CHARACTERIZATION OF ANTIBODY-DEFINED EPITOPES ON HLA-DRB1*04 MOLECULES

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Characterization of Antibody-Defined Epitopes on HLA-DRB1*04 Molecules

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Thesis Abstract

We have previously identified antibody-defined epitopes on the HLA-DRB1*04 molecules that are associated with development of rheumatoid arthritis (RA). The expression of a NFLD.D11-defined epitope ($D11^+0401$) on B cell lines (BCL) is dependent on HLA-DM. NFLD.D13 recognizes DRB1*0404, but not DRB1*0401 in DM^+ BCL ($D13^+0404$). However, in DM^- BCL, NFLD.D13 recognizes an epitope on HLA-DRB1*0401 molecules ($D13^+0401$). It was speculated that these epitopes arise either through acquisition of peptides from differentiation antigens or through differential processing and acquisition of a peptide from a common protein. Initially, we show that these epitopes were not detected on the surface of synovial fibroblasts from RA patients, despite the up-regulation of DR, DM molecules and compartments necessary for class II antigen processing and presentation. Further investigation showed that the expression of the $D11^+0401$ and $D13^+0404$ epitopes was restricted to professional antigen presenting cells, with the exception of the melanoma cell MDA MD 435.0401, which expressed $D11^+0401$. To analyze the intracellular mechanisms by which the $D11^+0401$ and $D13^+0404$ epitopes are generated, we treated normal and DM^- BCL with various inhibitors, including brefeldin A and protease inhibitors, and employed confocal and immunoelectron microscopy to dissect the compartments within the endocytic pathway where these epitopes form. We found that both the $D11^+0401$ and $D13^+0404$ epitopes form within $CD63^+CD82^+$ lysosomal compartments in normal BCL. The $D11^+0401$ epitope requires cytoplasmic and endosomal cysteine proteases, whereas $D13^+0404$ epitope expression is protease independent. Both epitopes appear to require tetraspan

protein-enriched microdomains for proper surface expression. Interestingly, the $D13^{+}0401$ epitope also required cysteine proteases for its expression, suggesting that a unique set of peptides are responsible for the formation of these DRB1*04 epitopes.

Acknowledgments

I wish to especially thank my supervisor, Dr. Sheila Drover for giving me the opportunity to work on this project, and for her expert guidance and patience throughout its duration. Also, other members of the lab, including Sharon Oldford, Dianne Codner, Allison Edgecombe and Nicole Winter deserve special thanks for making working in Dr. Drover's lab enjoyable. Thanks also to the faculty, staff and students of the Immunology Department at Memorial University for help throughout the years. Finally I would like to thank Graduate Studies at Memorial University and the CIHR for financial support.

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List of Abbreviations.

APC	Antigen presenting cells
BCL	B cell lines
BSA	Bovine serum albumin
CLIP	Class II-associated invariant chain peptide
CM	Complete Media
DC	Dendritic cells
ER	Endoplasmic reticulum
EBV	Epstein - Barr virus
EAE	Experimental allergic encephalomyelitis
FCS	Fetal calf serum
cFLS	Fibroblast-like synoviocytes
GILT	Gamma-interferon-inducible lysosomal thiol reductase
GAM	Goat anti-mouse
HBSS	Hank's balanced salt solution
T_H	Helper T cells
HEL	Hen egg lysozyme
IMDM	Iscove's modified Dulbecco's medium
IFN-γ	Interferon-γ
Ii	Invariant chain
LPS	Lipopolysaccharide
MΦ	Macrophages
MHC	Major histocompatibility complex
MIIC	MHC class II compartments
mAb	Monoclonal antibody
MCC	Moth cytochrome c
MBP	Myelin basic protein
OA	Osteoarthritis
PFA	Paraformaldehyde
pMHC	Peptide/MHC
PBS	Phosphate buffered saline
PA	Psoriatic arthritis
RA	Rheumatoid arthritis
TBS	Tris-buffered saline
TCR	T cell receptor
TNF-α	Tumor necrosis factor alpha
SE	The shared epitope

Table 1. Human Cell Lines Used in Thesis (In order of appearance in the text)

Cell Line	HLA-DRB1 Type	Description
T2.Dw4	Transfected DRB1*0401	B-T cell hybrid that does not express its class II genes.
T2.Dw4.DM	Transfected DRB1*0401	B-T cell hybrid that does not express its class II genes.
SAVC	DRB1*0401	Normal EBV-transformed B cell line.
MT14	DRB1*0404	Normal EBV-transformed B cell line.
8.1.6 0401	Transfected DRB1*0401 Endogenous DRB1*0301	Normal EBV-transformed B cell line.
9.5.3 0401	Transfected DRB1*0401 Endogenous DRB1*0301	HLA-DM negative EBV-transformed B cell line.
5.2.4 0401	Transfected DRB1*0401 Endogenous DRB1*0301	HLA-DM, HLA-DO negative EBV-transformed B cell line.
5.2.4 0404	Transfected DRB1*0404 Endogenous DRB1*0301	HLA-DM, HLA-DO negative EBV-transformed B cell line.
Daudi.Dw4	Transfected DRB1*0401	HLA class I null Burkitt's lymphoma cell line.
Daudi.Dw4.B_2M	Transfected DRB1*0401	HLA class I null Burkitt's lymphoma cell line.
BLS-1.Dw4	Transfected DRB1*0401	HLA class II negative BCL
SJO.Dw4	Transfected DRB1*0401	HLA class II negative BCL
DAP3*	Transfected DRB1*0401	Mouse fibroblast cell line
MDA MB 435.Dw4	Endogenous DRB1*0405, 13 Transfected DRB1*0401	Breast carcinoma cell line
T47D.Dw4	Endogenous DRB1*0102 Transfected DRB1*0401	Breast carcinoma cell line
MCF-7.Dw4	Endogenous DRB1*03, 15 Transfected DRB1*0401	Breast carcinoma cell line
BT20	DRB1*0404	Breast carcinoma cell line

HT29	DRB1*0402, 07	Intestinal epithelial cell line
1359 Mel	DRB1*0401, 0301	Melanoma cell line
DM331	DRB1*0401, 01	Melanoma cell line
DM13	DRB1*0404	Melanoma cell line
SN35	DRB1*0404	Synovial fibroblast cell line
SN41	DRB1*0101, 07	Synovial fibroblast cell line
SN50	DRB1*0401, 1502	Synovial fibroblast cell line
SN08	DRB1*0401, 07	Synovial fibroblast cell line
SN07	DRB1*0402, 0101	Synovial fibroblast cell line

* Mouse Fibroblast cell line.

Table 2. Monoclonal Antibodies Used in this Thesis

Monoclonal Antibody	Species Reactivity	Specificity	Source
NFLD.D1	Human	Recognizes a non-polymorphic epitope on the β_2 chain of all HLA-DR4 molecules.	Local
NFLD.D2	Human	Recognizes the amino acid sequence of QKRAA/QRRAA on the HLA-DRB1 chain.	Local
NFLD.D10	Human	Binds to HLA-DR molecules that express and Q at position 70 of the HLA-DRβ chain.	Local
NFLD.D11	Human	Recognizes a HLA-DM dependent epitope on HLA-DRB1*0401 molecules in B cell lines.	Local
NFLD.D13	Human	Recognizes an epitope on DRB1*0404 molecules in HLA-DM positive B cell lines. Also recognizes an epitope on HLA-DRB1*0401 molecules in HLA-DM negative B cell lines.	Local
HB15e	Human	Anti-CD83	BD Pharmingen
HI149	Human	Anti-CD1a	BD Pharmingen
EBM11	Human	Anti-CD68	DAKO
L243	Human	pan-HLA-DR	ATCC
NFLD.M15	Human	Anti-HLA-B	Local
MaP.DM1	Human	Anti-HLA-DM	BD Pharmingen
LN2	Human	Recognizes class II associated invariant chain.	BD Pharmingen

cerCLIP	Human	Recognizes class II associated invariant chain peptides (CLIP) associated with class II molecules.	BD Pharmingen
M-A712	Human	Anti-CD71	Cedarlane
50F11	Human	Anti-CD82	BD Pharmingen
CLB-180	Human	Anti-CD63	Cedarlane
M5E2	Human	Anti-CD14	BD Pharmingen
B-ly4	Human	Anti-CD21	BD Pharmingen
M-L233	Human	Anto-CD23	BD Pharmingen
HA58	Human	Anti-CD54	BD Pharmingen
H4A3	Human	Anti-CD107a	BD Pharmingen
To5	Human	Anti-CD35	DAKO
PG-M1	Human	Anti-CD68	DAKO
F8/86	Human	Anti-factor VIII	DAKO
5B5	Human	Fibroblast marker	DAKO
DAL-1	Human	Anti-CD80	Cedarlane
BU63	Human	Anti-CD86	Cedarlane
EA-5	Human	Anti-CD40	BD Pharmingen
2B11	Human	Anti-CD45	BD Pharmingen
PL3	Human	Recognizes HLA-DRB1*07 and HLA-DRB4*	A kind gift from Dr. Susan Radka
SFR16	Human	Specific for HLA-DRB1*07	A kind gift from Dr. Susan Radka
DOB.L1	Human	Recognizes an epitope on the HLA-DOB chain	A kind gift from Dr. Anne Vogt

Thesis Co-authorship Statement

This manuscript-style thesis is presented as a total of five Chapters. Chapter 1 is a general introduction and Chapter 5 is an integrated discussion of results presented in Chapters 2, 3 and 4. The manuscripts presented in Chapters 2, 3 and 4 contain original work by David Spurrell under the supervision of Dr. Sheila Drover at Memorial University of Newfoundland. Each manuscript Chapter involves varying amounts of collaboration with other scientists within and outside Memorial University.

The work described in Chapter 2 represents the most collaborative effort. The original study was done with Dr. Bodil Larsen, who provided the synovial tissues from which the synovial fibroblasts were derived. Initial experiments with cultured synovial fibroblasts were done by Toby Frost and presented within his B.Sc.(Hons) thesis at Memorial University. The project was expanded with the help of other members of Dr. S. Drover's lab, including Sharon A. Oldford (immunohistochemical studies referred to within B.Sc.(Hons) thesis at Memorial University), Allison Edgecombe (RT-PCR) and Dianne Codner (cell culture and RT-PCR). The author is responsible for the majority of data presented in Chapter 2, including flow cytometry and immunocytochemical studies to characterize the synovial fibroblasts, kinetic studies, and manuscript preparation.

Chapter 2 manuscript is in press within *Human Immunology,* and is entitled:

(1) Spurrell D R, T Frost, B Larsen, S A Oldford, D Codner, A Edgecombe, and S Drover. 2004. Discordant Expression of HLA Class II Associated Chaperones and HLA-DRB Alleles in Cultured Fibroblast-like Synoviocytes.

The majority of the work described in Chapter 3, as well as manuscript preparation were done by the author. Exceptions include experiments shown in Figures 1 and 5, which were done by D. Codner and the experiment shown in Figure 8 which was done by D. Codner and N. Winter. This manuscript is in preparation for submission to *International Immunology* and is entitled:

Cellular Expression of HLA-DM Dependent and Independent HLA-DRB1*04 Epitopes.

The author also contributed Chapter 3-related data published in:

(2) Patil, N. S., F. C. Hall, S. Drover, D. R. Spurrell, E. Bos, A. P. Cope, G. Sonderstrup, and E. D. Mellins. 2001. Autoantigenic HCgp39 epitopes are presented by the HLA-DM-dependent presentation pathway in human B cells. *J.Immunol.* 166:33-41.

All experiments shown in Chapter 4, including data analysis and manuscript preparation were done entirely by the author, under the supervision of Dr. Sheila Drover.

1. Introduction

1.1 The Major Histocompatibility Complex

1.1.1 Genetic Organization

The major histocompatibility complex (MHC), a tightly linked cluster of genes involved with the adaptive and innate immune systems, is located on the short arm of chromosome 6 (band p21.3) in humans and on chromosome 17 in mice [1]. The human MHC genomic region, known as human leukocyte antigen (HLA) is 3.6 Mb in length with approximately 224 coding and non-coding sequences [1-4]. The HLA region comprises several loci that encode structurally homologous proteins which are classified into HLA class I (HLA-A, -B, and C) and class II (HLA-DR, -DQ, and DP) antigens in relation to their structure, tissue distribution and function. MHC molecules were originally identified because of their role in tissue rejection following transplantation, but upon further analysis their biological function was shown to be involved in presenting exogenous and endogenous peptides to T cells [5-8]. The hallmark of MHC molecules is their allelic polymorphism, which increases the total chance of an immune response against a given microorganism. This way at least a subset of individuals within a given population will respond to the microbe, ultimately increasing the total population's chance of survival against infection.

Based on the search for HLA compatibility required for proper tissue transplantation between individuals, the large polymorphism amongst HLA molecules was first detected by serology, using typing reagents derived from the sera of multiparous women, or blood donors that had received multiple blood transfusions. More recently,

DNA based typing techniques have further advanced our knowledge of the extensive diversity of the HLA complex. For example, at the time of writing, according to the European Bioinformatics Institute (EMBL-EBI-www.ebi.ac.uk/imgt/hla) the 100 serologically-defined HLA-A, -B, -C, -DR, -DQ and –DP specificities now comprise more than 1700 alleles defined at the DNA level.

In humans the MHC is organized into three distinct, non-overlapping gene-rich regions that are designated from centromere to telomere as class II, III and I regions (Figure 1). In addition to the six major groups of loci mentioned above, the MHC is distinguished from other regions of the human genome by the high degree of gene density, with about 40% of the genes found within the MHC encoding for proteins with immunological functions [9]. There are several functional categories of MHC gene products that are expressed, including those involved with antigen processing and presentation (HLA-DM, HLA-DO, LMP2, LMP7, and TAP), those involved in innate immunity, inflammation and immune regulation, such as the complement components C2 and C4, TNF and HSP70, as well as genes whose function is other than the immune response (Fig 1).

The HLA class I region, found on the telomeric end of the MHC region is 1.8 Mb and encodes for 18 HLA class I genes (6 coding and 12 pseudogenes) and several non-HLA related genes whose functions range from cell growth, DNA replication and repair and regulation of transcription [9]. The function of many of the genes within the HLA class I region is still unknown. As mentioned above, this region includes genes that encode for the heavy chains of the classical class I molecules HLA-A, B and C.

Currently, there are 303 known HLA-A alleles, 609 known HLA-B alleles and 150 known HLA-C alleles [EMBL-EBI]. The HLA class I heavy chain, also known as the α chain, pairs with a smaller molecule known as β_2-microglobulin (β_2M), encoded on chromosome 15, to form membrane glycoproteins found on the surface of almost all nucleated cells [4]. These molecules function by presenting short peptide antigens which are mainly derived from endogenous proteins, to $CD8^+$ T lymphocytes [4]. The HLA class I region also encodes for non-classical MHC molecules, also known as class Ib molecules, such as HLA-G, -F and –E. These molecules, which are structurally similar to classical HLA class I molecules, also function by presenting peptides to $CD8^+$ T cells and have been suggested to have a role in immune recognition [10].

