively, epitope expression could be evaluated on B cells isolated from inflamed synovium or synovial fluid from RA patients. To determine the potential therapeutic use of these mAbs, their ability to block autoreactive T cell activation could be measured using ELISPOT or flow cytometry-based T cell activation/proliferation assays by stimulating peripheral T cells

from RA patients *in vitro* using overlapping peptide pools of known RA-causing autoantigenic proteins in the presence of blocking NFLD.D11 and NFLD.D13 mAbs. If these epitopes are indeed responsible for the activation or expansion of autoreactive T cells in RA or other autoimmune disorders, then a potential therapeutic use of these antibodies would be invaluable.

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Appendices

Preamble

The results presented in the Appendices include mostly preliminary data that requires further follow with additional experiments. The author did not feel that these results warranted inclusion in Chapter 3 or 4, as the data would likely be excluded from a future manuscript prepared from these chapters. Appendices D, E and F show results from several independent experiments and are meant to compliment the data presented in Figures 4.6A, 4.6B, and 4.9, respectively, which display mean values compiled from the independent experiments.

Appendix A. The effect of additional protease inhibitors on surface DR epitope expression.

Figure A.1. SAVC cells were treated with several protease inhibitors or with the appropriate diluent control and surface expression of HLA-DR (L243), DRB1*04 (NFLD.D1), D11-0401 (NFLD.D11), CLIP (cerCLIP), and Ii (LN2) was measured by CELISA. Brefeldin A prevents transport of proteins from the ER to the Golgi apparatus. Chloroquine prevents acidification of late endosomes and thus lysosomal degradation of proteins. Leupeptin is an inhibitor of cysteine, serine, and threonine peptidases. E64d is an inhibitor of a wide range of cysteine peptidades including papain, cathepsin B, cathepsin L, and calpain. Pepstatin inhibits aspartyl proteases. Calpeptin is a calpain inhibitor. Bafilomycin is an endosomal acidification and late autophagy inhibitor. Lactacystin is an inhibitor of the proteasome. Results are displayed as a ratio of protease inhibitor-treated expression values to untreated controls. Results are from at least two independent experiments ($n \geq 2$). Error bars indicate standard error. DRB1*04, D11-0401, and CLIP expression was compared to DR using a paired t-test and asterisks indicate significant differences (* = $p < 0.05$; ** = $p < 0.01$).

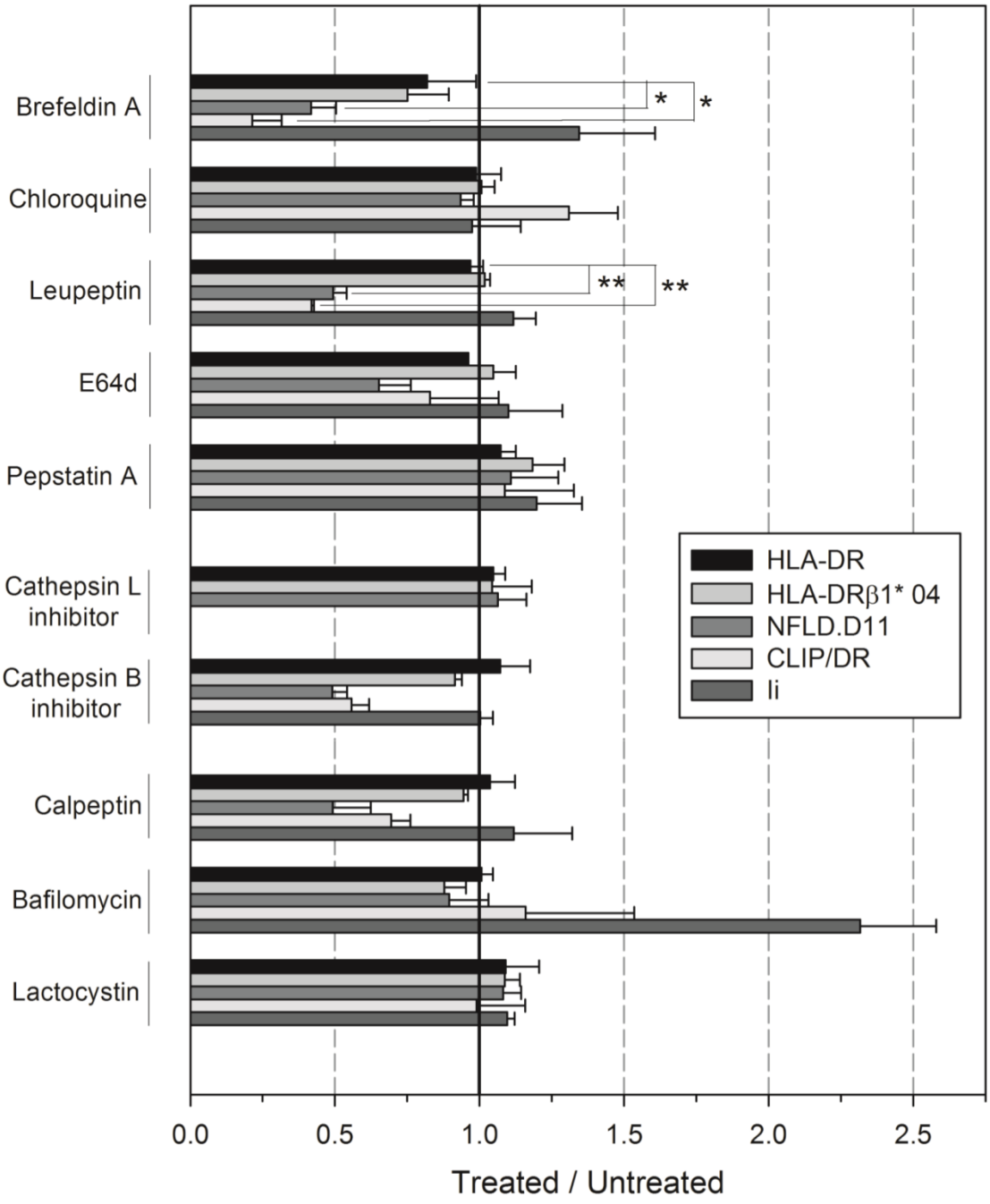
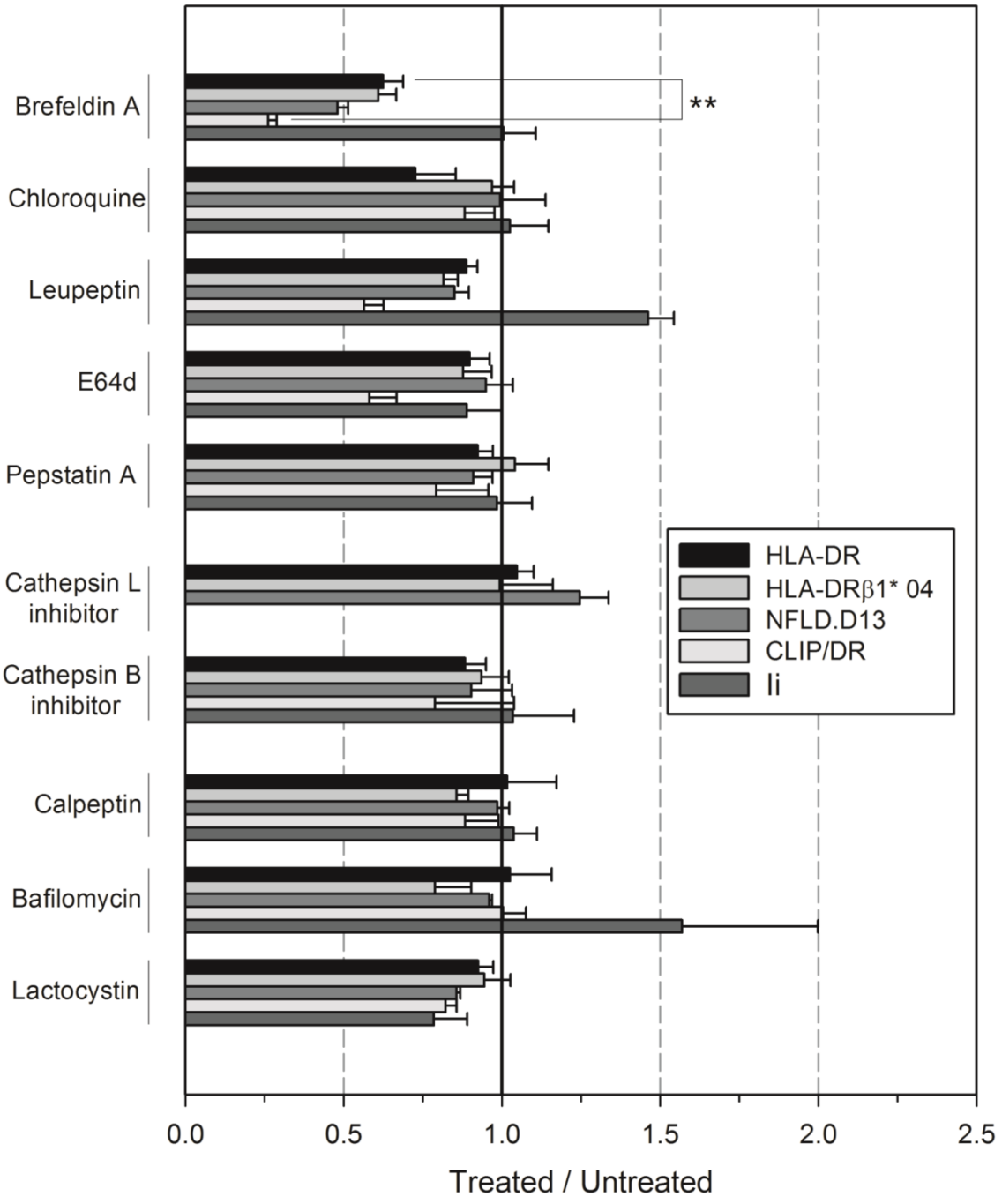