(a)

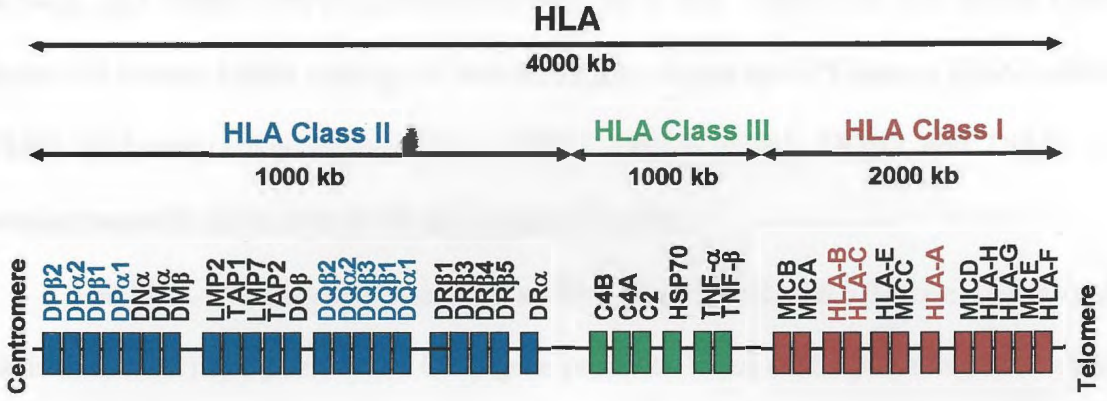

Figure 1. Diagram of the genomic map of the human HLA region located on chromosome 6, spanning 4000 kb. The HLA class I genetic region is shown in red, the HLA class II is shown in blue, and the HLA class III region is shown in green. (Adapted from Kuby Immunology, 4th edition, © W.H Freeman and Company).

The HLA class II region is located on the centromeric end of the MHC and covers about 0.7 Mb, encoding for 19 HLA class II genes (11 coding and 8 pseudogenes). This region includes genes that encode for the classical MHC class II molecules HLA-DP, DQ and DR in addition to genes that encode for proteins with a variety of immunological functions, including HLA-DM and HLA-DO, two non-classical HLA class II molecules involved in antigen processing and presentation (Fig 1). HLA-DP and HLA-DQ proteins

are formed from the pairing of either the DPA1 and DPB1 or DQA1 and DQB1 gene products, and exhibit less polymorphisms then HLA-DR. There are 108 known DPB1 alleles, 20 known DPA1 alleles, 56 known DQB1 alleles and 25 known DQA1 alleles [EMBL-EBI-www.ebi.ac.uk/imgt/hla]. DPA2, DPA3, DPB2, DQB2 and DQA2 are pseudogenes also found within the HLA class II region.

The HLA-DR region encodes for a DRA gene, which has 3 known alleles (only one is functional) and pairs with a DRB gene product. Much of the polymorphism within HLA-DR molecules stems from the DRB1 gene, for which there are approximately 363 known alleles [EMBL-EBI-www.ebi.ac.uk/imgt/hla] at time of writing. Another reason for the increased complexity of the HLA-DR region is due to the fact that a second DRB gene may be expressed, depending on the particular MHC haplotype. All individual haplotypes contain, with the exception of DRB1*01, *10 and *08, a second DRB allele in addition to the DRB1 allele. For example, DRB3 (DR52) is expressed in individuals that are DRB1*11, *12, *13, *14, *17 or *18; DRB4 (DR53) is expressed in individuals that are DRB1*4, *7 or *9; and DRB5 (DR51) is expressed in DRB1*15 and *16 individuals. This further increases the number of HLA-DR molecules that can be expressed on the cell surface as the DRα chain can pair with either of the two DRβ chains [8].

This complexity is reflected in the changes in nomenclature for the HLA over the past 20 years. International collaboration has allowed the definition and official designation of all HLA specificities (serology) and alleles (DNA), providing a universally accepted system. This is necessary since most of the serologically defined specificities can be subdivided into many continually growing allelic subtypes. For example,

serotypically two people can both be DR4, but contain different DR4 alleles (i.e. ranging from HLA-DRB1*0401 through to HLA-DRB1*0438). Despite this diversity, all HLA-DR molecules are formed from a DRα chain and a DRβ chain, which form heterodimeric, membrane glycoproteins. These molecules are found constitutively on antigen presenting cells (APC) such as B cells, macrophages (MΦ) and dendritic cells (DC), and can be up-regulated on other cell types where they function by presenting peptides derived from exogenous antigens to $CD4^+$ T lymphocytes.

The MHC class III region is located centrally between the class I and class II regions. This region is the most gene rich within the human genome, with 62 genes containing more than 500 exons over 706 kb [4]. Although this region does not contain classical HLA class I or class II-like molecules, HLA class III genes do encode for proteins that have a variety of immune functions such as the inflammatory mediators tumor necrosis factor-α (TNF-α), heat shock protein 70 as well as components of the complement system, such as C4A.

1.1.2 General Structure of HLA Class I and Class II Molecules

HLA class I and class II molecules are closely related membrane bound glycoproteins that bind short antigenic peptides and present them to CD8+ and CD4+ T cells respectively. Class I molecules (HLA-A, B and C) consist of a large polymorphic α chain (45 kD) associated with a smaller β_2-microglobulin molecule (12 kD) (Figure 2). The α chain can be further divided into three smaller domains known as α1, α2, and α3, along with a transmembrane domain and a cytoplasmic domain. The α1 and α2 domains

form a peptide binding groove, which consists of eight antiparallel β strands on the bottom and two α-helical regions on the sides. This structure is then able to accommodate an appropriate antigenic peptide in its groove. Since the peptide binding groove is closed at both ends, class I molecules require peptides between 8 and 10 amino acids in length [4, 8]. The α3 has an immunoglobulin-like domain and associates non-covalently with β_2-microglobulin. The CD8 molecule on the T cell contacts the α2 and α3 domains of the HLA class I molecule, as well as having some contact with β_2-microglobulin.

HLA class II molecules (HLA-DP, DQ and DR) have a similar basic structure, although the class II heterodimer consists of a 33-35 kD α-subunit combined with a 25-30 kD β-subunit [8]. Both the α and the β subunits contain two domains. The α2 and β2 domains are closest to the cell membrane and like the class I α3 domain, have homology to the immunoglobulin fold structure. The membrane-distal α1 and β1 domains form a large peptide binding groove, similar to that seen in the class I molecules. However, unlike class I molecules, the peptide binding groove in class II molecules is open at both ends, and can therefore accommodate slightly longer peptides consisting of 13-25 amino acids [8]. The CD4 molecule on the T cell contacts the HLA class II molecule via the β2 domain. The allelic polymorphisms in HLA-DR molecules are located primarily in the β1 domain, whereas polymorphism in HLA-DQ and HLA-DP molecules are located in both the α1 and β1 chains.

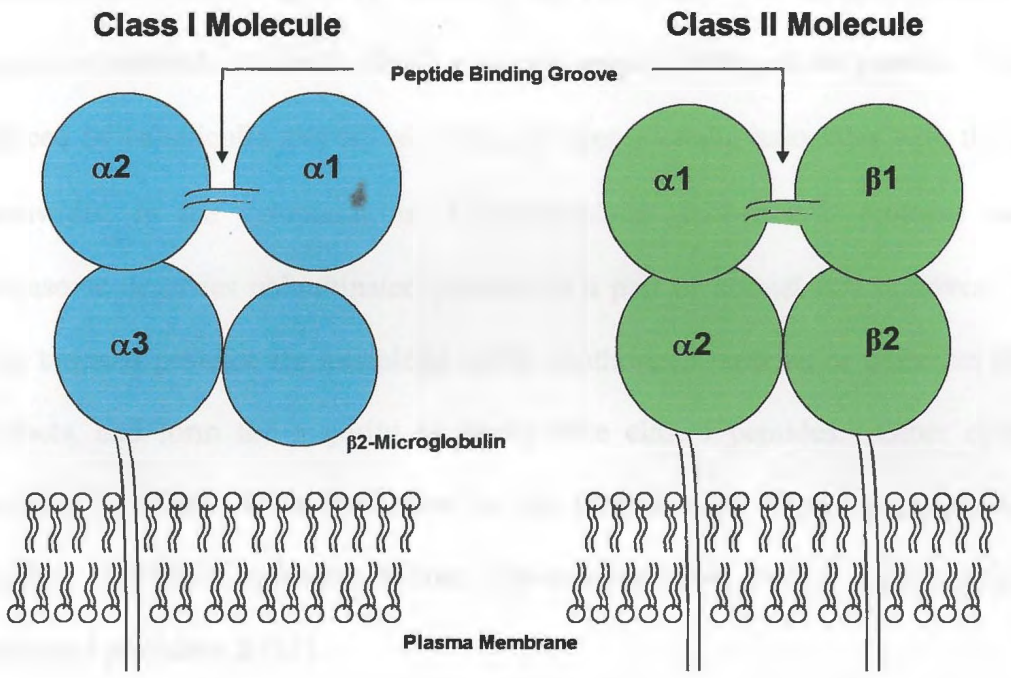

Figure 2. Diagram of the structure of HLA class I and class II molecules. Both molecules contain external domains, transmembrane segments, cytoplasmic tails and a peptide binding cleft which is formed by the α2 and α3 domains for HLA class I and the α1 and β1 domains for HLA class II. (Adapted from Kuby Immunology, 4th edition, © W.H Freeman and Company).

1.1.3 Generation of Cell Surface HLA Class I and II Molecules

1.1.3.1 HLA Class I

HLA class I molecules present peptides, derived mostly from intracellular polypeptides, to $CD8^+$ T lymphocytes. The assembly of mature, peptide-bound class I molecules occurs in the endoplasmic reticulum (ER) and involves several chaperone and

accessory molecules (Figure 3). Initially, the HLA class I α chain associates with the chaperone molecule calnexin, which promotes proper folding of the protein. Calnexin is replaced by calreticulin and tapasin when β_2-microglobulin associates with the α chain. Meanwhile, in the cytoplasm an ATP-dependent multi-subunit protease called the proteasome degrades ubiquitinated proteins as a part of normal cell turnover. Most of these targeted proteins are misfolded newly synthesized proteins or defective ribosomal products, and form the majority of steady-state class I peptides. Other cytoplasmic proteases have also been implicated in the formation of class I peptides, including calpains, bleomycin hydrolase, thimet oligoendopeptidase, leucine aminopeptidase, and tripeptidyl peptidase II [11].

Peptides of the appropriate length (8-11 amino acids) are then transported from the cytoplasm to the ER by a heterodimeric protein known as transporters associated with antigen processing (TAP). This process requires the hydrolysis of ATP and preferentially favors the transport of peptides with either hydrophobic or basic carboxyl-terminal amino acids, which are preferred as anchor residues for class I molecules. The complex of the class I α chain, β_2-microglobulin and calreticulin are brought into close proximity to TAP by tapasin, forming the class I peptide-loading complex. The close physical association of the molecules in this complex favors the binding of appropriate peptides to the class I molecule. After stable binding, the MHC/peptide complex is transported from the ER through the Golgi complex to the plasma membrane through small vesicles [8].

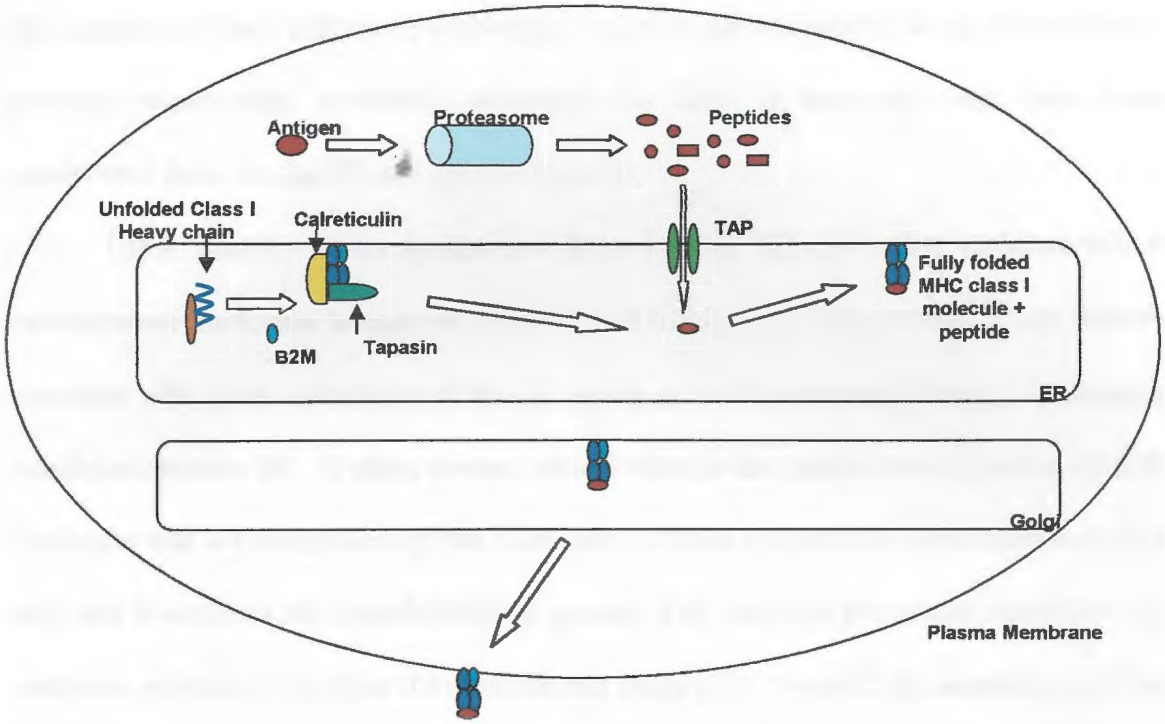

Figure 3. Overview of the HLA class I antigen presentation pathway. First, Ubiquitinated proteins are degraded by the multi-subunit proteasome to generate individual peptides. These peptides are then transported into the ER by the TAP complex. Meanwhile HLA class I molecules are associate with various chaperone molecules until loaded with the appropriate peptides (Adapted from Kloetzel, Nature Reviews Molecular and Cellular Biology, 2001, **2**:179-186. © Nature Publishing Group, Macmillan Publishers).

1.1.3.2 HLA Class II

HLA class II molecules are expressed constitutively on specialized professional APC such as B cells, macrophages (MΦ) and dendritic cells (DC). In the presence of inflammatory mediators such as interferon-γ (IFN-γ), expression of these molecules can be induced on the surface of a wide variety of cell types collectively known as non-

professional APC [8]. The formation of HLA class II/peptide complexes is the result of the encounter of two trafficking pathways. Antigens are transported along the endocytic pathway where they eventually encounter the class II molecules that have been transported there via the ER and golgi (Figure 4).

Class II molecules are synthesized *de novo* in the ER where they associate with a co-chaperone molecule known as the invariant chain (Ii). Three class II αβ dimers associate with three molecules of the Ii, which is a non-polymorphic type II integral membrane protein [8]. Ii plays several critical roles in the production of mature class II molecules that are recognized by the TCR; first, Ii binds to the class II molecule in such a way that it occupies the peptide-binding groove. This prevents premature association of irrelevant peptides with class II in the ER and golgi [12]. Second, the association of the Ii with newly synthesized class II molecules ensures that the molecular complex is targeted to the endocytic pathway due to a double leucine motif in the cytoplasmic tail of the Ii [12]. Because of this, class II/Ii complexes can leave the ER and travel to the Golgi apparatus, towards intracellular vesicles within the endocytic pathway, with the majority finally accumulating within the low pH environment of late endosomal (multivesicular structure) and lysosomal (multilaminar structure) compartments collectively known as MHC class II compartments (MIIC) [13-15]. This ensures that class II molecules interact with antigenic peptides that are derived from proteins degraded in the endocytic vesicles. This process is not 100% efficient though, and a fraction of class II/Ii complexes are delivered to the plasma membrane directly. These complexes are believed to recycle through early endosomes where they can encounter antigenic peptides [16].