Brefeldin A
Chloroquine
Leupeptin
E64d
Pepstatin A
Cathepsin L inhibitor
Cathepsin B inhibitor
Calpeptin
Bafilomycin
Lactocystin
*
*
**
**
HLA-DR
HLA-DRβ1* 04
NFLD.D11
CLIP/DR
Ii
0.0
0.5
1.0
1.5
2.0
2.5
Treated / Untreated

Figure A.2. MT14B cells were treated with several protease inhibitors or with the appropriate diluent control and surface expression of HLA-DR (L243), DRB1*04 (NFLD.D1), D13-0404 (NFLD.D13), CLIP (cerCLIP), and Ii (LN2) was measured by CELISA. Brefeldin A prevents transport of proteins from the ER to the Golgi apparatus. Chloroquine prevents acidification of late endosomes and thus lysosomal degradation of proteins. Leupeptin is an inhibitor of cysteine, serine, and threonine peptidases. E64d is an inhibitor of a wide range of cysteine peptidades including papain, cathepsin B, cathepsin L, and calpain. Pepstatin inhibits aspartyl proteases. Calpeptin is a calpain inhibitor. Bafilomycin is an endosomal acidification and late autophagy inhibitor. Lactacystin is an inhibitor of the proteasome. Results are displayed as a ratio of protease inhibitor-treated expression values to untreated controls. Results are from at least two independent experiments ($n \geq 2$). Error bars indicate standard error. DRB1*04, D13-0404, and CLIP expression was compared to DR using a paired t-test and asterisks indicate significant differences (* = $p < 0.05$; ** = $p < 0.01$).


Brefeldin A
Chloroquine
Leupeptin
E64d
Pepstatin A
Cathepsin L inhibitor
Cathepsin B inhibitor
Calpeptin
Bafilomycin
Lactocystin
**
HLA-DR
HLA-DRβ1* 04
NFLD.D13
CLIP/DR
Ii
0.0
0.5
1.0
1.5
2.0
2.5
Treated / Untreated

Appendix B. Surface and intracellular expression of membrane proteins assessed on exosomes.

Figure B.1. 8.1.6 0401 cells were analyzed for surface and intracellular expression of antigen presentation molecules, DRB1*04 epitopes, and exosomal markers using flow cytometry. Isotype control staining was performed in parallel and had an MFI ≤ 10.