HLA class II molecules present peptides derived mainly from the exogenous processing pathway. More specifically, the protein source for class II peptides are generally proteins that have been taken up by the cell via clathrin-dependent receptor-mediated endocytosis, clathrin-independent macropinocytosis or receptor-mediated phagocytosis [8]. The antigen then enters the endocytic pathway and is transported along a series of compartments from early endosomes (pH 5.5-6.5), to late endosomes (pH 4.5-5.5), and finally to lysosomes and MIIC (pH 3.0-4.0) [15]. Within these low pH compartments the antigen is unfolded and degraded by a variety of proteases. Further analyses of natural MHC class II ligands have shown that most class II-associated peptides are derived from cellular proteins, predominantly from proteins expressed on the plasma membrane [17], such as the transferrin receptor, which recycles constantly through the early endosomes from the plasma membrane, Na^+K^+ATPase and the IL-3 receptor [17]. Peptides derived from MHC class I molecules are also abundantly expressed in the peptide binding groove of MHC class II molecules [17]. Finally, a considerable portion of class II peptide ligands originate from proteins found within the endosomal and lysosomal compartments, with the majority of them derived from the invariant chain [17]. Peptides derived from lysosomal proteases, such as cathepsin H and cathepsin E have also been observed [Reviewed in 17].

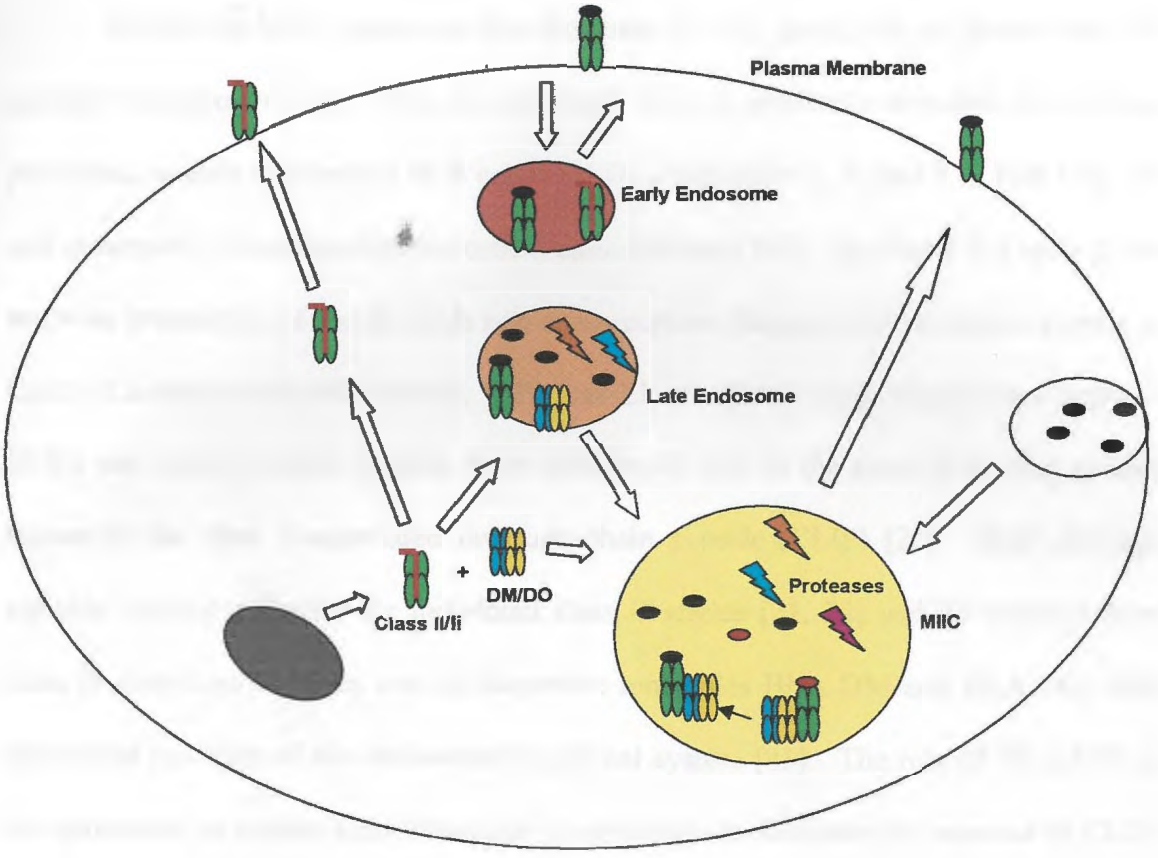

Figure 4. The HLA class II antigen processing and presentation pathway. HLA class II molecules associate with the Ii and are transported either to the late endosomes or to the MHC class II compartments (MIIC). Here the Ii is degraded and CLIP is removed by HLA-DM, being replaced by antigenic peptides that are formed from the breakdown of exogenous proteins. These complexes are then transported to the cell surface in order to interact with CD4+ T lymphocytes. Alternatively, Class II/Ii complexes can be transported directly to the cell surface where they can recycle through early endosomal compartments. The relative pH levels of each of the intracellular compartments in the endocytic pathway are represented by the appropriate litmus color. (Adapted from van Ham *et al*, Immunogenetics 2000, **51(10)**:765-70. © Springer-Verlag Heidelberg).

Within the MIIC numerous key processes for the generation of proper class II-peptide complexes occur. First, the invariant chain is gradually degraded by cysteine proteases, notably cathepsin S in B cells and DC, cathepsins L, S and F in MΦ [18, 19] and cathepsin L in murine thymic cortical epithelial cells [18]. As shown in Figure 5, the stepwise proteolysis of the Ii yields numerous peptide fragment intermediates known as Lip23 (Leupeptin-induced peptide; LIP) and Lip10 (Small leupeptin-induced peptide; SLIP) and finally a short peptide from residues 88-102 in the class II binding groove known as the class II-associated invariant chain peptide (CLIP) [20]. CLIP displays variable binding affinities for individual class II alleles [21, 22] and its removal from class II molecules involves two co-chaperone molecules HLA-DM and HLA-DO, both permanent residents of the endosomal/lysosomal system [23]. The role of HLA-DM in the generation of mature class II/peptide complexes is to facilitate the removal of CLIP, while promoting the binding of more stably binding peptides [23-27]. HLA-DM can also remove peptides that bind to class II with low affinity, thereby selecting peptides that bind to the class II molecules with higher affinity. This process seems to be due to conformational changes in the class II-DM complex, which is different in the presence of low and high affinity peptides. As a co-chaperone molecule, HLA-DM also stabilizes the peptide-free conformation of the HLA class II molecule, preventing irreversible damage in the low pH environment of the MIIC.

The role of HLA-DO is more controversial. It associates with HLA-DM in heterodimeric complexes within the endocytic pathway and is expressed mainly in B cells. Although it is clear that HLA-DO modifies the peptide exchange activity of HLA-

DM, the physiological relevance of the DM-DO association is still unclear [24]. The influence of DO on DM activity appears to be pH-dependent, such that DO inhibits the activity of DM at pH above 5.5 [23, 28]. Kinetic analysis has shown that DM-DO complexes bind more tightly to class II molecules compared to DM alone, suggesting another potential mechanism of DO function.

Investigation of pMHC complexes within the endocytic pathway and on the cell surface has revealed that the interaction between APC and T cell results in a protein reorganization on the surface of both cells known as the immunological synapse. MHC molecules can be incorporated into two types of membrane microdomains; cholesterol and glycosphingolipid-enriched domains, known as lipid rafts, and microdomains made up of 10-30 tetraspan proteins such as CD9, CD63, CD81, and CD82 [15-16]. Lipid raft microdomains are smaller in size compared to tetraspan microdomains, and differ from other membrane areas in their high cholesterol and high lipid to protein content. Tetraspan microdomains, defined by the B cell activation marker CDw78 may contain up to 30 proteins, including non-tetraspan proteins such as HLA-DM. These microdomains have been shown to contain class II molecules that are enriched with particular peptides, probably due to the presence of DM [15]. Although lipid rafts and tetraspan microdomains are distinct structures, they may exist together or separately on the cell surface, even though both function to concentrate relevant pMHC complexes for interaction with TCR on the surface of T cells.

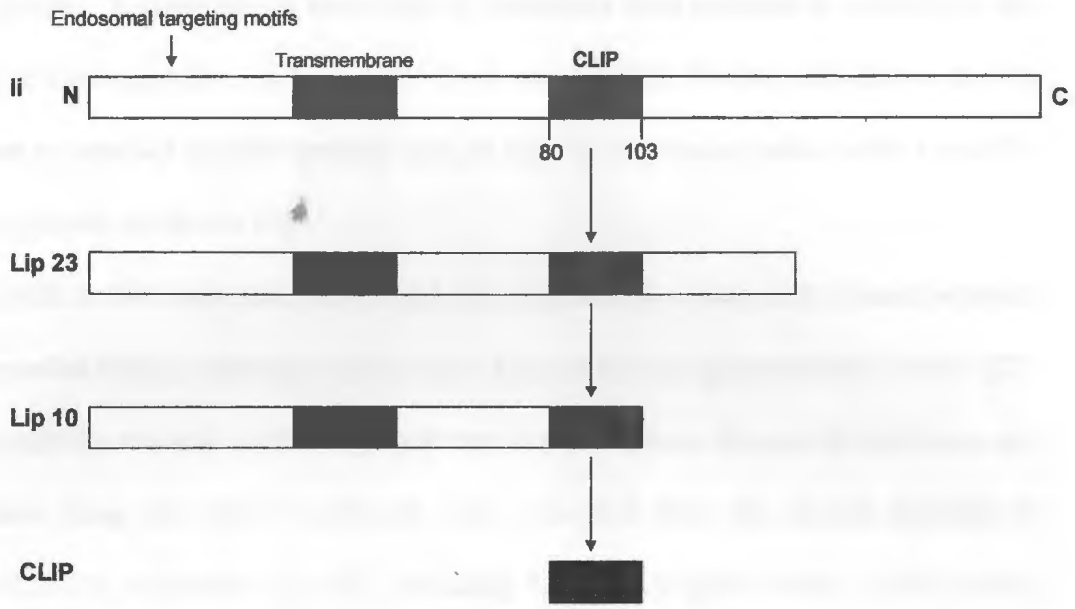

Figure 5. Diagram of the stepwise breakdown of the invariant chain (Ii). Full-length Ii is shown at the top, followed by the initial breakdown product, Lip23 (LIP), which is further degraded into Lip10 (SLIP) and finally into CLIP. (Adapted from Riese and Chapman, 2000; Current Opinion in Immunology **12**:107-113. © Elservier Science Ltd).

1.1.4 HLA-DR Structure and Peptide Binding

As stated above, the primary function of HLA molecules is to bind short peptides and present them to T cells. This interaction will influence the selection of the peripheral T cell repertoire as well as the specific T cell response to certain antigens. The binding capacity of any given peptide to a MHC class II molecule is dependent on the primary sequence of the peptide and on the allelic variation of the residues within the peptide

binding groove. Knowledge of how class II molecules bind peptides in addition to the sequence of these peptides has been aided by *in vitro* peptide binding and elution studies in addition to detailed crystallographic data of class II molecules bound with a specific peptide [reviewed in 29 and 30].

For HLA-DR molecules, the peptide is bound within a long cleft formed between two antiparallel helical structures and a floor formed by an eight-stranded β-sheet [27-29]. The peptides are held in place by hydrogen bonds between the peptide backbone and the residues along the class II molecule itself. As seen from the crystal structure of several HLA-DR molecules [31-33], including HLA-DR4 (DRA*0101, DRB1*0401) complexed with a human collagen II peptide [31], class II molecules bind peptides of varying lengths, but each peptide contains a core binding region of 7-10 amino acid residues, designated P1 through P10. Bound peptide residues that project downwards into the peptide binding groove are accommodated by allele-specific pockets found within the class II molecule. It is the class II pocket shape and composition that determines the amino acid make-up of the peptides that can bind to the class II molecules, and these qualities differ greatly amongst HLA-DRB alleles.

From the crystal structures of several HLA-DR molecules associated with known peptides, it has been observed that most MHC class II molecules have 4 major binding pockets in their peptide binding groove [31-33]. The majority of the HLA-DR allele-specific polymorphic residues are located on the surface of the pockets, and this selects for a distinct peptide repertoire amongst different HLA-DR molecules due to the amino acid composition and the electrostatic charge of the individual amino acids. Pocket 1 is

made up from DRβ chain amino acids 85, 86, 89 and 90; pocket 4 contains β-chain amino acids 13, 44, 70, 71 and 78; pocket 6 is formed from β chain amino acids 11 and 13; pocket 7 is formed from β chain amino acids 28, 47, 61, 67 and 71, and pocket 9 is formed from β chain amino acids 9 and 57 [32]. Both non-polymorphic and polymorphic residues contribute to peptide binding though, with non-polymorphic residues binding to main chain atoms of the bound peptide and polymorphic residues within the different pockets binding to the peptide side chains [29].

Analysis of the sequence motifs of peptides that bind to HLA-DR molecules revealed that as many as 80% of the peptides show an enrichment of aromatic residues near the amino terminus [17, 34, 35]. This amino acid residue at position 1, known as P1 is the general anchor residue. P4 is known as the second general anchor residue, and aligned peptide pools from class II molecules show an enrichment of hydrophobic residues [34]. The third anchor residue is at P6, which is generally occupied by small amino acids such as alanine and glycine for DRB1*0101 or threonine and serine for DRB1*0401 [34]. P6 also has an important role in determining allelic specificity of peptide binding to HLA-DR molecules. P9 also forms an anchor residue which is directed inwards towards the class II peptide binding groove pockets. Therefore amino acids residues at peptide positions P1, P4, P6 and P9 occupy the class II binding cleft, whereas positions P2, P3, P5, P7 and P8 face away from the cleft, and are available for interaction with the T cell receptor. In fact, amino acid residues P4-P8 are the most diverse, mainly because they interact with HLA-DR molecules in areas with the highest degree of polymorphism [35]. Of these positions, the P5 environment is the most

polymorphic and contains the particularly variable positions β11, β13, β30, β71 and β74 of the DRB1 chain. P5, along with P7 have been suggested to play an important role in T cell recognition [35].

Promiscuity is a common feature of peptides bound to HLA class II molecules, meaning that certain peptides are able to bind several or more DR alleles. It is thought that this promiscuous binding is because the HLA-DR molecule has both conserved and allele-specific residues in the peptide binding groove for the putative peptide to interact with. Since HLA class II molecules exhibit both allele-specific and non-specific residues, they are able to bind a large range of peptides in both a promiscuous and allele-specific manner. A good example of peptides that bind HLA class II molecules in a promiscuous manner are the invariant chain-derived peptides which bind at variable abilities to different class II alleles, since these peptides often exhibit mismatches to the allele-specific residues in the peptide binding groove.

1.1.5 Proteases Involved in the Generation of HLA Class II-peptide Ligands

As outlined in section 1.3.2, cysteine proteases are responsible for the degradation of Ii in various cell types. This makes HLA class II molecules receptive for interaction with HLA-DM, which catalytically removes CLIP. Various proteases found within the endocytic pathway are also responsible for degrading protein antigens into short peptides. If these peptides contain the correct structural motifs, they should bind the relevant class II molecules. Generation of class II peptide ligands within the endocytic pathway is thought to be mainly due to cathepsins, which are proteases that are optimally active at

acidic pH [20, 36, and 37]. The main categories of cathepsins include cysteine, aspartyl and serine proteases, depending on their substrate specificity. In APC the most prominent endocytic cysteine proteases are cathepsin B, S and L, whereas the most common endocytic aspartyl proteases are cathepsin D and E [20].

The breakdown of a particular antigen does not involve one single protease at a time, but requires the interplay of the diverse group of endosomal proteases. For example, cathepsins D, E, L and S all have potent endoprotease activities and may therefore be important in revealing and releasing antigens. Cathepsins B and H have weak endoprotease activities, but do have potent carboxypeptidase and aminopeptidase activities, respectively [38]. The involvement of a diverse group of proteases implies that the loss of a particular protease might be tolerated by an organism. This, in fact, has been shown in mice deficient for either cathepsin B, D or L [18, 39]. These mice have normal T cell development, both in cell numbers and diversity of the T cell response, indicating the dispensability of individual proteases in class II peptide ligand generation [18, 39]. Recent evidence has suggested that class II peptide ligands can also be generated by proteases that are active at neutral pH, namely the proteasome and calcium dependent cytoplasmic cysteine proteases collectively known as calpains [40].