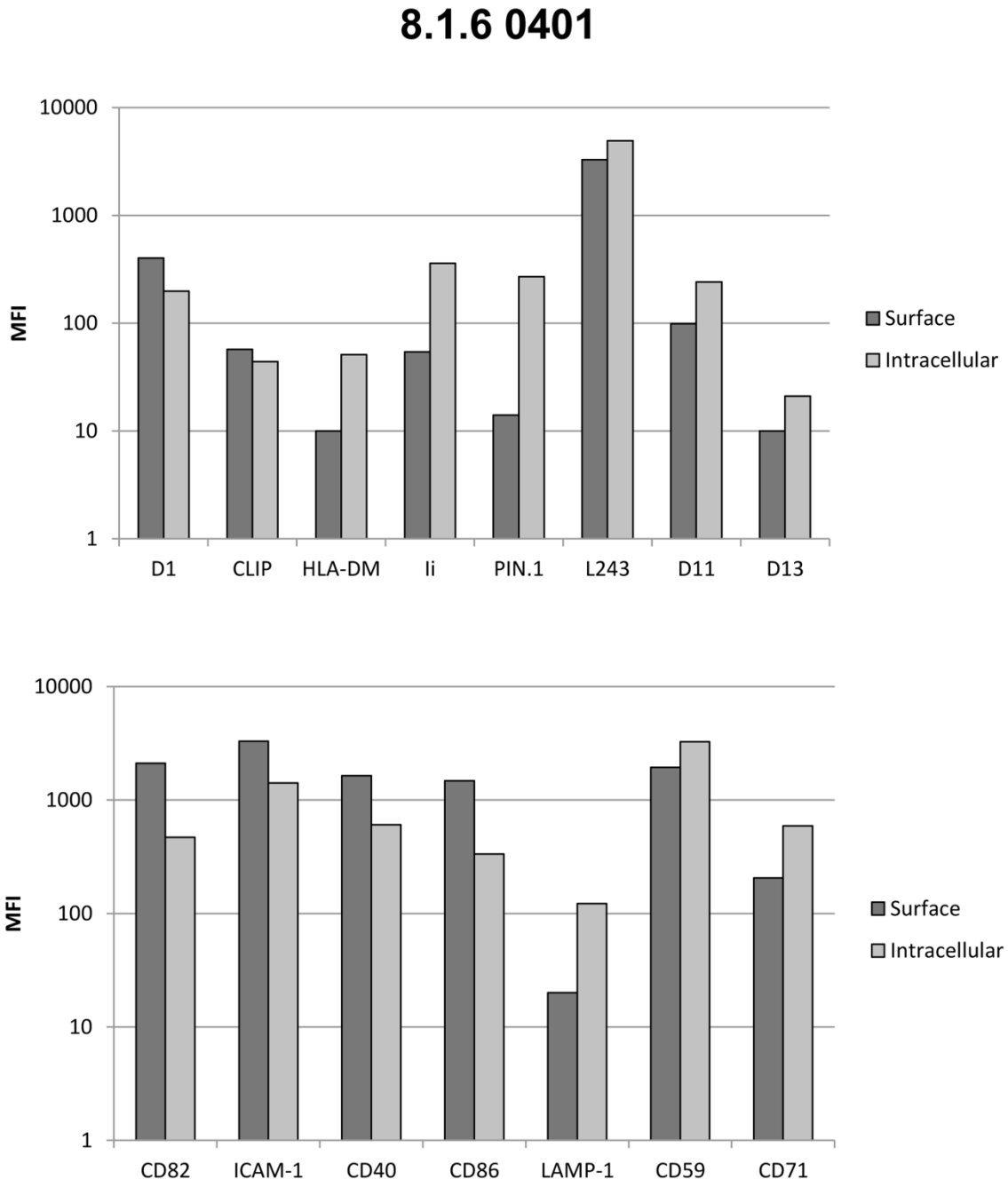
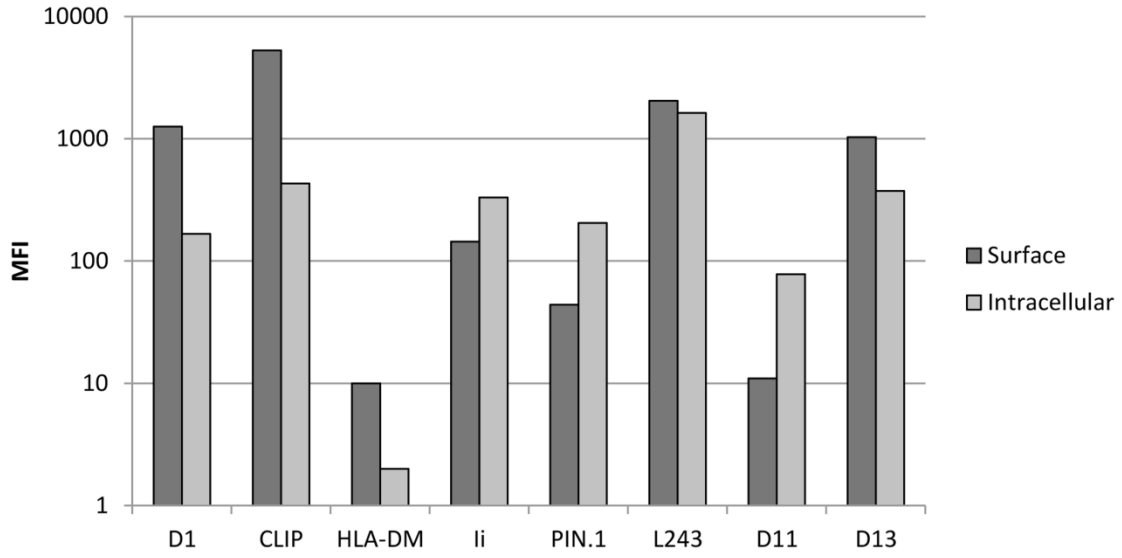
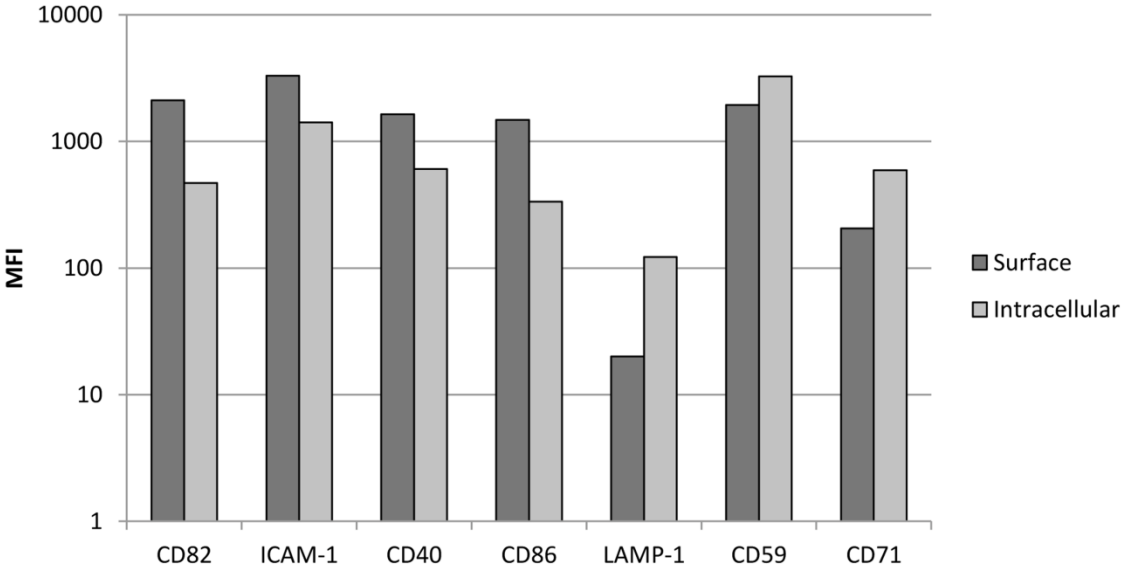

8.1.6 0401
10000
1000
100
10
1
MFI
D1
CLIP
HLA-DM
Ii
PIN.1
L243
D11
D13
Surface
Intracellular
10000
1000
100
10
1
MFI
CD82
ICAM-1
CD40
CD86
LAMP-1
CD59
CD71
Surface
Intracellular

Figure B.2. 9.5.3 0401 cells were analyzed for surface and intracellular expression of antigen presentation molecules, DRB1*04 epitopes, and exosomal markers using flow cytometry. Isotype control staining was performed in parallel and had an MFI ≤ 10.

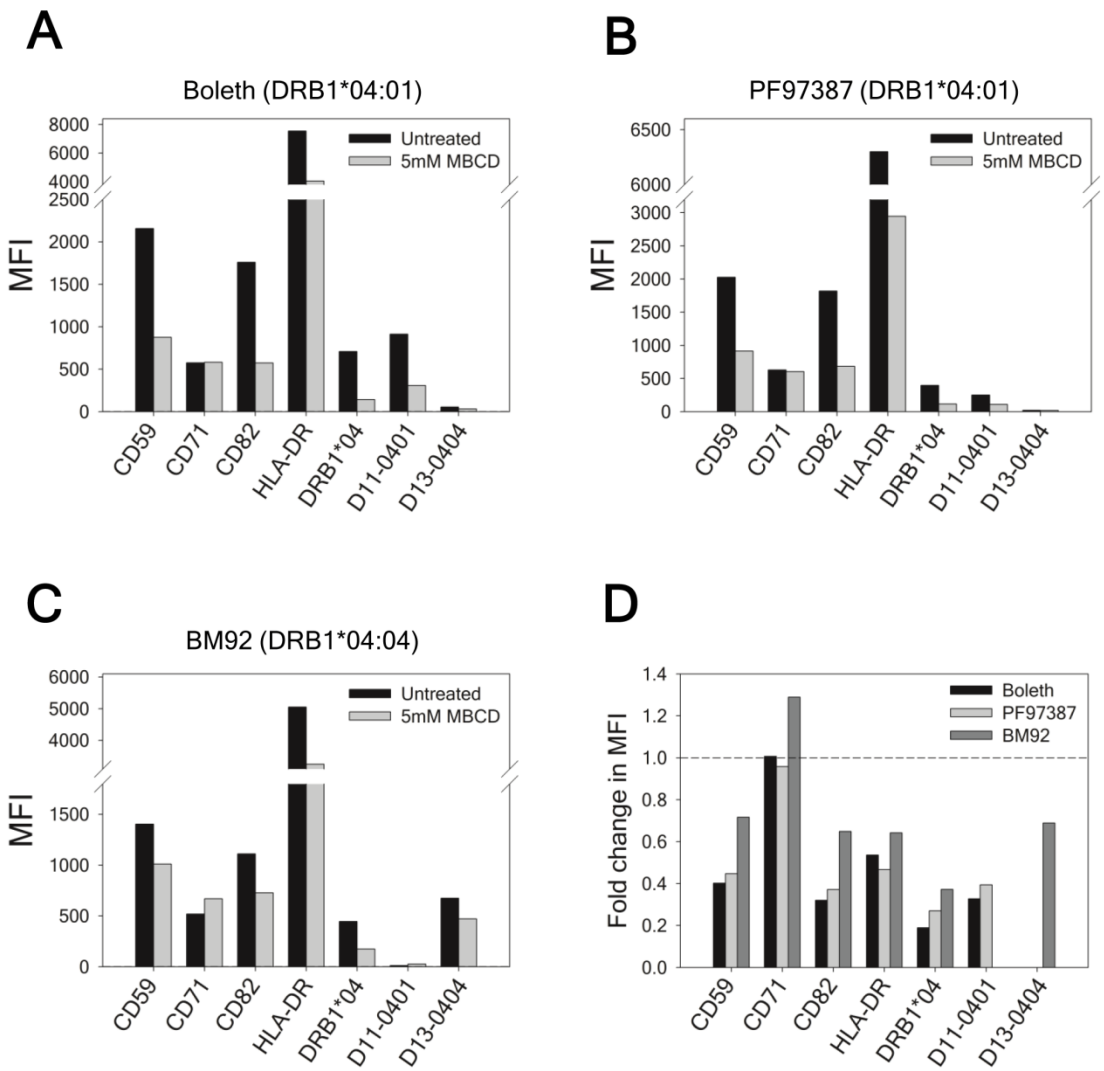
9.5.3 0401


10000
1000
100
10
1
MFI
D1
CLIP
HLA-DM
Ii
PIN.1
L243
D11
D13
Surface
Intracellular


10000
1000
100
10
1
MFI
CD82
ICAM-1
CD40
CD86
LAMP-1
CD59
CD71
Surface
Intracellular

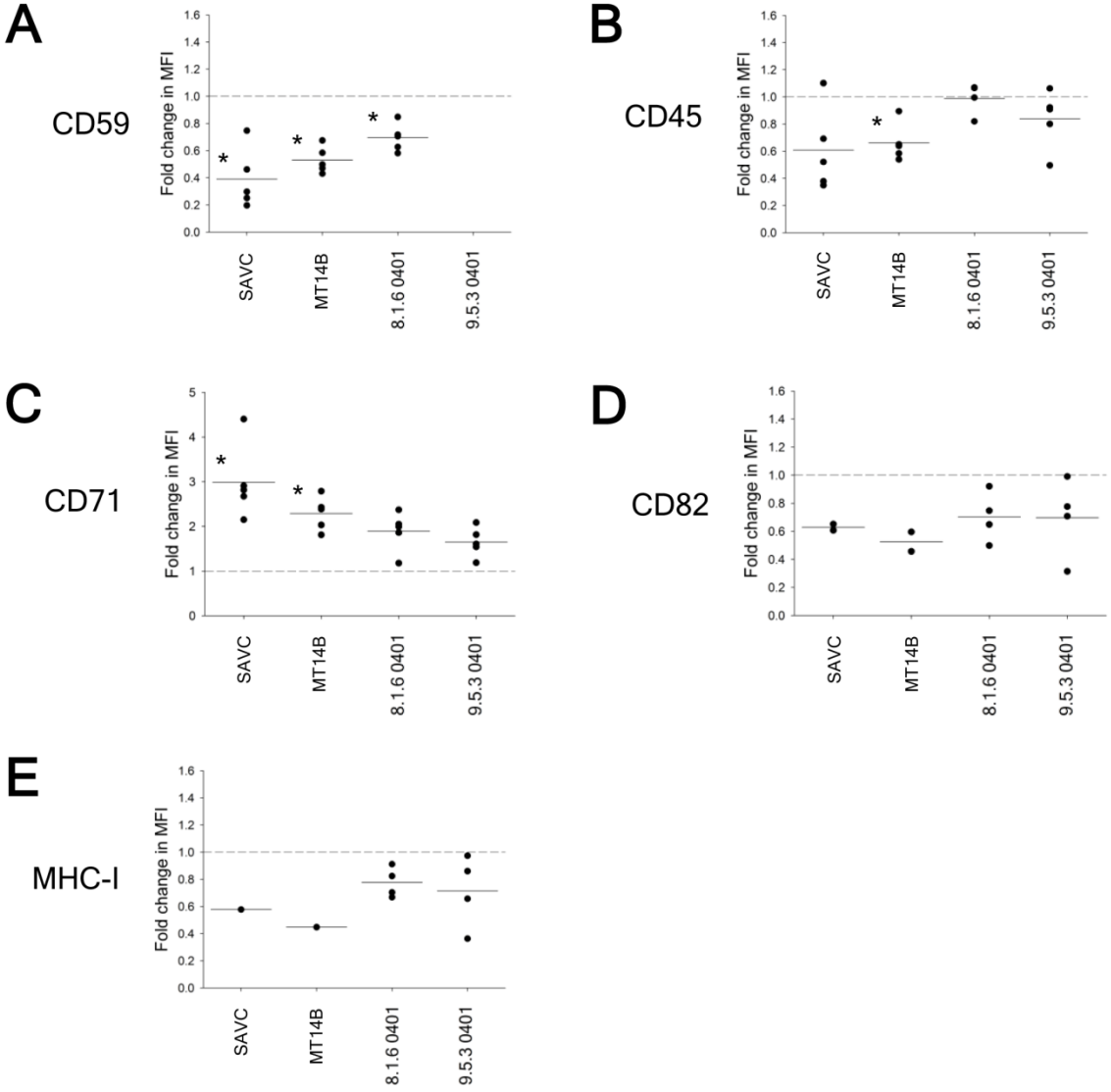
Appendix C. The effect of lipid raft disruption on the surface expression of raft markers and DRB1*04 epitopes in additional DRB1*04 B cell lines.