Once loaded with an appropriate peptide ligand, the class II-peptide complex travels to the cell surface, either through direct fusion of the MIIC with the plasma membrane, or through a series of smaller transport vesicles.

1.1.6 HLA-DRB1*04 Molecules and Rheumatoid Arthritis

Because of their role in presenting peptides to T cells via the T cell receptor (TCR), and since various HLA class II alleles have been associated with autoimmune disease in humans it has been hypothesized that HLA class II molecules play an important role in the development of autoimmune disease [41, 42]. For example, HLA-DQB1*0302 and HLA-DQB1*0201 are associated with autoimmune type 1 diabetes and Celiac disease. HLA-DR molecules are associated with diseases such as autoimmune hepatitis, multiple sclerosis and rheumatoid arthritis (RA) [43]. All RA-susceptible alleles possess a homologous short stretch of amino acids (LLEQ(K/R)RAA) at positions 67-74 of the β1 chain, which is often referred to as the shared epitope [41]. In Caucasian populations, the major HLA-DR alleles that are associated with increased risk to RA are DRB1*0401 and DRB1*0404, which differ in the shared epitope region, at positions β71 (Lys to Arg) and β86 (Gly to Val).

Studies of Mediterranean and Jewish populations showed that although DRB1*0402 was the most common DR4 allele, it was not associated with RA. Instead, DRB1*01 and *10 were found to be associated with RA [42-44]. Amino acid sequencing of these β chains showed that DRB1*01 and *10 carried the shared epitope, whereas B1*0402 differed conservatively from B1*0401 and B1*0404 by negatively charged residues at positions β70D and β71E. Interestingly, HLA-DRB1*0402 confers resistance to the development of RA. The amino acids at positions 67, 70 and 71 which line pocket 4 appear to be particularly important in disease progression. For example, HLA-DRB1*0401 molecules prefer a negatively charged residue to bind in pocket 4 due to a

lysine at position β71. In contrast, HLA-DRB1*0402 molecules have negatively charged residues (β70-D, β71-E) surrounding pocket 4, causing selection of peptides that have a positively charged residue at this position. Positions β70 and β71 have also been shown to be important for interacting with the TCR. Therefore it is possible that HLA molecules that carry the shared epitope may select a particular population of T cells in the thymus that lead to RA disease progression [43, 44].

Several mAbs that are specific for various epitopes on HLA-DRB1*04 molecules were produced and characterized by Drover and Marshall [45-51]. They include NFLD.D1, a mAb that recognizes an epitope on the β_2 chain of all HLA-DR4 molecules [48] and mAbs that recognize the shared epitope. NFLD.D2 recognizes the amino acid sequence of QKRAA/QRRAA on the DRB1 chain [48] and NFLD.D10 binds to HLA-DR molecules that express and Q at position 70 of the β chain [49], with the exception of HLA-DRB1*03 and DRB3 products. These mAbs have been useful in studying the expression of HLA-DR4 and the shared epitope on a variety of P-APC cell types, including HLA-DM negative mutants and NP-APC including synovial fibroblasts isolated from patients that have RA.

Two other mAbs, whose epitopes are the main focus of this thesis, are NFLD.D11 and NFLD.D13. NFLD.D11 was previously shown to bind DRB1*0401 molecules on EBV-transformed B cell lines [47, 50, 51]. In addition to binding strongly to B cell lines, NFLD.D11 was also shown to bind weakly to peripheral blood B cells from normal controls and RA patients [47] The formation of this epitope is dependent on the presence of the co-chaperone molecule HLA-DM [50 and Chapter 3], as B cells that do not express

HLA-DM fail to express the NFLD.D11 epitope [50, 51]. NFLD.D13 has an intriguing specificity. It recognizes DRB1*0404, but not DRB1*0401 in HLA-DM positive EBV-transformed B cell lines. However, in B cells that do not express HLA-DM, NFLD.D13 recognizes an epitope on HLA-DRB1*0401 molecules [51 and Chapter 3]. Both the NFLD.D11 epitope on DRB1*0401 molecules and the NFLD.D13 epitope on DRB1*0404 molecules are also expressed on activated macrophages, but not on a wide variety of NP-APC, suggesting that an activated phenotype is necessary for their formation. This may be due to specific proteases or antigen processing compartments present only in P-APC.

The critical role of HLA-DM in the formation of these DRB1*04 epitopes suggests that a different set of peptides may be involved in their generation. Alternatively, since the NFLD.D11 and NFLD.D13 epitopes are preferentially expressed on P-APC, differential antigen processing pathways may be required for their generation. For the work described in this thesis, several approaches were used to dissect the cellular processes which are involved in the formation of the NFLD.D11 and NFLD.D13 epitopes. To understand further how these DRB1*04 epitopes may be used to investigate basic immunological processes, the following sections will discuss how other mAb specific to pMHC have been defined experimentally and used in different aspects of immunological research.

1.2 MHC Class II/Peptide Specific mAbs.

The first MHC class II-peptide (pMHC) specific antibody was described initially in 1989 by Charles Janeway's group and was designated YAe. It recognizes a complex formed by a self peptide ($E\alpha_{56-73}$) bound to $I\text{-}A^b$ molecules [52] and is expressed on B cells, medullary thymic cells and DC [52, 53]. Studies using YAe have led to important insights about the antigen processing of endogenous proteins within the endocytic pathway in a variety of cell types. Since its description several other pMHC-specific mAbs have been described, with each contributing to our overall knowledge of basic cellular and immunological processes.

First, since these mAbs recognize endogenous peptides in the context of MHC class II, they have proved useful in assessing the abundance of endogenous pMHC complexes on a variety of cell types, including both professional APC and non-professional APC. For example, identification of specific endogenous pMHC expressed within the thymus contributed to our understanding of the role of self peptides in positive and negative selection of T cells. By comparing the expression of thymus-expressed peptides within peripheral sites, such as the spleen and lymph nodes, these studies highlighted the importance of endogenous pMHC in the maintenance of peripheral tolerance in a healthy, non-self reactive immune system.

Second, since many of the described mAbs apparently bind to the pMHC complex in a manner analogous to T cell receptor (TCR) recognition of the same complex, they have been useful in studying the TCR-MHC interaction both in structural terms and in quantifying the number of MHC/specific peptide complexes required for T cell

activation. Third, given that these mAbs are specific for a single pMHC complex, the intracellular generation of these complexes can be assessed within different cell types using subcellular fractionation and various microscopical methods. Lastly, if the mAb is directed towards a known autoantigenic pMHC complex, then the possibility of specific immunotherapy of the autoimmune disease in question by blocking the autoreactive T cell response is tantalizing. This was partly addressed by Aharoni *et al*, 1991 [54] who showed that mAbs specific for myelin basic protein (MBP) peptides in the context of I-A^s inhibited the onset of experimental allergic encephalomyelitis (EAE) in mice. Unfortunately these promising results presented by the authors have not been followed up in a more recent publication.

1.2.1 Relative Abundance of Self-Peptide/MHC Complexes.

1.2.1.1 Endogenous Antigen

Originally it was thought that the majority of cell surface MHC class II molecules contained peptides derived from exogenous antigen. Sequencing of peptides eluted from class II molecules revealed that the peptide repertoire is actually generated from endogenous as well as exogenous proteins [55].

Specifically, Rudensky and colleagues found that the majority of peptides isolated from the MHC class II molecules (I-A and I-E) from the murine B-cell lymphoma line LB27.4 (H-2^{bxd}) were derived from endogenously synthesized membrane proteins [56]. The Y-Ae mAb confirmed that class II molecules are loaded with peptides derived from endogenously synthesized proteins. Y-Ae, which was generated by immunizing

histoincompatible mice with lipopolysaccharide (LPS) activated spleen cells from B10.A(5R) (H-2^b) mice [52] was found to bind specifically to splenic B cells, and medullary thymic cells, in addition to MΦ and DC [52, 53]. Further analysis of the peptides eluted from Y-Ae purified molecules showed that the I-A^b molecules recognized by Y-Ae contained a major self-peptide from another class II molecule; I-E^b [53]. This peptide, $E_{\forall 52\text{-}68}$ was found to be present in 10-15% of all I-A^b molecules on the surface of peripheral B lymphocytes [56], and was found to be one of four or five major self peptides presented on the surface of I-A^b+ I-E^b+ B cell lines [52].

Another major study investigating the percentage of class II molecules bound with a known peptide involved mAb 30.2, generated against the Ii processing intermediate CLIP bound to I-A^b [57]. Its binding was shown to be dependent on a lysine residue at position 90, flanking a conserved region of the Ii in human, mouse and rat [58, 59]. Similar to the high expression of endogenous $E_{\alpha 52\text{-}68}$ peptide in the contex of I-A^b recognized by Y-Ae, Eastman *et al*, 1996 found that 30.2 bound to 13% of I-A^b molecules on the surface of the human B cell line T1 tranfected with the I-A^b gene [57].

A major self-peptide bound to class II was detected with the UL-5A1 mAb [60]. In this case, UL-5A1 recognized a conformational epitope on HLA-DR1(DRA/DRB1*0101) molecules bound with endogenously-derived HLA-A2 peptides or from exogenously provided HLA-A2 peptide 105-117 [60]. It was shown that this mAb recognizes an epitope found primarily on activated cells, such as EBV-transformed B cell lines.

1.2.1.2 Exogenous Antigen

The mAb Aw3.14, generated against murine I-A^k molecules bound with peptide 48-62 from the exogenous antigen hen egg lysozyme (HEL), was shown to bind to the peptide binding groove, contacting both the α-helices and the bound peptide [61]. Aw3.14 was also found to block recognition of HEL_{48-62} by specific T cell hybridomas [61]. The authors showed that when the murine B lymphoma cell line M12-A^k was incubated overnight with whole HEL, about 9% of the surface A^k molecules became loaded with the HEL_{48-62} peptide, as measured by Aw3.14. By calculating this as a percentage of the total class II molecules on the surface of M12-A^k, this amount of occupancy of the HEL_{48-62} peptide corresponded to 2×10^5 out of a possible 2.2×10^6 molecules on the cell surface [61]. Taken together, these results supported the notion that a small number of abundant peptides, either from endogenous or exogenous proteins are present within the peptide-binding grooves of class II molecules. Studies of peptides eluted from class II molecules have shown that out of the total numbers of peptides, there are about 12 peptide species that are present at high levels. In addition to these peptides, a cell can present thousands of less abundant MHC/peptide complexes.

1.2.2 Conformational Changes in MHC Class II Molecules Measured by pMHC mAbs.

MHC class II molecules interact with the co-chaperone molecules HLA-DM and HLA-DO in compartments along the endocytic pathway and replace CLIP with antigenic peptides [22-26]. This exchange of peptides can be visualized by formation of sodium

dodecyl sulphate (SDS)-stable dimers such that class II molecules loaded with CLIP are SDS instable and termed floppy. MHC class II molecules loaded with the correct antigenic peptides are more stable and do not fall apart in the presence of SDS. The question of whether or not stable class II molecules exist in different conformations on the cell surface, and if these conformations affect the interaction with T cells has been addressed in studies using pMHC-specific mAbs.

The specificities of some pMHC-specific mAbs are dependent on a certain conformation of the class II molecules [62, 63]. A study involving a peptide-dependent mAb 25-9-17 [62], and the peptide-specific Y-Ae mAb addressed whether the nature of the bound peptide affects the conformation of MHC class II molecules [62]. The epitope recognized by the 25-9-17 mAb is dependent on amino acids β65-67 and β70, with the β65-67 being Pro-Glu-Ile, a sequence which is located in a kink in the α-helix of the β chain. 25-9-17 appeared to recognize two different conformations, depending on the peptide that was bound in the groove of I-A^b, due to this β65-67 region. For example, it did not recognize the I-A^b-Eα_{52-68}, even though it did recognize I-A^b-CLIP.

Knowing that the I-A^b-Eα_{52-68} exists in "tight conformations" on the cell surface, and I-A^b-CLIP exists in "loose conformations" on the cell surface, the authors described two separate 25-9-17-recognizable conformations [62], which were dependent on the peptide which was available to bind in the class II groove. Using a large panel of peptides the 25-9-17 mAb was subsequentially shown to discriminate between SDS-stable (25-9-17$^-$) and SDS-unstable (25-9-17$^+$). This was further extended by using 25-9-17 to block the activation of several T cell hybridomas which were specific for various

naturally processed I-A^b peptides [62]. It was found that 25-9-17 blocked the activation of some but not all of the T cell hybridomas, suggesting that class II molecules acquire 25-9-17^+ and 25-9-17^- conformations, and that T cells are sensitive to these two I-A^b conformations.

The results of this study had broad implications for thymic selection and autoimmunity, since at the time it was unclear whether T cells were selected on the basis of MHC class II structure. According to the 25-9-17 data, T cell recognition is directly dependent on the nature of the peptide and the conformation that the MHC molecule adopts. According to the studies done by Unanue and colleagues, the I-A^{g7} molecules, which are implicated in the development of autoimmune diabetes in the NOD mouse, exists in a "floppy" conformation [64]. Thus, it is more likely to positively select for autoreactive T cells that fail negative selection and this may underlie the increased susceptibility to autoimmune disease.

Another well-studied mAb, 16.23 which recognizes a conformational epitope on DR3 molecules, was first described as DR3-specific [65], and was later shown to be dependent on HLA-DM [24, 63]. It was noticed that 16.23 did not bind to the DM negative B cell lines such as .174 [66]. Transfection of DR3 into the cell line T2, which does not express HLA class II genes, did not reconstitute the 16.23 epitope and subsequent efforts showed that DM was absolutely required for the generation of the 16.23 epitope [63]. However, although it initially was thought that 16.23 recognizes specific peptide(s) presented in the context of DR3, recent data has shown that 16.23 actually recognizes a DM-induced conformational change in the DR3 molecule in a

peptide independent fashion [63]. This situation is reminiscent of the NFLD.D11 mAb which does not bind to DRB1*0401 in DM negative B cells, but which can be restored with the introduction of DM [50].

1.2.3 Insights into MHC Class II Antigen Processing and Presentation

1.2.3.1 The Relationship of CLIP and HLA-DM in Antigen Presentation

MHC class II molecules bind short peptides found within the endocytic pathway for presentation to CD4+ T_H cells. As described in section 1.3.2 above, these proteins enter the endocytic pathway via numerous mechanisms, including pinocytosis, phagocytosis and receptor-mediated endocytosis. Considerable work has been done to dissect the processing of a protein internalized from via these different mechanisms [67, 68, and 69]. In addition to the classical endocytic pathway, an alternative processing pathway is utilized for the production of endogenously-derived peptides for presentation on MHC class II molecules [38, 70].

The co-chaperone molecule HLA-DM catalytically replaces CLIP with peptides that have higher affinity for the class II peptide binding groove. This process was first demonstrated using a panel of mutant B cell lines that were defective in their ability to present protein antigen to $CD4^+$ T cells, despite normal amounts of class II expression [71]. The ability to present antigen to T cells improved after the cells were given exogenous peptides. Despite the presence of DR3, these cell lines were not recognized by 16.23, a result that provided novel insights into the function of HLA-DM [71, 72]. Further genetic analysis of these cells showed that the defect in these cells mapped to the

class II region of the MHC [72, 73] and the deficiency could be rescued by transfection of functional HLA-DM genes [74, 75].