Figure C.1. Lipid rafts were disrupted by membrane cholesterol depletion using MBCD to treat **(A)** Boleth, **(B)** PF97387, and **(C)** BM92 and expression of CD59 (MEM-43), CD71 (M-A712), CD82 (TS82b), total DR (L243), DRB1*04 (NFLD.D1), D11-0401, and D13-0404 was compared to untreated control cells. Results from one flow cytometry are shown. **D.** The fold change in expression was calculated in by dividing the MFI values of MBCD-treated cells by the MFI values of untreated controls. The broken line in the graph represents no change in expression caused by MBCD.


A
Boleth (DRB1*04:01)
Untreated
5mM MBCD
MFI
8000
6000
4000
2500
2000
1500
1000
500
0
CD59
CD71
CD82
HLA-DR
DRB1*04
D11-0401
D13-0404
B
PF97387 (DRB1*04:01)
Untreated
5mM MBCD
MFI
6500
6000
3000
2500
2000
1500
1000
500
0
CD59
CD71
CD82
HLA-DR
DRB1*04
D11-0401
D13-0404
C
BM92 (DRB1*04:04)
Untreated
5mM MBCD
MFI
6000
5000
4000
1500
1000
500
0
CD59
CD71
CD82
HLA-DR
DRB1*04
D11-0401
D13-0404
D
Boleth
PF97387
BM92
Fold change in MFI
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CD59
CD71
CD82
HLA-DR
DRB1*04
D11-0401
D13-0404

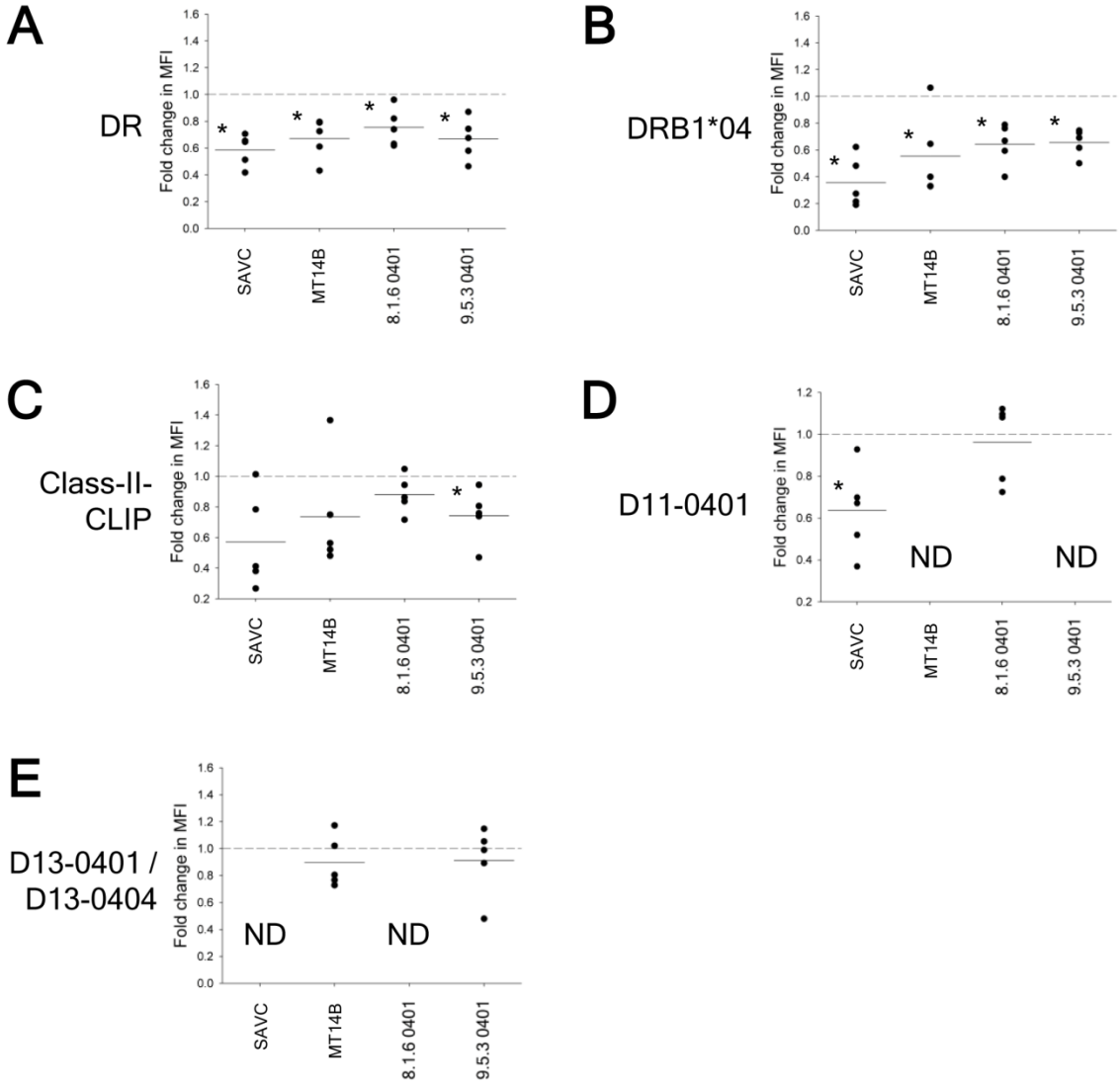
Appendix D. The effect of lipid raft disruption on the surface expression of transmembrane proteins.

Figure D.1. The effect of lipid raft disruption on the surface expression of **(A)** CD59 (MEM-43), **(B)** CD45 (HI30), **(C)** CD71 (M-A712), **(D)** CD82 (TS82b), and **(E)** MHC-I (W6/32) on SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 was determined as displayed in Figure 4.6A. The fold change in expression caused by MBCD treatment (Fold change in MFI) for each individual experiment is shown and was calculated by dividing the MFI expression value of MBCD-treated cells by the MFI of untreated controls. Each dot denotes an individual experiment and the solid lines indicate the mean fold change in MFI. The broken line in each graph represents no effect of MBCD-treatment on expression. A paired t-test was performed to compare expression between MBCD-treated and untreated control cells for a given cell line. Significant differences are indicated by an asterisk ($p < 0.05$).


A
CD59
Fold change in MFI
SAVC
MT14B
8.1.6 0401
9.5.3 0401
B
CD45
C
CD71
D
CD82
E
MHC-I

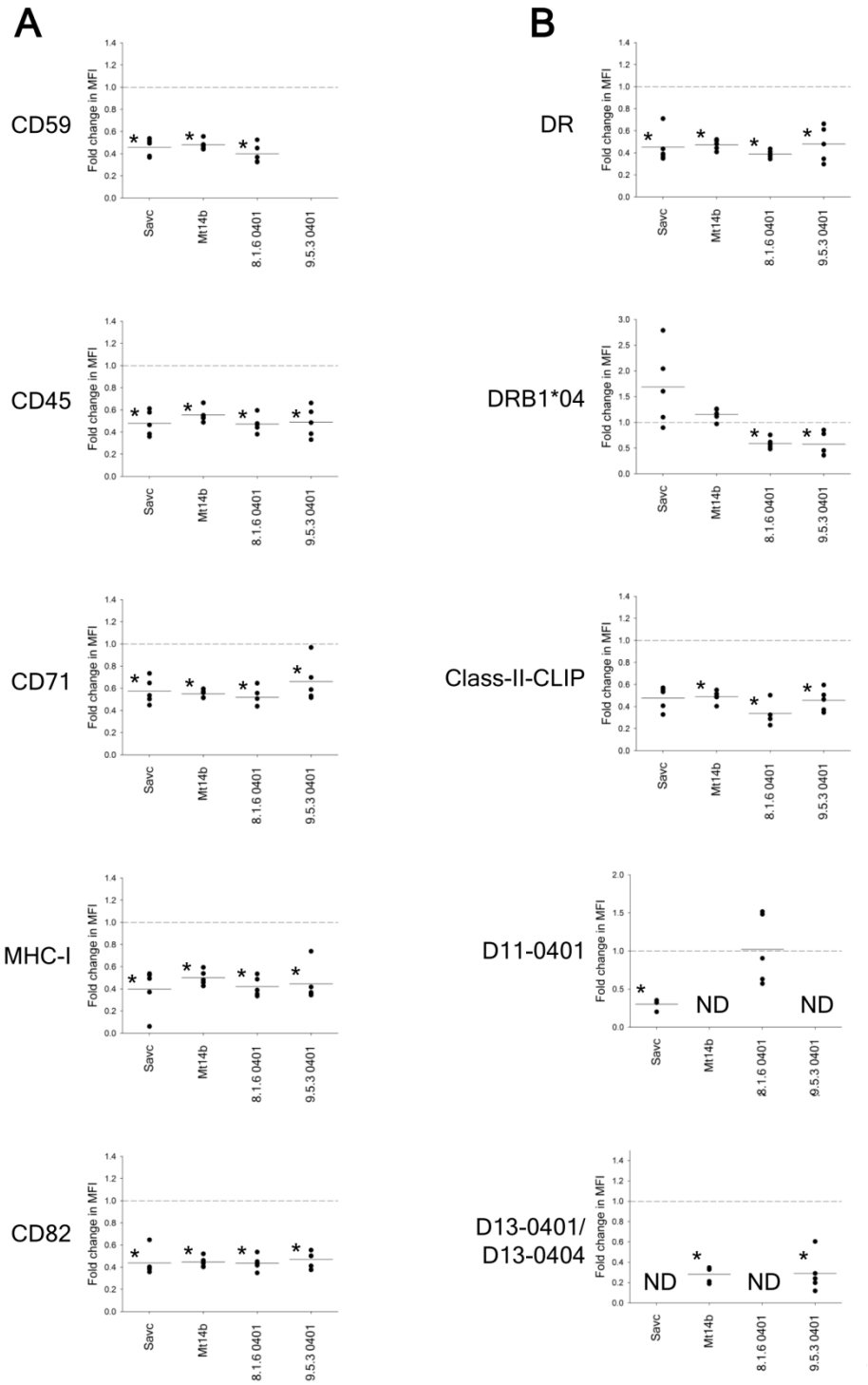
Appendix E. The effect of lipid raft disruption on the surface expression of DRB1*04 epitopes.

Figure E.1. The effect of lipid raft disruption on the surface expression of **(A)** total DR (L243), **(B)** DRB1*04 (NFLD.D1), **(C)** class-II-CLIP (cerCLIP), **(D)** D11-0401, and **(E)** D13-0401/D13-0404 on SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 was determined as displayed in Figure 4.6B. The fold change in expression caused by MBCD treatment (Fold change in MFI) for each individual experiment is shown and was calculated by dividing the MFI expression value of MBCD-treated cells by the MFI of untreated controls. Each dot denotes an individual experiment and the solid lines indicate the mean fold change in MFI. The broken line in each graph represents no effect of MBCD treatment on expression. A paired t-test was performed to compare expression between MBCD-treated and untreated control cells for a given cell line. Significant differences are indicated by an asterisk ($p < 0.05$). ND, not determined.


A
DR
B
DRB1*04
C
Class-II-CLIP
D
D11-0401
ND
ND
E
D13-0401 / D13-0404
ND
ND
Fold change in MFI
SAVC
MT14B
8.1.6 0401
9.5.3 0401

Appendix F. The effect of TEM disruption on the surface expression of transmembrane proteins and DRB1*04 epitopes.

Figure F.1. The effect of saponin treatment on the surface expression of **(A)** transmembrane proteins and **(B)** DRB1*04 epitopes on SAVC, MT14B, 8.1.6 0401, and 9.5.3 0401 was determined as displayed in Figure 4.9. The fold change in expression caused by saponin treatment (Fold change in MFI) for each individual experiment is shown and was calculated by dividing the MFI expression value of saponin-treated cells by the MFI of untreated controls. Each dot denotes an individual experiment and the solid lines indicate the mean fold change in MFI. The broken line in each graph represents no effect of saponin-treatment on expression. A paired t-test was performed to compare expression between saponin-treated and untreated control cells for a given cell line. Significant differences are indicated by an asterisk ($p < 0.05$). ND, not determined.


A
B
CD59
DR
CD45
DRB1*04
CD71
Class-II-CLIP
MHC-I
D11-0401
CD82
D13-0401/
D13-0404
Fold change in MFI
Savc
Mt14b
8.1.6 0401
9.5.3 0401
ND
ND
ND
ND

Appendix G. Flow cytometric assay of detergent resistance.

G.1 Flow cytometric assay of differential detergent resistance

As an alternative to sucrose density gradient ultracentrifugation, a unique flow cytometric assay of differential detergent resistance (FCDR) was used to analyze whether DRB1*04 epitopes associate with detergent resistant membranes. Similar to sucrose density gradient ultracentrifugation, the FCDR assay is based on the principle that lipid rafts and associated proteins are resistant to solubilisation in nonionic detergent such as Triton X-100 (1-2). Association of DRB1*04 epitopes with rafts was measured by their differential sensitivity to Triton X-100 before and after cholesterol depletion using MBCD. Since this method involves intact cells instead of cell lysates, the chance that proteins become associated with rafts after cell lysis are not of concern.