Another mAb specific for pMHC, called 30-2 has also been useful in elucidating various components of the class II antigen presentation pathway, including the organelles where pMHC ligands are formed, and the relevant chaperone and proteases. Rudensky's group, using the mAb 30-2, specific for CLIP:I-A^b complexes, confirmed previous studies that showed high levels of these complexes on the surface of HLA-DM negative cells. For this they used the human antigen presentation mutant cell line T2 transfected with I-A^b, which contained significant amounts of CLIP bound to class II due to the absence of HLA-DM. They also investigated whether the general cysteine protease inhibitor leupeptin, which causes the partial inhibition of Ii degradation and the accumulation of LIP and SLIP, modulated the number of 30-2^+ complexes on T2- I-A^b. They found that leupeptin did not decrease the number of 30-2^+ complexes, and actually increased the number of these complexes [58] indicating that 30.2 can also recognize I-A^b bound with LIP and SLIP.

1.2.3.2 Intracellular Generation of pMHC Complexes.

The C4H3 mAb, developed by Germain's group has been used extensively in the study of class II processing and presentation [76]. They produced several mAbs specific for various Hen egg lysozyme (HEL) peptides presented in the context of I-A^k, with C4H3 being specific for a subset of these molecules that bind $HEL_{46\text{-}61}$, and weakly cross reacting with $HEL_{116\text{-}129}$ [76]. To study the class II processing and presentation pathway

in more detail, they used confocal microscopy to localize the direct intracellular formation of the I-A^k-HEL_{46-61} complex after administration of exogenous HEL. C4H3 staining gave a bright vesicular pattern in A^k expressing B cells, co-localizing mainly with the lysosomal marker LAMP-1, and not with a marker for early endosomes, the transferin receptor (CD71). This indicates that the major site of generation of this specific pMHC complex from exogenous source of antigen is the low pH lysosomal compartments and not the early endosomes [76].

The Y-Ae mAb was similarly used to study the formation of complexes of class II molecules loaded with endogenous peptide [77]. They found that like class II molecules loaded with peptides from exogenous protein, the Y-Ae epitope accumulates within MHC-enriched compartments before reaching the cell surface of B lymphocytes.

1.3 Thesis Rationale

The work presented in this thesis is based on the use of several HLA-DRB1*04-specific mAbs that were produced by S. Drover and W.H Marshall [45-50]. These include NFLD.D11, specific for HLA-DRB1*0401; NFLD.D13, specific for HLA-DRB1*0404; NFLD.D1, specific for HLA-DRB1*04; and NFLD.D2 and NFLD.D10, which both recognize determinants on the shared epitope sequence.

Since NFLD.D11 and NFLD.D13 recognize HLA-DRB1*0401 and DRB1*0404 molecules respectively, which are strongly associated with the development and progression of RA, we questioned whether these epitopes were expressed on synovial fibroblasts derived from patients with RA. We know that inflamed synovial tissues contain abundant numbers of macrophages and activated transformed-like fibroblasts, which secrete a myriad of tissue degrading enzymes [78], but it is presently unclear whether HLA-DR expression on fibroblasts plays any role in the immunopathogenesis of RA. The presence of the D11 and D13 epitopes on a subset of DR4 molecules within synovial fibroblasts from RA patients could hold insights into factors necessary for T cell activation within the synovium, whether or not these epitopes are truly peptide dependent or dependent on the presence of tetraspan microdomains.

Objectives for Chapter 2

(1) To phenotype the cultured fibroblast-like synoviocytes (cFLS), derived from patients with various forms of inflammatory arthritis.

(2) To investigate the expression of specific HLA-DRB1*04 epitopes, along with the shared epitope on cFLS.

(3) To characterize the class II pathway in synovial fibroblasts.

Chapters 3 and 4 deal more extensively with DRB1*04 epitopes defined by the mAbs NFLD.D11 and NFLD.D13. NFLD.D11 was previously shown to bind a subset of DRB1*0401 molecules on EBV-transformed B cell lines [50, 51]. The formation of this epitope is dependent on the presence of the co-chaperone molecule HLA-DM [50] as B cells that do not express HLA-DM fail to express the NFLD.D11 epitope [51]. Its absence on fibroblasts that up-regulate DM suggested that factors other than DM were required for its generation. NFLD.D13 recognizes DRB1*0404, but not DRB1*0401 in HLA-DM positive EBV-transformed B cell lines. However, in B cells that do not express HLA-DM, NFLD.D13 recognizes an epitope on HLA-DRB1*0401 molecules [51].

Based on previous results, there are at least two hypotheses to explain the restricted pattern of the DRB1*04 epitopes recognized by NFLD.D11 and NFLD.D13:

(a) The epitopes are formed by a combination of allele-specific residues and cellular-restricted peptides such as those derived from proteins expressed exclusively by P-APC.

(b) The putative peptides that form these epitopes are derived from common proteins that are either differentially processed or loaded in P-APC.

Objectives for Chapters 3 and 4

(1) To extend the characterization of DRB1*04 molecules recognized by NFLD.D11 and NFLD.D13 on cell types that include P-APC and NP-APC (Chapter 3).

(2) To characterize the intracellular pathway and processing mechanisms that are involved in generating the NFLD.D11 and NFLD.D13 epitopes in BCL (Chapter 4) using confocal and immunoelectron microscopy as well as inhibitors of the MHC class II pathway.

(3) To determine if cellular-restricted peptides are involved in forming these epitopes by examining the effect of protease inhibitors on the formation of these epitopes on BCL (Chapter 4).

We believe that the study of how these class II epitopes are generated will yield valuable information for our understanding of fundamental immunological processes such as the proteolytic generation, and intracellular trafficking of specific HLA class II epitopes.

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CHAPTER 2

Discordant Expression of HLA Class II Associated Chaperones and HLA-DRB Alleles in Cultured Fibroblast-like Synoviocytes

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Abstract

HLA-DR positive synovial fibroblasts are frequently observed in rheumatoid arthritis (RA) and may be implicated in the autoimmune reaction since RA is associated with certain HLA-DRB1* alleles. The question of whether specific DRB alleles and components of the class II antigen presentation pathway are efficiently expressed by synovial fibroblasts is germane to this hypothesis. To address this, cultured fibroblast-like synoviocytes (cFLS), were analyzed for constitutive and IFN-γ induced expression of specific DRB alleles and class II associated co-chaperones. RT-PCR showed DRB alleles were differentially expressed in two RA-cFLS, with one also expressing CIITA and DRA; none were detected in non-RA-cFLS. Ii and HLA-DOB were detected in all, while HLA-DMA and DMB were additionally present in RA-cFLS. IFN-γ induction of Ii, DM and DR molecules was observed in all cFLS, but expression of specific DR allotypes was variable. Interestingly, DM-modulated epitopes on RA-associated DR molecules were either absent or delayed, despite strong DM-expression and a paucity of MHC/CLIP complexes. In summary, cFLS discordantly express class II genes *de novo*, but display co-ordinate IFN-γ induction. Altered expression of specific peptide-dependent epitopes on RA-associated HLA-DR molecules suggests differences in antigen presentation by cFLS, which may have implications for the immunopathogenesis of RA.

2.1 INTRODUCTION

Synovial tissues from patients with rheumatoid arthritis (RA), psoriatic arthritis (PA) and certain forms of osteoarthritis (OA) are characterized by a progressive inflammatory reaction [1-3]. Although their etiologies are poorly understood, RA and PA are hypothesized to have autoimmune elements partly because of large numbers of T-cells in the inflammatory infiltrates and partly because both diseases are associated with particular HLA-DR genes [4,5]. The strongest DR-association is for RA in which most patients express an amino acid motif, QKRAA/QRRAA, known as the shared epitope (SE). It is found on a subset of HLA-DRβ1*04, 01 and 14 molecules at β-chain positions 70-74, which are crucial for antigen presentation and T-cell recognition [5,6]. The inflamed synovial tissues also contain abundant numbers of macrophages and activated transformed-like fibroblasts, which secrete a myriad of tissue degrading enzymes and which have up-regulated DR-expression [7]. Although DR-expression on non-conventional antigen-presenting cells (APC) has long been hypothesized as a mechanism for developing and/or perpetuating autoimmune diseases [8], the contribution of DR expression on synovial fibroblasts to the etiology of autoimmune arthritis has remained controversial.

Several studies have generated adherent cell lines from inflamed synovial tissues, and although generally characterized as fibroblast-like, they often possess diverse phenotypes and properties including those of pannocytes, follicular dendritic cells and nurse cells [7,9-11]. Most cultured fibroblast-like synoviocytes (cFLS) are initially DR-positive, but lose expression after a few passages and re-express if treated with IFN-γ

[7,12-14]. Thus *in vivo* DR-expression on synovial fibroblasts may be a bystander effect of IFN-γ production in the inflamed synovium. An alternative explanation, based on a more recent study demonstrating *de novo* class II expression on long term cultured fibroblasts from RA patients [15], is that synovial fibroblasts may already have switched on their class II gene expression machinery, thereby potentially functioning as APC in a self reactive immune response.

Conventional APC, which include B-cells, macrophages and dendritic cells, express or up regulate various co-stimulatory molecules and possess a highly efficient class II antigen presentation pathway (reviewed in [16]). In this pathway endocytosed antigens are degraded in the acidic protease-rich endosomal/lysosomal vesicles into peptides suitable for loading onto class II molecules. Targeting of HLA class II molecules to the endosomes is aided by the invariant chain (Ii), which also blocks the peptide binding groove, thereby preventing premature binding of peptides en route from the endoplasmic reticulum to the endocytic pathway. As HLA class II/Ii complexes traffic through the endocytic vesicles, Ii is progressively degraded leaving only a fragment called the class II associated invariant chain peptide (CLIP), bound to the peptide-binding groove. Removal of CLIP and its exchange for a stable antigenic peptide is facilitated by HLA-DM [17], which in some APC is further regulated by another endosomal resident protein, HLA-DO [18].

Differences in antigen presenting capabilities of APC from RA patients have been reported including reduced HLA-DM expression in B-cells [19], and increased expression of RA-associated alleles [20]. Although some studies have examined the

ability of HLA class II-positive fibroblasts to activate T-cells [21], little is known regarding the presence or function of various components of the class II pathway in cFLS. To address this, we have examined cFLS for expression of the HLA class II co-chaperones Ii, DM and DO, as well as selective expression of individual HLA-DRB alleles. To determine the efficiency of the class II pathway, we further examined cFLS for their ability to express peptide-dependent and HLA-DM modulated epitopes on the RA-associated HLA-DR molecules.

2.2 METHODS

2.2.1 Patient Samples

Synovial tissues were obtained by BL, with approval of the local Human Investigation Committee, from arthritis patients who were undergoing hip or knee surgery. Tissues used for isolation of cFLS were SN41 (hip) and SN50 (knee) from rheumatoid arthritis (RA) patients; SN08 (hip) from a psoriatic arthritis (PA) patient; and SN07 (hip) from an osteoarthritis (OA) patient. A portion of each synovial sample was stored at -70°C and the remainder was digested as described below. HLA class II types of cFLS, determined by DNA-typing using Micro SSP generic HLA Class II DNA typing kits (One Lambda, Canoga Park, CA, USA), were as follows: SN41, (DRB1*0101, 07; DRB4*; DQB1*05, 02); SN50, (DRB1*0401, 1502; DRB4*; DRB5*; DQB*0301, 06); SN08, (DRB1*0401, 07; DRB4*; DQB1*0301, 02); and SN07, (DRB1*0402, 0101; DRB4*; DQB1*0302, 05).

2.2.2 Preparation of cFLS

Tissue samples were minced and digested with an enzyme cocktail containing 0.05% type IV collagenase, 0.1% hyaluronidase, and 0.01% deoxyribonuclease in 1.0% Hank's balanced salt solution (HBSS) with 2.5% Hepes (all from Sigma, Oakville, ON, Canada) for 3 hr at 37°C. Mixtures were centrifuged at 20 X g for 3 min to remove excess cellular debris. The cell-rich supernatants were collected and washed 2X in HBSS at 400 X g for 7 min, and resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated fetal calf serum (FCS), 100U penicillin/ml, 100μg

streptomycin/ml, 0.25 μg amphotericin B/ml and 2mM L-glutamine (all from Gibco BRL, Rockville, ML, USA). Cells were seeded into 25-cm^2 tissue culture flasks (Fisher Scientific, Halifax, NS, Canada) and incubated in humidified air containing 5% CO_2 at 37°C. Non-adherent cells were removed after 24 hr by extensive washing and adherent cells were grown to confluency.

2.2.3 Cell Culture and Interferon-γ Treatment

cFLS were harvested at confluency using 0.25% trypsin (Gibco BRL) in phosphate buffered saline (PBS), pH 7.4. A portion was frozen and the remainder was passaged by splitting at a ratio of 1:3. For experiments, cFLS were plated in 7 ml CM at 1 X 10^5 cells/25 cm^2 flask or in 0.4 ml CM at 5 X 10^3 cells/well in 8-well chamber slides (Nalge Nunc International, Naperville, IL, USA) and allowed to adhere overnight. Cells were either left untreated or treated with 500U interferon-γ/ml (Pharmingen, San Diego, CA, USA) for 6 days at 37°C. Human B cell lines (BCL): SAVC (HLA-DRB1*0401; DQB1*0302), Jesthom (DRB1*0101; DQB1*0501), PLH (DRB1*0702; DQB1*0201) and E418 (DRB1*1502; DQB1*0601), obtained through the 10^{th} International Histocompatibility Workshop (IHW) [22], were maintained in IMDM supplemented with 10% FBS and antibiotics and used as positive controls.

2.2.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from cFLS using Trizol (Gibco BRL) according to the manufacturer's instructions and treated with DNA-free (Ambion, Austin, Texas, USA) to

remove any contaminating DNA. cDNA was prepared from 1 μg RNA using the First Strand cDNA Synthesis Kit from Pharmacia Biotech (Baie d'Urfe, PQ, Canada), according to manufacturer's instructions. All primers (Table 1) with the exception of DRB1*04 [23], (a kind gift from D. Haegert) were synthesized by Gibco BRL. These included DRA [24], CIITA [25], Ii [26] and DRB1*01, 07, 15 allele specific primers [27] and β-actin [28]. HLA-DMA, HLA-DMB, HLA-DOA, and HLA-DOB primers were devised using sequences acquired from Genbank. The accession numbers of the gene sequences used for primer construction were: X76775 for DMA, X76776 for DMB, M26039 for DOA and L29472 for DOB. Potential primer sequences were entered into the Amplify computer program to ensure primer pairs were compatible and potentially proficient for a particular gene amplification. Primer sequences were analyzed using the OligoTech program to determine the primer G + C content and melting temperature (Tm).

PCR amplification was performed in a total volume of 50μl. PCR Buffer consisted of 20mM Tris-HCl (pH 8.4), 50mM KCl, 0.2mM dNTPs, 1 Unit of Taq DNA polymerase (all from Gibco) and 1:1 of cDNA or RNA. All PCR reactions used 1.5 mM $MgCl_2$ with the exception of DRB1*04 which required 1.25mM $MgCl_2$. PCR conditions and primer concentrations are given in Table 1.

Table 1. Primers and experimental conditions used to amplify HLA class II genes by RT-PCR

Primer	Primer Sequence 5'-3'	PCR Conditions	Size
DRA Sense DRA Antisense	cga gtt cta tct gaa tcc tga cca gtt ctg ctg cat tgc ttt tgc gca	94°C-1min, 55°C -1min, 72°C -3min x 30	643
DRB1*01 Sense DRB1*01 Antisense	ttg tgg cag ctt aag ttt gaa t ctg cac tgt gaa gct ctc ac	94°C -1min, 55°C -1min, 72°C -1min x 35	255
DRB1*04 Sense DRB1*04 Antisense	gtt tct tgg agc agg tta aac ccg ctg cac tgt gaa gct ct	95°C -30sec, 60°C -30sec, 72°C -1min x 30, 72°C -5min	263
DRB1*07 Sense DRB1*07 Antisense	cct gtg gca ggg taa gta ta ccc gta gtt gtg tct gca cac	94 °C -1min, 55 °C -1min, 72 °C -1min x 30	232
DRB1*15 Sense DRB1*15 Antisense	ttc ctg tgg cag cct aag agg ccg ctg cac tgt gaa gct ct	95 °C °C -30sec, 60 °C -30sec, 72 °C -1min x 30, 72 °C -5min	261
Ii Sense Ii Antisense	tcc caa gcc tgt gag caa gat g cca gtt cca gtg act ctt tcg	94 °C -1min, 55 °C -1min, 72 °C -3min x 30	340
DMA Sense DMA Antisense	cca atg tgg cca gat gac ctg c cgc tga aca ctt cag cga tag	94 °C -1min, 60 °C -1min, 72 °C -3min x 30	303
DMB Sense DMB Antisense	gca gaa gtg act atc acg tgg ccg cca gct gat cac acc aag	94 °C -1min, 60 °C -1min, 72 °C -3min x 30	296
DOA Sense	cca cat ggg ctc cta cgg acc	94 °C -1min, 55 °C -1min, 72 °C -3min x 30	446

Primer	Primer Sequence 5'-3'	PCR Conditions	Size
DOA Antisense	ggc agg tag tgg aac ttg cgg		
DOB Sense	ctg cac tgc tct gtg aca ggc	94 °C -1min, 55 °C -1min, 72 °C -3min x 30	406
DOB Antisense	gga cct tag cat gac tga gg		
CIITA Sense	caa gtc cct gaa gga tgt gga	94 °C -1min, 60 °C -1min, 72 °C -3min x 30	447
CIITA Antisense	acg tcc atc acc cgg agg gac		
β-actin Sense	atc tgg cac cac acc ttc tac aat gag ctg cg		
β-actin Antisense	cgt cat act cct gct tgc tga tcc aca tct gc		

2.2.5 Antibodies

Expression of differentiation and cell lineage markers by cFLS was determined using commercially available monoclonal antibodies (mAbs). These included CD14 (clone M5E2); CD21 (B-ly4), CD23 (M-L233), CD54 (HA58), CD107a (H4A3) (Pharmingen); CD35 (To5), CD68 (EBM11 and PG-M1), factor VIII (F8/86) and fibroblast marker (5B5) (DAKO Mississauga, ON, Canada); CD63 (CLB-180), CD80 (DAL-1), CD86 (BU63) (Cedarlane, Hornby, ON, Canada); CD40 (EA-5) (Calbiochem, San Diego, CA, USA); CD45 (2B11) (Medicorp, Montreal, PQ, Canada). HLA-class II expression was determined using L243, pan HLA-DR (ATCC); NFLD.D1, pan HLA-DRB1*04 [29]; NFLD.D2 and NFLD.D10, overlapping determinants on the shared epitope [30]; NFLD.D11, DRB1*0401-specific [31]; PL3, HLA-DRB1*07 + HLA-DRB4* [32] and SFR16 [33], pan HLA-DRB1*07, a kind gift from Dr. Susan Radka. Components of the HLA class II processing pathway were determined using DOB.L1, anti-human HLA-DOB chain, kindly provided by A. Vogt [18]; MaP.DM1, anti-human HLA-DM, kindly provided by P. Cresswell [17]; and LN2, invariant chain (Ii) (Pharmingen). Isotype-matched non-specific mAbs NS "IgM", NS "IgG1", NS "IgG2a" and NS "IgG2b" were kindly provided by Terra Nova Biotechnology, St. John's, NF, Canada. Secondary antibodies included a peroxidase labeled polymer conjugated to anti-mouse and anti-rabbit Igs (DAKO Envision) for immunocytochemistry; R-Phycoerythrin (PE)-labeled goat anti-mouse (GAM) IgG Fc and PE-labeled GAM IgM μ chain specific (both from Jackson Immunoresearch, West Grove, PN, USA) for cytofluorometry.

2.2.6 Immunocytochemistry

cFLS grown in chamber slides were washed in PBS, fixed in acetone for 15 min at 4°C, air dried for 1hr and rehydrated in PBS. Endogenous peroxidase activity was blocked using 1% hydrogen peroxide in PBS. Slides were washed in PBS containing 0.5% bovine serum albumin (Sigma) + 0.05% Tween 20 (BIO-RAD, Hercules, CA, USA), blocked for non-specific binding for 1 hr using 15% human AB serum in PBS, and incubated with primary mAbs (optimally diluted in wash buffer) for 1 hr at room temperature (RT) in a humid chamber. Slides were washed, incubated with horseradish peroxidase labeled conjugate (DAKO) for 30 min at RT, washed again and developed with diaminobenzidine + H_2O_2 (Sigma) to detect peroxidase, then counterstained with Mayer's haematoxylin. The following coding scheme, used by two readers (DS and SD), was based on percentage of positive cells and relative staining intensity compared to the negative isotype matched control; negative, (0-5% of cells positive, any intensity); (+/-, (6-30% weak intensity); +, (6-30% strong intensity or 31-60% moderate intensity or 61-100% weak intensity); ++, (31-60% strong intensity or 61-100% moderate intensity); +++, (61-100% strong intensity).

2.2.7 Cytofluorometry

Antibody binding assays were done as previously described [30]. Briefly, trypsin-harvested cFLS or controls BCL at 1-5 X 10^5 cells/tube were incubated with appropriate mAbs for 30 min at 4°C. Antibody binding was detected with GAM-PE conjugate (Jackson Immunoresearch), followed by fixation in 1.0% paraformaldehyde (Sigma), and

analysis of 5000-10,000 cells using a FACStarPlus flow cytometer (Becton Dickinson, Mississauga, ON, Canada).

2.3 RESULTS

2.3.1 Characterization of Cultured Cell Lines Derived from Synovial Tissues

Adherent cell cultures, prepared from synovial tissues (SN07, SN08, SN41 and SN50), morphologically resembled synovial fibroblasts as described by others [7,12]. To confirm and more completely characterize their lineage, they were analyzed with a panel of mAbs against several differentiation markers. None expressed CD14, CD45, CD21, CD23 or CD35 (data not shown), indicating they were not bone marrow derived or follicular dendritic cells. As shown in Table 2 and Figure 1, they lacked the endothelial cell marker, Factor VIII, but strongly expressed both the 5B5-defined fibroblast marker, prolyl 4-hydroxylase and the EBM11-defined macrophage marker CD68. However, they were negative for other macrophage markers, PG-M1 and non-specific esterase staining (Table 2 and Fig 1a), suggesting they are unlikely to be derived from the type A macrophages that are present in the synovial lining.

Further analysis revealed moderate to strong constitutive expression of the adhesion molecule ICAM-1, which was further up regulated by IFN-γ on SN41 and SN50. Low levels of the co-stimulatory molecule CD40, present in all untreated cells, were moderately to strongly up regulated by IFN-γ treatment. In contrast, the co-stimulatory molecules, CD80 and CD86, found on macrophages and other APC, were neither present nor inducible by IFN-γ (Table 2). Of note, CD63 and LAMP-1, markers of endosomal/lysosomal vesicles in which peptide loading of MHC class II molecules occurs, were strongly and constitutively present in all cFLS.

Table 2. Immunocytochemical characterization of cFLS using antibodies against differentiation antigens

Differentiation Antigen	SN07		SN08		SN41		SN50	
	UT[a]	IFN-γ	UT	IFN-γ	UT	IFN-γ	UT	IFN-γ
5B5	+++[b]	+++	+++	+++	+++	+++	+++	+++
Factor VIII	-	-	-	-	-	-	-	-
CD68 (EBM11)	+++	+++	+++	+++	+++	+++	+++	+++
CD68 (PG-M1)	-	-	-	-	-	-	-	-
ICAM-1	+++	+++	+++	+++	++	+++	++	+++
CD40	+	++	+/-	+++	+	+++	+	++
CD80	-	-	-	-	-	-	-	-
CD86	-	-	-	-	-	-	-	-
CD63	+++	+++	+++	+++	+++	+++	+++	+++
LAMP-1	+++	+++	+++	+++	+++	+++	+++	+++

[a] Untreated.
[b] codes are: -, 0-5% cells positive; +/-, 6-30% weak intensity; +, 6-30% strong intensity or 31-60% moderate intensity or 61-100% weak intensity; ++, 31-60% strong intensity or 61-100% moderate intensity; +++, 61-100% strong intensity.

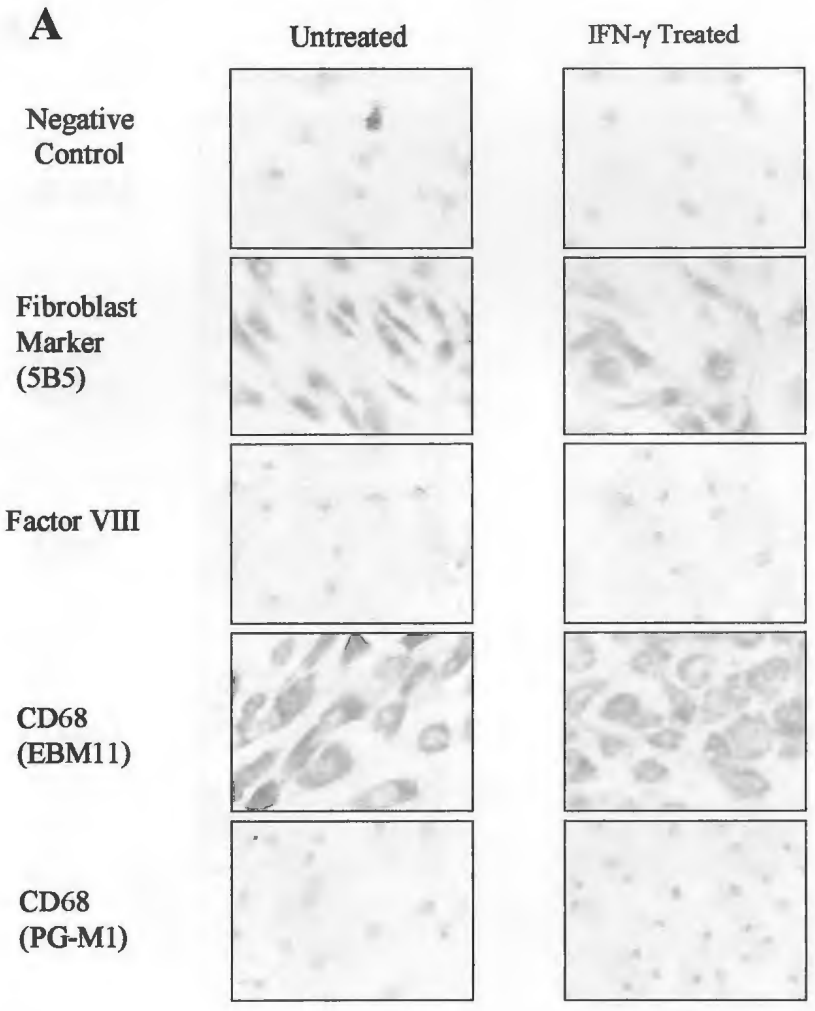


Figure 1A. Differentiation antigens expressed by cFLS: cFLS, untreated or treated with 500U IFN-γ/ml for 6 days in chamber slides, were fixed and analyzed by immunocytochemistry using antibodies that detected lineage-associated markers. Negative control is irrelevant mouse IgG; representative example is SN08, with original magnification X 400. Similar results were obtained in three independent experiments.

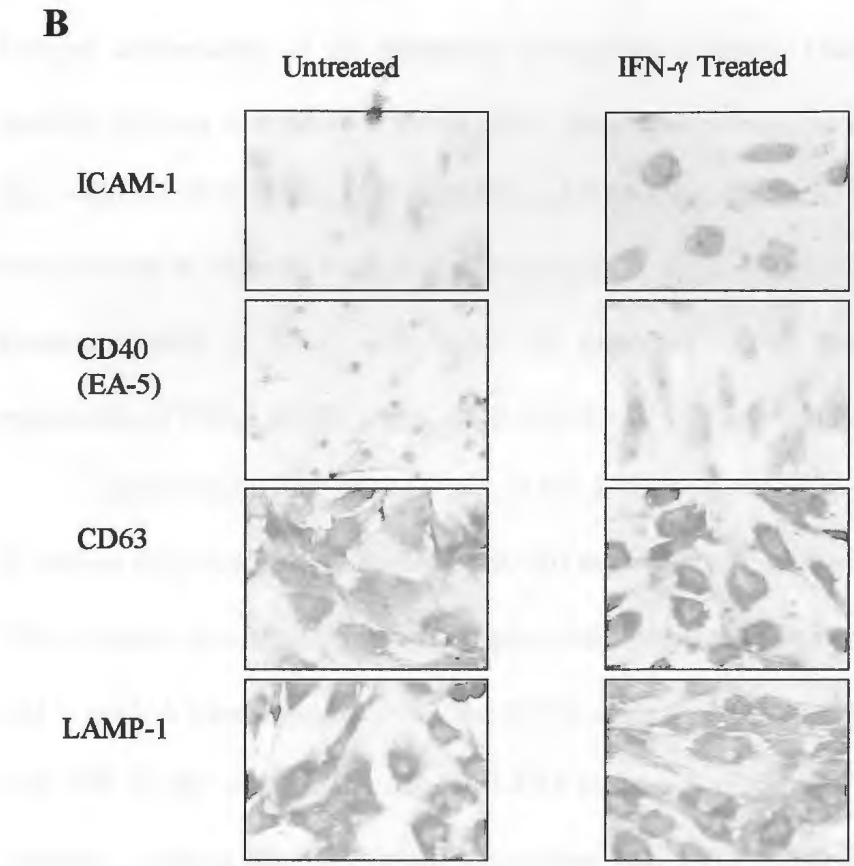
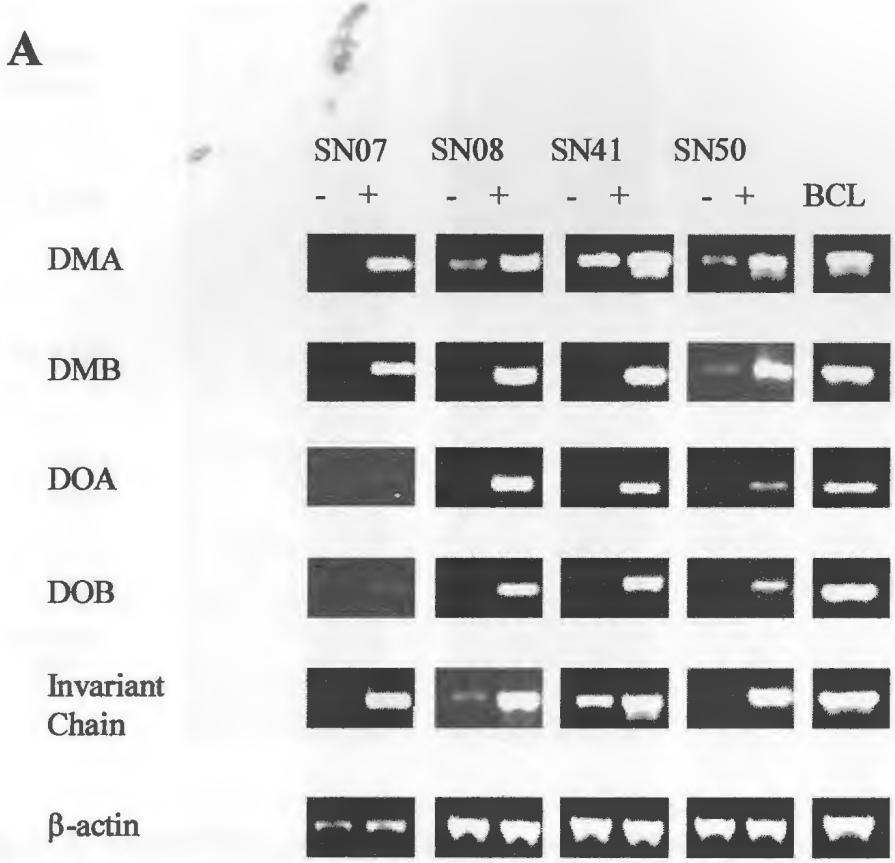


Figure 1B. Differentiation antigens expressed by cFLS: cFLS, untreated or treated with 500U IFN-γ/ml for 6 days in chamber slides, were fixed and analyzed by immunocytochemistry using antibodies that detected antigens typically expressed by conventional antigen presenting cells. Negative control is irrelevant mouse IgG as in **A**; representative example is SN08, with original magnification X 400. Similar results were obtained in three independent experiments.