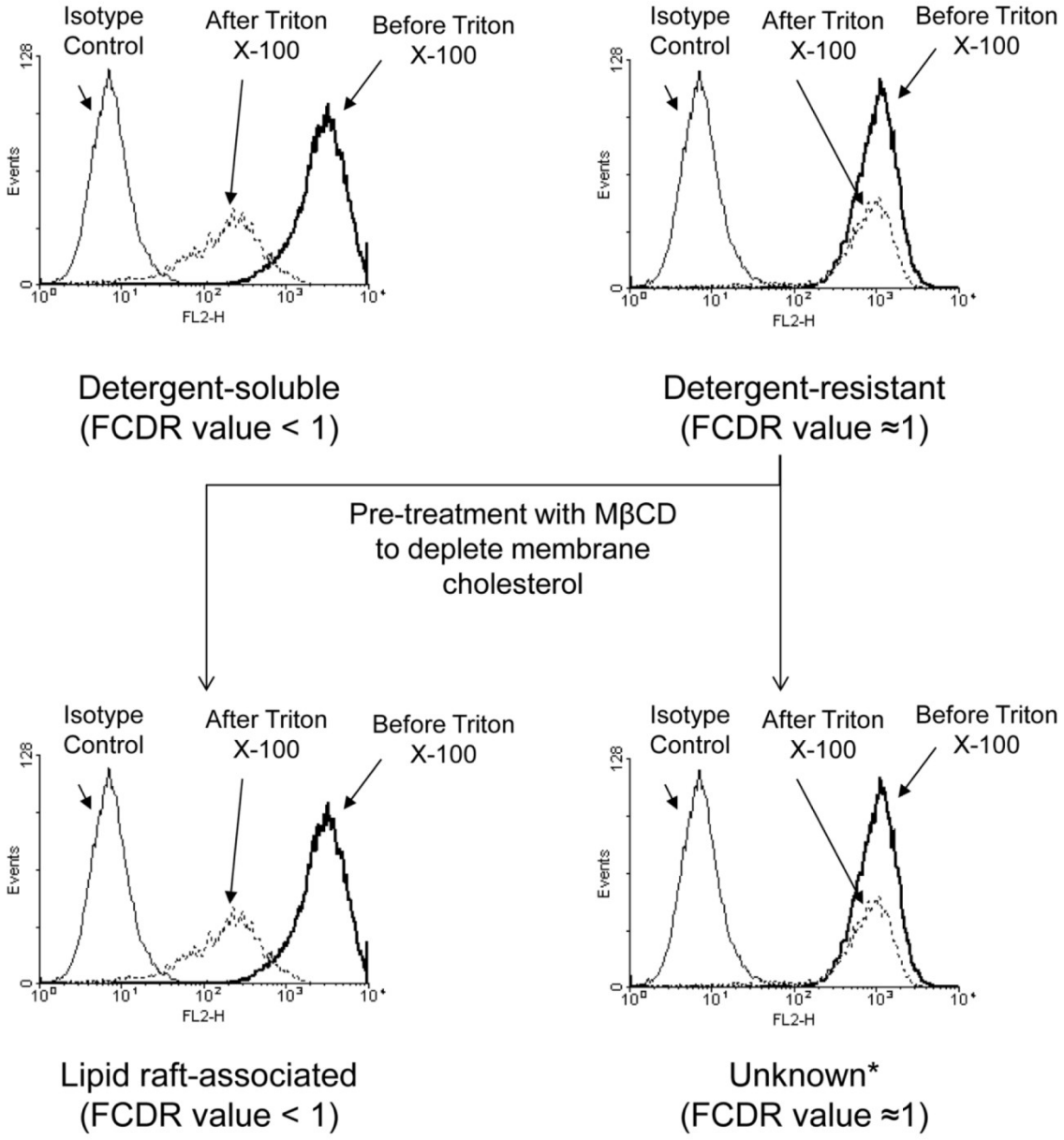
The FCDR protocol has been previously described in detail (1). Freshly harvested cells (5×10^5/test) were washed with RPMI media and incubated in media for 15 minutes at 37^{o}C. The cells were then washed with media followed by a wash with FACS buffer (PBS containing 0.2% FCS and 0.02% sodium azide). Cells were then labeled with the appropriate primary and secondary antibody as previously described for flow cytometry and analyzed using a BD FACSCalibur flow cytometer. After a baseline level of expression was acquired, cells were treated with 0.1% ice-cold Triton X-100 in PBS for 5 minutes on ice, followed by a second analysis of surface expression by flow cytometry. Analysis of flow cytometric data was performed using FlowJo 7.6 software.

Detergent resistance was quantified using the mean fluorescence intensity (MFI) values of the following fluorescence histograms as previously described (1): MFI of labeled untreated cells (MFI_{UT}), MFI of labeled cells treated with 0.1% Triton X-100 for 5 minutes (MFI_{TX}), MFI of isotype control untreated cells (MFI_{UTiso}), MFI of isotype control cells treated with 0.1% Triton X-100 for 5 minutes (MFI_{TXiso}). The extent of detergent resistance was calculated using the below equation:

$$\text{FCDR value} = (MFI_{TX} - MFI_{Txiso}) / (MFI_{UT} - MFI_{UTiso})$$

Therefore, the FCDR value is approximately less than 0.5 if a membrane protein is solubilized by Triton X-100 treatment and is approximately equal to 1 in the case of a typical detergent resistant protein. For a detergent resistant protein, pretreatment of the cells with 10mM MBCD in media for 15 minutes at 37^{o}C can provide insight into the cause of the detergent resistance. For example, a significant decrease in the FCDR value after cholesterol depletion with MBCD indicates that the observed detergent resistance is likely caused by the association of the membrane protein with lipid rafts. When a detergent resistant FCDR value does not significantly change after MBCD treatment, it indicates that factors other than lipid raft association are responsible for the observed detergent resistance. The possible outcomes of the FCDR assay and the interpretations are summarized in Figure G.1.

Figure G.1. Summary of potential outcomes and interpretation of the FCDR assay. Immunostaining of cells for a particular protein is first performed followed by analysis of surface expression by flow cytometry before and after treatment with Triton X-100. If surface expression substantially decreases after Triton X-100 treatment (upper left histogram), the protein is considered detergent soluble and likely not associated with membrane rafts. If expression is unaffected by Triton X-100 (upper right histogram), the protein is considered detergent resistant. The change in expression due to Triton X-100 treatment is quantified using the FCDR value, where a value markedly less than 1 indicates detergent-solubility and a value approximately equal to 1 indicates detergent-resistance. Once a protein is considered detergent resistant by this method, pretreatment of the cells in culture with MBCD to reduce membrane cholesterol before performing the FCDR assay can determine whether the observed detergent resistance is caused by association with membrane rafts. If cholesterol depletion with MBCD results in a decrease of the FCDR (bottom left histogram), this indicates that the observed detergent resistance is likely due to lipid raft association. If MBCD treatment has no effect on the FCDR (bottom right histogram), addition cellular factors other than raft association (i.e. cytoskeletal attachment) are most likely responsible for the observed detergent resistance.


Isotype Control
After Triton X-100
Before Triton X-100
Events
FL2-H
Detergent-soluble (FCDR value < 1)
Detergent-resistant (FCDR value ≈1)
Pre-treatment with MβCD to deplete membrane cholesterol
Lipid raft-associated (FCDR value < 1)
Unknown* (FCDR value ≈1)

G.2 Determination of detergent solubility of DRB1*04 epitopes using the FCDR assay

The association of DRB1*04 epitopes with lipid rafts was measured by their differential sensitivity to the nonionic detergent Triton X-100 using the FCDR assay as described above. The detergent resistance of CD59, CD71, and CD82 was first analyzed. As expected, CD71 on SAVC and MT14B cells displayed a high sensitivity to treatment with detergent, resulting in FCDR values of 0.27 and 0.25 for SAVC and MT14B respectively, indicating that CD71 was almost completely solubilized from the membrane with 0.1% Triton X-100 (Figure G.2). Similar results were observed in 8.1.6 0401 and 9.5.3 0401 cells (Table G.1). The tetraspanin CD82 also exhibited a high sensitivity to detergent treatment with FCDR values of 0.15 and 0.22 in SAVC and MT14B respectively, suggesting that this protein is not associated with membrane rafts in these cells (Figure G.2). In contrast, the raft marker CD59 was mostly detergent resistant in both SAVC and MT14B, with FCDR values of 0.73 and 0.88 respectively (Figure G.2). In addition, CD59 displayed detergent resistance in 8.1.6 0401 cells with a FCDR value of 0.76 (Table G.1). The detergent resistance of CD59 in 9.5.3 0401 was not performed as these cells were previously shown not to express this protein (Figure 4.4).

Since the detergent solubility of CD59 and CD71 corresponded to their known raft-association properties, the FCDR assay was used to analyze the detergent resistance of total DR and DRB1*04 epitopes. Total DRαβ dimers (L243), MHC-II/CLIP, and total DRB1*04 (NFLD.D1) were largely detergent soluble in SAVC, MT14B, 8.1.6 0401, and

Figure G.2. Total DR and D11-0401 are detergent soluble while D13-0404 is detergent resistant measured using the FCDR assay.