2.3.2 HLA-Class II Associated Co-Chaperones are Differentially Expressed in cFLS

cFLS were examined for mRNA and protein expression of Ii, DM and DO, which are integral components of the endocytic processing pathway. Using RT-PCR and locus specific primers, constitutive Ii and DMA transcripts were detected in all cFLS (Figure 2a), while DMB was detected in SN41 and SN50, but not in SN07 or SN08. DOA was not detected in any cFLS while DOB transcripts were evident in SN08 and SN41, but scarcely visible in SN07 and SN50. As expected, IFN-γ treatment induced strong expression of DMA, DMB, DOA, DOB and Ii mRNA transcripts in all cFLS (Figure 2a).

Immunocytochemical analysis of cFLS showed that proteins for DM, DO and Ii as well as HLA-DR (Table 3 and Figure 2b) were moderately or strongly expressed in the IFN-γ treated samples. However, despite constitutive expression of HLA-DMA, DMB, and Ii mRNA transcripts in SN41 and SN50, their respective proteins were noted in less than 6% of the untreated cells. HLA-DO protein was present in all untreated cFLS, probably because the DO mAb recognizes the DOβ chain, which is discordantly expressed in a variety of cells [34].



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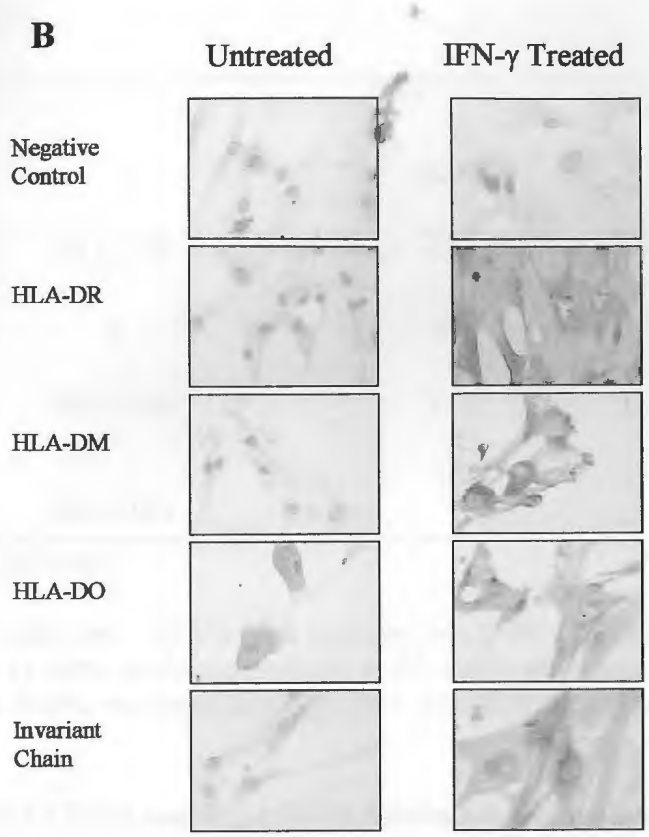


Figure 2. Constitutive and inducible expression of HLA class II chaperone genes in cFLS: (A) RT-PCR was used to analyze DMA, DMB, DOA, DOB and Ii mRNA expression in untreated (-) or IFN-γ (500U/ml) treated (+) cFLS or control BCL; β-actin served as an internal control. **(B)** Intracellular expression of HLA-DR and class II co-chaperones by cFLS, grown on chamber slides for 6 days was ascertained by immunocytochemistry: Negative control (irrelevant mouse IgG); DR (L243); DM (MaP.DM1); DO (DOB.L1); and Ii (LN2). The representative example is SN41, with original magnification X 400. Similar results were obtained in three independent experiments.

Table 3. Expression of HLA-DR and HLA class II associated chaperone molecules in cFLS

	SN07		SN08		SN41		SN50	
	UT[a]	IFN-γ	UT	IFN-γ	UT	IFN-γ	UT	IFN-γ
HLA-DR	-[b]	+++	-	+++	-	+++	-	+++
Ii	-	++	-	+++	-	++	-	++
HLA-DM	-	+++	-	+++	-	++	-	++
HLA-DO	++	++	++	++	++	++	+	++

[a] Untreated.

[b] codes are: -, 0-5% cells positive; +/-, 6-30% weak intensity; +, 6-30% strong intensity or 31-60% moderate intensity or 61-100% weak intensity; ++, 31-60% strong intensity or 61-100% moderate intensity; +++, 61-100% strong intensity.

2.3.3 CIITA and HLA-DRB Alleles are Discordantly Expressed in cFLS

Most studies have found that HLA-DR is not expressed in long-term cultured synovial fibroblasts [7,12], an exception being a study by Navarrete Santos *et al.*, which showed constitutive CIITA and HLA-DR expression in long-term cultured fibroblasts from RA synovium [15]. We also detected a number of transcripts in RA-derived SN41 and SN50, but not in non-RA-derived SN08 (Figures 3a and 3b) nor in SN07, which was only available for one assay (data not shown). CIITA and DRA transcripts (Figure 3a) were detected only in SN41, which additionally expressed DRB1*01 (Figure 3c). However, only trace amounts of DRB1*07 and no transcripts of its associated gene, DRB4* (DR53), were detected in SN41 (Figure 3c and data not shown). DRB1*0401 and its

associated DRB4* gene were not detected in SN50, whereas DRB1*15 and its associated DRB5* gene were well-expressed (Figure 3d and data not shown) despite the absence of detectable CIITA. Interestingly, DQA and DQB were also detected in SN50, but not in SN41 (data not shown), suggesting a CIITA-independent mechanism of class II regulation in those cells. Although SN41 expressed CIITA, DRA and DRB mRNA transcripts, insignificant amounts of DR proteins were detected in untreated cells by immunocytochemistry, whereas high levels were detected in all cells treated with IFN-γ (Table 2).

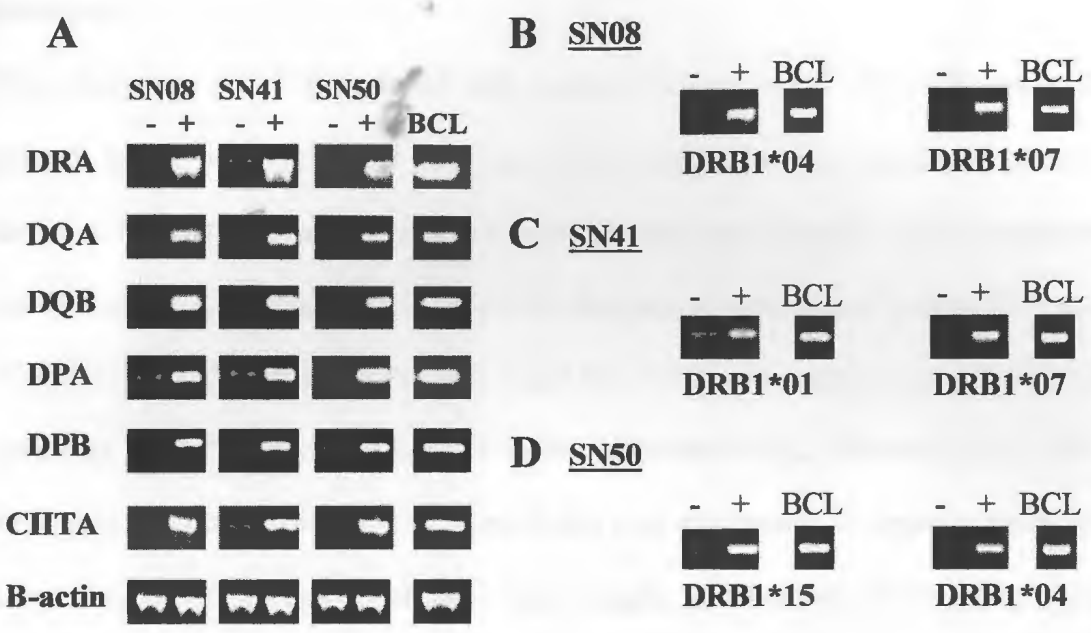


Figure 3. Analysis of mRNA expression of HLA class II genes in cFLS using RT-PCR: (A) cFLS, untreated (-) or treated with 500U IFNγ/ml (+) for 6 days and control BCL were analyzed for DRA and CIITA transcripts with β-actin as an internal positive control. Transcripts for specific HLA-DRB1 alleles were determined using allele specific primers - **(B)** SN08, DRB1*04 and 07; **(C)** SN41, DRB1*01 and *07; **(D)** SN50 DRB1*04 and DRB1*15. Control BCL were SAVC (DRB1*0401) and PLH (DRB1*07) for SN08; Jesthom (DRB1*0101) and PLH (DRB1*07) for SN41; and E418 (DRB1*15) and SAVC (DRB1*04) for SN50. Results are representative of 2-3 independent experiments per cFLS.

2.3.4 IFN-γ Teatment of cFLS Induces Variable Expression of Specific HLA-DRB1* Molecules

IFN-γ treatment of cFLS induced cell surface expression of HLA-DR molecules, although levels were relatively low on SN07 (Figure 4). To assess expression of individual HLA-DRB1* alleles, mAbs capable of discriminating HLA-DR allotypes and cytofluorometry were used. HLA-DRB1*04 molecules, determined by pan-DR4 mAb, NFLD.D1, were up regulated on both SN08 and SN50 with approximately 80% cells expressing DRB1*0401 compared to 95% for the control BCL. However, only 11% of SN07 cells expressed DRB1*0402, a result that was comparable to separate experiment analyzed by CELISA (data not shown). Interestingly, treatment of SN07 with IFN-γ for 14 days (Figure 3 inset) resulted in significantly increased DRB1*0402 expression (MFI: 503 on day 14, compared to MFI of 22 on day 6). By contrast, DRB1*01 molecules, ascertained by NFLD.D10, were expressed almost as well as total DR on SN07. Strong NFLD.D10 binding to SN41 indicated abundant expression of DRB1*01, while its binding to SN08 and SN50 reflected known crossreactivity of this mAb with DRB1*0401 and 1502 [29]. DRB1*07 expression by SN08 and SN41 could not be definitively determined as PL3 mAb also binds DR53, both of which are present in these cells. However, in subsequent assays using an anti-DRB1*07 mAb SFR16 [33], kindly provided by Dr. Susan Radka, DRB1*07 was found to be well expressed (data not shown). Due to lack of discerning mAbs, DRB1*1502 and DR51 expression by SN50 could not be assessed.

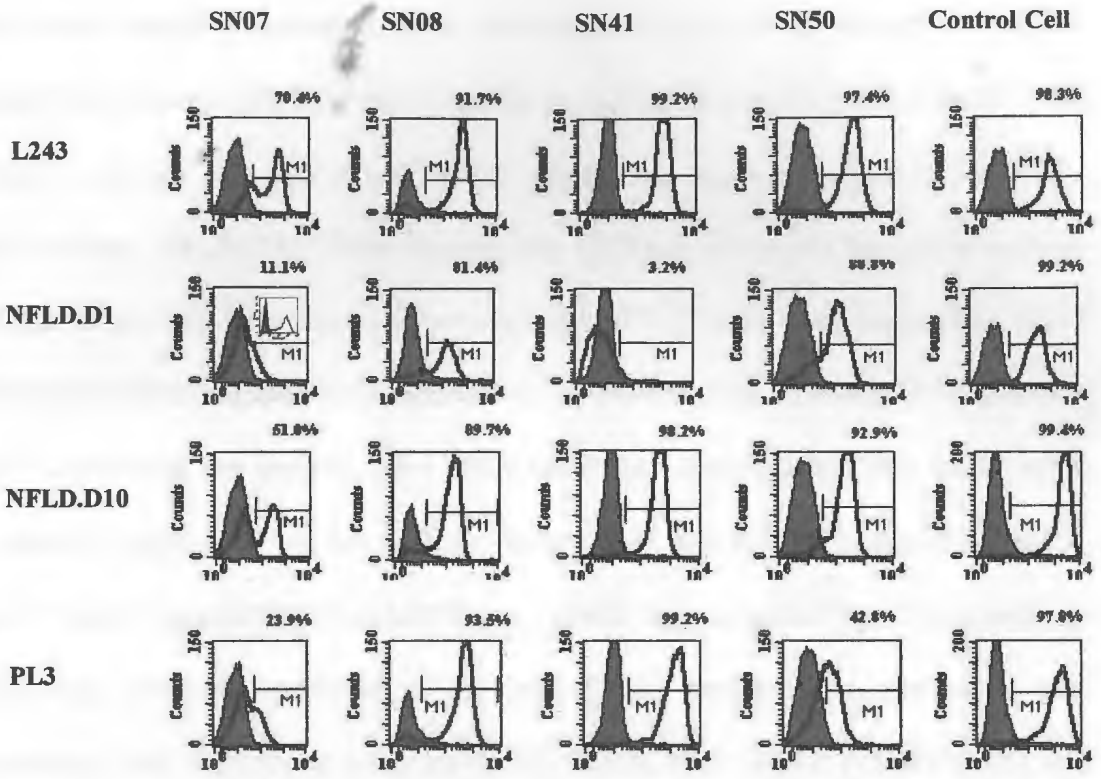


Figure 4. Cell surface expression of specific HLA-DRB alleles by cFLS is induced with IFN-γ. cFLS were treated with 500U IFN-γ/ml for 6 days and analyzed by cytofluorometry using mAbs: L243, pan-HLA-DR; NFLD.D1, pan DRB1*04 (SN07, SN08 and SN50); NFLD.D10, DRB1*01 (SN07 and SN41); and DRB1*0401 (SN08 and SN50). Inset shows NFLD.D1 binding to SN07 after a 14 day treatment with 500U IFN-γ/ml. Open histograms = test mAb; filled histograms = IgG negative control; percentages at the top indicate the % of positive cells. Control BCL was SAVC (DRB1*0401, DR53). Similar results were obtained in two or three independent experiments.

2.3.5 Peptide-Dependent Epitopes on HLA-DRB1* Molecules Are Inefficiently Expressed on cFLS

As an indirect means of ascertaining the functionality of the class II pathway in cFLS, we analyzed IFN-γ-treated cFLS for their capacity to express peptide-dependent and/or DM-modulated epitopes on their HLA-DRB1* molecules. Interestingly, a DRB1*0401-specific epitope, NFLD.D11 which requires the QKRAA version of the shared epitope and which is strongly expressed on conventional APC [35] was not detected on SN08 and SN50 by cytofluorometry or by immunocytochemistry, despite strong DR expression (Figure 5a and data not shown). Even when SN50 was treated with IFN-γ for 14 days, there was no expression of this epitope. In contrast, the NFLD.D2-specific broadly defined "shared epitope" QKRAA/QRRAA, which is modulated by DM-expression [30,35], was detected on SN08, SN50 and SN41; however, its expression was comparatively less than levels achieved on SE^+ B-cell lines, SAVC (DRB1*0401) and Jesthom (DRB1*0101) (Figure 5).

To determine whether reduced SE-expression is time dependent and/or related to accumulation of intracellular co-chaperones, SN41 and SN50 were cultured with IFN-γ for 12 or 15 days, and at 3-day intervals, they were analyzed for cell surface expression of DR molecules and SE. Since CLIP complexes accumulate when DM-loading is deficient, we also determined CLIP expression as well as intracellular expression of Ii, DM and DO.