The association of membrane proteins with lipid rafts in SAVC and MT14B cells was analyzed by measuring detergent resistance using the FCDR assay. Surface expression of CD59, CD71, CD82, DRαβ (L243), class-II-CLIP, DRB1*04, and D11-0401 or D13-0404 on live SAVC and MT14B cells was measured before (thick histogram) and after (broken histogram) treatment with 0.1% Triton X-100 to determine the detergent resistance of this proteins. Isotype controls (thin histogram) were adjusted to a MFI equal to 10. The calculated FCDR value is shown to the right of each histogram.

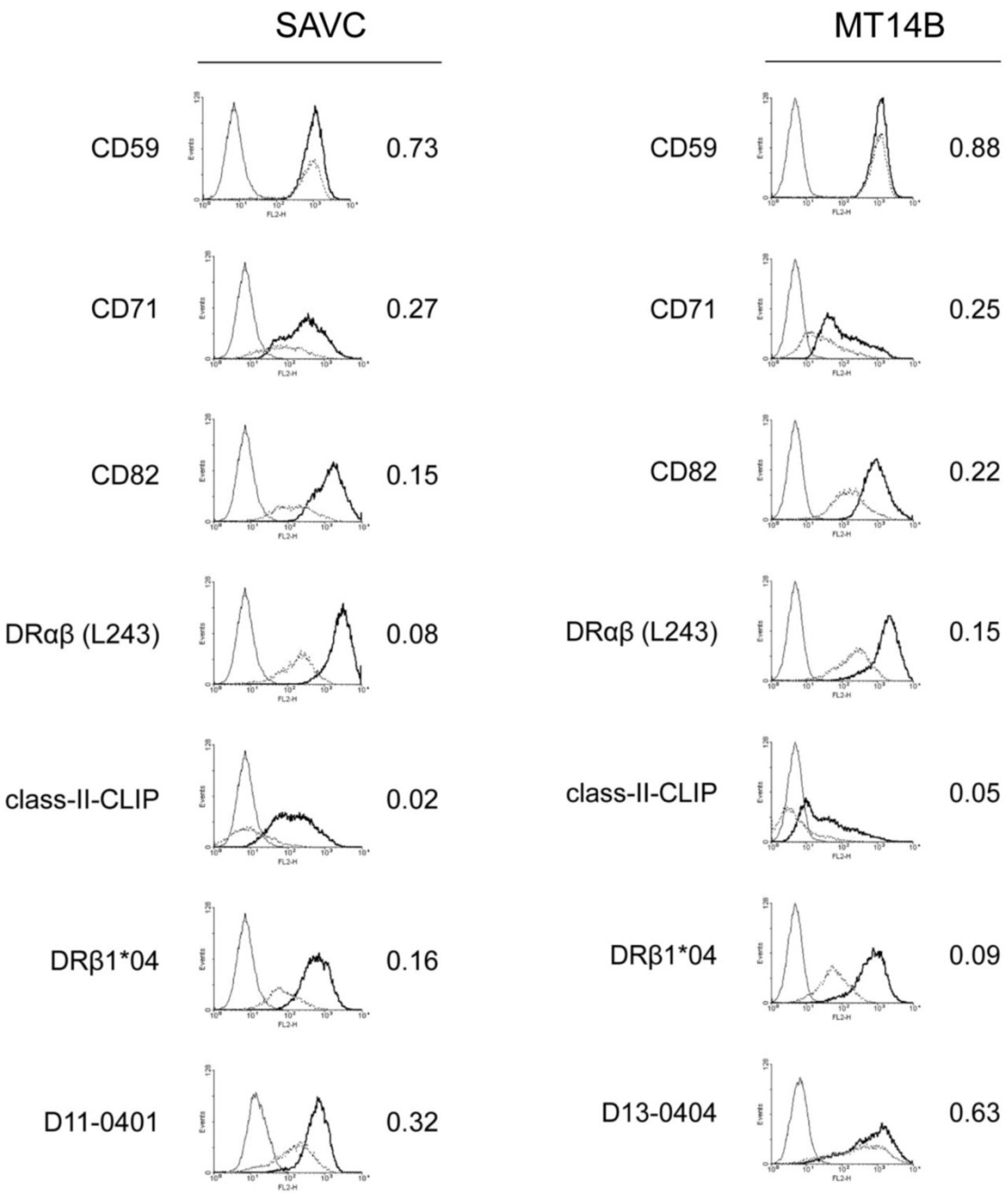

SAVC
MT14B
CD59
0.73
CD59
0.88
CD71
0.27
CD71
0.25
CD82
0.15
CD82
0.22
DRαβ (L243)
0.08
DRαβ (L243)
0.15
class-II-CLIP
0.02
class-II-CLIP
0.05
DRβ1*04
0.16
DRβ1*04
0.09
D11-0401
0.32
D13-0404
0.63

Table G.1. Summary of FCDR values for various molecules and DRB1*04 epitopes.

The association of membrane proteins with lipid rafts in 8.1.6 0401 and 9.5.3 0401 cells was analyzed by measuring detergent resistance using the FCDR assay. Surface expression of CD59, CD71, CD82, DRab (L243), class-II-CLIP, DRB1*04, D11-0401 and D13-0401 on live 8.1.6 0401 and 9.5.3 0401 cells was measured before and after treatment with 0.1% Triton X-100 to determine the detergent resistance of this proteins. The calculated FCDR values are displayed, along with those from SAVC and MT14B for comparison. NT, not tested.

	SAVC	MT14B	8.1.6 0401	9.5.3 0401
CD59	0.73	0.88	0.76	NT
CD71	0.27	0.25	0.30	0.37
CD82	0.15	0.22	NT	NT
DRαβ (L243)	0.08	0.15	0.07	0.05
class-II-CLIP	0.02	0.05	0.01	0.14
DRβ1*04	0.16	0.09	0.04	0.04
D11-0401	0.32	NT	0.13	NT
D13-0401	NT	NT	NT	0.43
D13-0404	NT	0.63	NT	NT

9.5.3 0401 cells, with FCDR values ranging from 0.01 to 0.16 (Figure G.2, Table G.1). Similarly, D11-0401 was found to be detergent soluble in SAVC and 8.1.6 0401 cells with FCDR values of 0.32 and 0.13 respectively (Figure G.2, Table G.1). D13-0401 in 9.5.3 0401 was also detergent soluble with an FCDR value of 0.43 (Table G.1). In contrast to the other DRB1*04 epitopes, D13-0404 exhibited partial detergent resistance in MT14B cells with an FCDR value of 0.63 (Figure G.2), suggesting that this molecule partially resides in lipid rafts.

In total, these FCDR results suggest that the majority of total DR, D11-0401, and D13-0401 are not associated with lipid rafts, while a portion of D13-0404 molecules may be raft-associated. These findings are not in agreement with the MBCD disruption and sucrose density gradient ultracentrifugation experiments, but instead suggest the exact opposite concerning the raft-association of these epitopes. Furthermore, it is well documented in the literature that a substantial fraction of total surface pMHC-II resides in lipid rafts (3). Given these discrepancies, it is apparent that further validation of this assay is required before its results can be correctly interpreted.

G.3. References

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