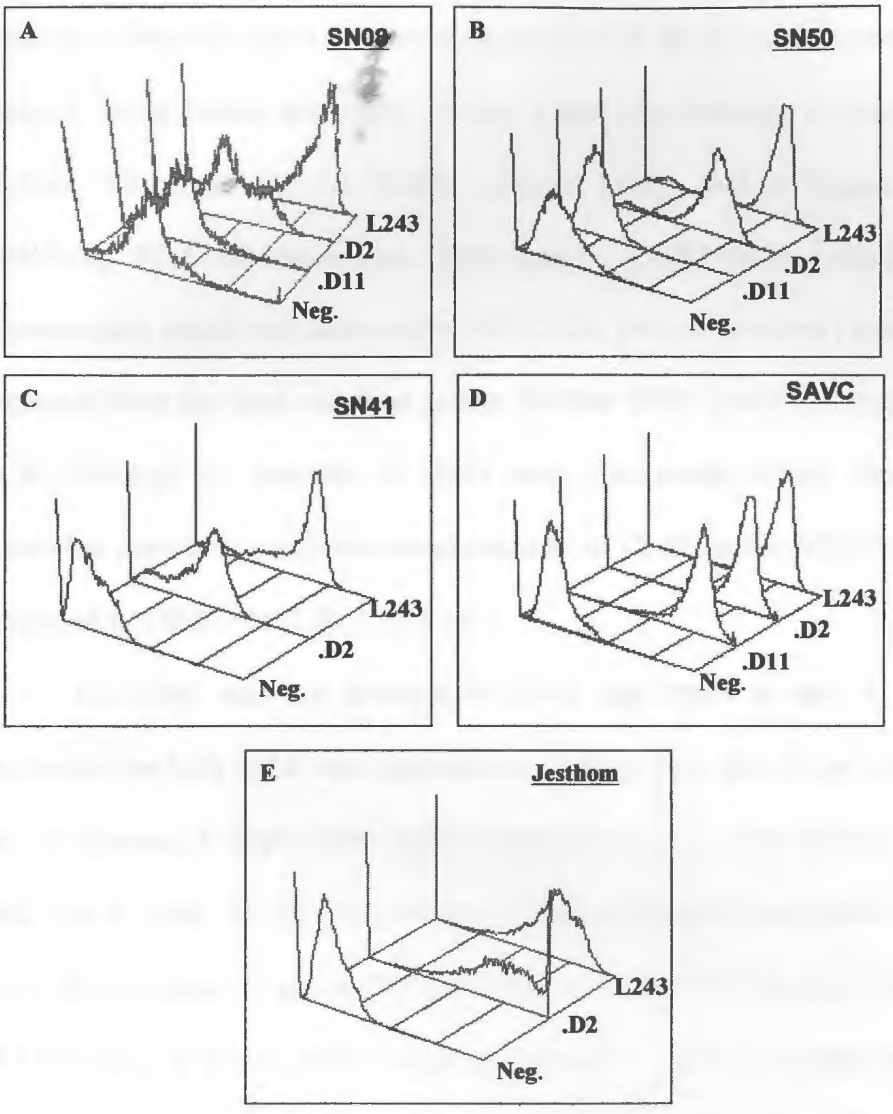
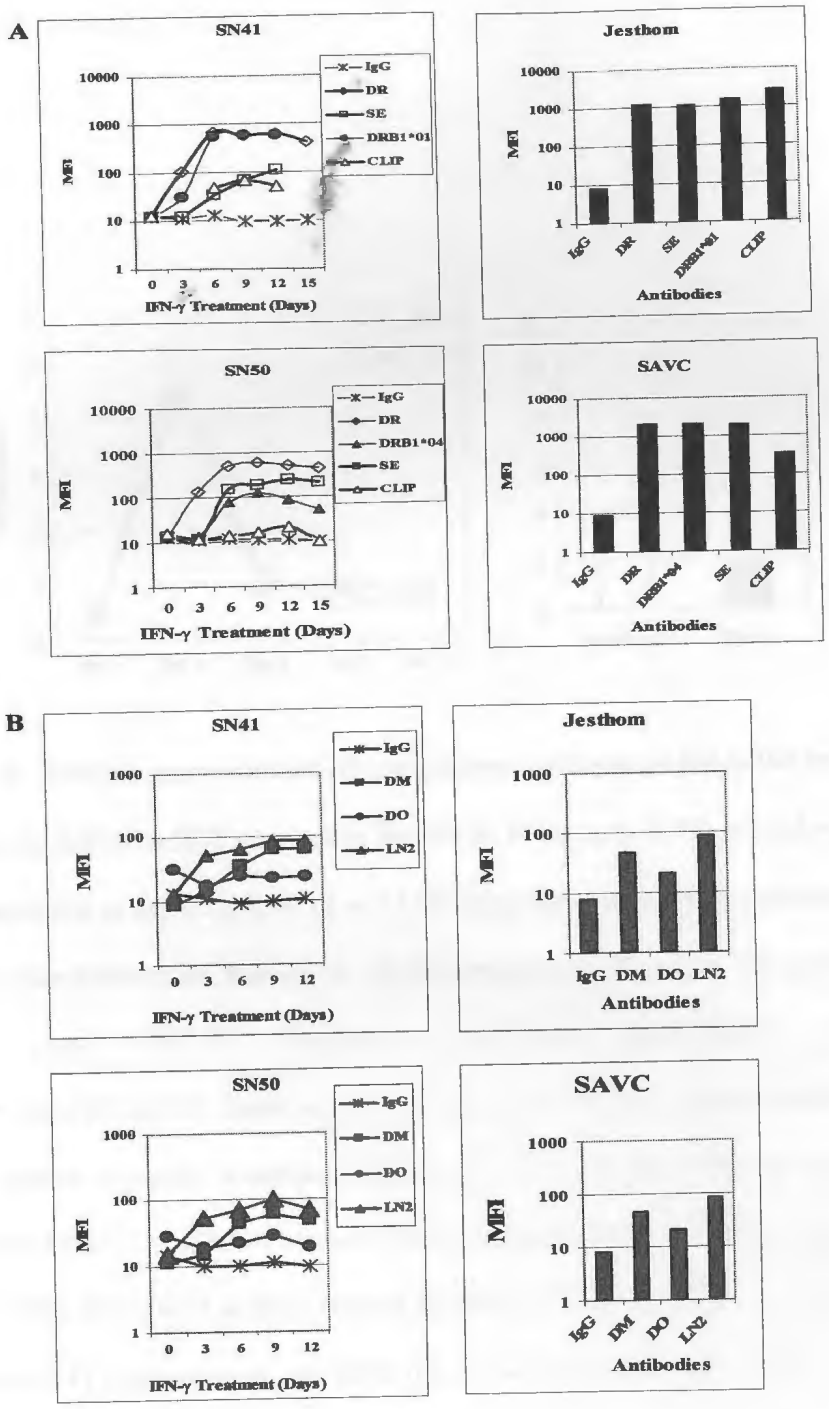


Figure 5. Cell surface expression of HLA-DM modulated epitopes on SE-bearing HLA-DRB molecules expressed by cFLS: cFLS (A-C), treated with 500U IFN-γ/ml for 6 days and control BCL, (D-E) were analyzed by cytofluorometry using mAbs: L243, pan-HLA-DR; NFLD.D2, SE-specific and NFLD-D11 (DRB1*0401 specific). Similar results were obtained in two or three independent experiments.

As shown in Figure 6a, DRB1*0101, which carries the SE sequence in SN41, paralleled total DR expression, peaking by Day 6 and maintaining high levels for the duration of the experiment. However, expression of SE on these molecules was markedly delayed, being barely detectable at day 6 and not reaching a plateau by day 12. In contrast, SE expression on SN50 occurred earlier and at higher levels, generally paralleling HLA-DR-expression. Interestingly, DRB1*0401, which carries the SE sequence and which was detected by NFLD.D1, peaked between Days 6 and 9, but then decreased over the next two time points. Neither SN41 nor SN50 expressed appreciable CLIP, although the amounts on SN41 were consistently higher than those on SN50, somewhat correlating with increased amounts of CLIP on the DRB1*01 BCL (Jesthom) compared to DRB1*0401 BCL (SAVC).

HLA-DM was not detected in SN41 and SN50 at day 3, but by day 6 its expression on both cFLS was equivalent or greater than that of the control BCL (Figure 6b). In contrast, Ii levels were easily detectable at Day 3 but did not reach BCL levels until Day 9. Thus, Ii:DM ratio (Figure 6c) was maximal in both SN41 and SN50 at Day 3, but then dropped to about the same ratio as for SAVC, but less than that of Jesthom. The hierarchy of Ii:DM, SN41>SN50 and Jesthom>SAVC, correlates directly with CLIP expression, perhaps explaining the markedly delayed and reduced expression of SE on DRB1*0101 (SN41). As for DO expression, we confirmed low constitutive levels previously observed by immunocytochemistry (Figure 2b), and indeed its expression changed little during the duration of the experiment. Similar expression levels on BCL, suggest that it is not implicated in the expression of these DR epitopes.



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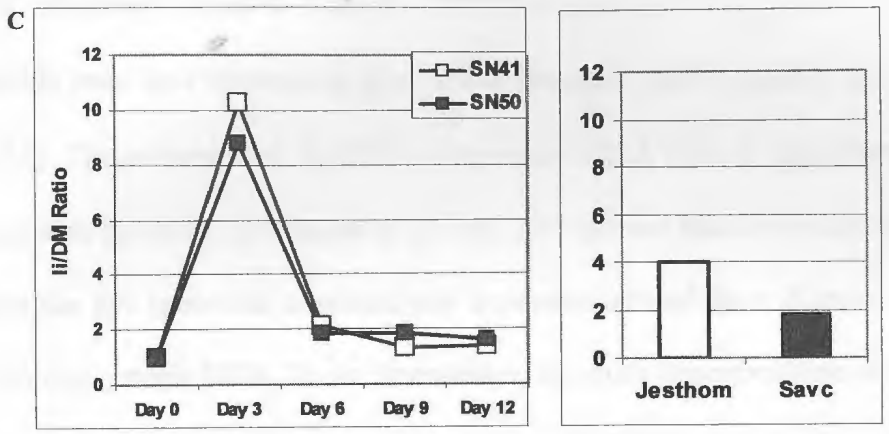

Figure 6. Delayed expression of DM-modulated epitopes on HLA-DR molecules in cFLS is not due to deficient DM expression by cFLS: SN41 and SN50, treated with 500 U of INF-(were harvested at day 0, 3, 6, 9, 12 or 15 (X-axis) and analyzed by cytofluorometry (MFI, mean fluorescence intensity on Y-axis). **A.** Surface expression of generic DR (L243), SE (NFLD.D2), MHC class II/CLIP complexes (cerCLIP); DRB1*0101 (NFLD.D10) and DRB1*0401(NFLD.D1); irrelevant mouse IgG, served as a negative control; BCL, Jesthom and SAVC served as positive controls for SN41 and SN50, respectively. **B.** Intracellular expression of DM (MaP.DM1), DO (DOB.L1) and Ii (LN2). Controls were as described above. **C.** Ratio of Ii to DM = (MFI of Ii - MFI of IgG) divided by (MFI of DM - MFI-IgG) at various time points (X-axis) for SN41 (open squares) and SN50 (closed squares) compared to BCL, Jesthom and SAVC.

2.4 DISCUSSION

HLA-class II expression on synovial fibroblasts in inflammatory arthritis is believed to be a consequence of IFN-γ, produced by the infiltrating T-cells. In support of this, fibroblasts isolated from the inflamed synovium and grown in culture medium, quickly lose their expression after a few passages, but re-express if treated with IFN-γ [7,12]. The existence of an IFN-γ independent HLA class II regulatory mechanism was suggested by Naverrate Santos *et al.* [15] who showed that long term cultured fibroblasts from the RA synovium constitutively expressed several class II genes including CIITA, DRA and generic DRB. To our knowledge, the study described here is the first to explore constitutive and inducible expression of specific DRB alleles and associated class II co-chaperones in cFLS. Our findings generally support *de novo* class II transcription in the RA-derived FLS, but additionally demonstrate discordant transcription of DRB alleles. Furthermore, various class II co-chaperone genes were transcribed in both RA and non-RA cFLS, with a greater number present in RA cFLS. However, with the exception of HLA-DOB, we observed only trivial expression of class II proteins using either cytofluorometry or immunocytochemistry in any of the cFLS.

The explanation for differential mRNA expression of HLA-DRB and class II co-chaperones by cFLS is unclear, but it is unlikely an artifact of culture conditions as similar results were obtained at both early passages (passage 2-4) and late passages (greater than 16). Although all cFLS displayed similar markers, their growth characteristics varied with SN07 being the least proliferative and eventually becoming senescent. Diverse phenotypes have been ascribed to RA-derived synovial cell lines

including properties of follicular dendritic cells, nurse cells and transformed-like cells [7,9,11,14,36]. The latter property is associated with expression of over amplified proto-oncogenes, such as cFos [7,9], which has previously been shown to play a role in class II regulation [37]. Although we did not investigate expression of oncogenes, it was noted that the least contact-inhibited and most proliferative cFLS (SN41) expressed the most class II genes and was the only one with detectable CIITA.

Differential expression of HLA-DRB alleles has been reported in various cell types and diseases [38,39]. Particularly interesting is a report by Kerlan-Candon *et al.* [20] that showed SE-bearing alleles (DRB1*04 and B1*01) were more highly expressed in B-cells from RA patients than in those from healthy individuals. Here we found that DRB1*0101 was constitutively transcribed in SN41, whereas DRB1*15 and DR51, and not DRB1*0401, were detected in SN50. Differences in HLA-DR expression in cFLS could reflect allele specific polymorphisms in the promoter regions, which are known to influence regulation [40-42]. In fact, DRB1*04 has been reported to be less transcriptionally active than alleles such as DRB1*01 and B1*15 [39,40]. An alternative explanation for the lack of DRB1*04 transcription in SN50 is that it may be more dependent on expression of CIITA, than are DRB1*15 and DR51.

In addition to the CIITA-independent expression of DRB1*15 and DRB5*, we also noted constitutive expression of DQA and DQB transcripts in SN50 (data not shown). Although no CIITA transcripts were detected in three separate experiments in these cells, it is possible that the standard RT-PCR used in these experiments is not sufficiently sensitive to detect very low levels of CIITA. Constitutive expression of Ii,

DMA and DOB in the three CIITA-negative cFLS was not totally unexpected, as although CIITA is required for maximal expression, CIITA-independent expression class II genes has been previously reported [43,44]. The HLA molecule markedly expressed in untreated cFLS was HLA-DO which is also expressed on thymic epithelial cells in the absence of HLA-DR expression [34]. Although its role in B-cells is to regulate DM activity [16,18] its function in cFLS and other non-conventional APC remains unclear.

Several observations in this study support the notion that cFLS can function as APC. Abundant levels of constitutive CD63 and LAMP-1 indicate the presence of endosomal and lysosomal vesicles, while strongly up regulated Ii and DM molecules suggest normal class II trafficking and peptide loading. Indeed levels of DM protein in IFN-γ induced cFLS were significantly higher than those observed for control B-cell lines. Unfortunately it was not possible for us to compare DM levels in B-cells from the same patients, but these observations are nonetheless intriguing in light of a report showing reduced DM levels in B-cells from RA patients compared to those from healthy controls [19]. In view of the importance of DM in displacing CLIP from the groove and facilitating peptide binding to class II molecules [16,17], the paucity of MHC II/CLIP complexes on DR-positive cFLS show that DM is functioning as in conventional APC. This is further supported by the expression, albeit delayed, on both SN50 (DRB1*0401) and SN41 (DRB1*01) of the DM-modulated SE defined by NFLD.D2 [30]. Its delayed induction may be due to the initially high Ii:DM ratios observed between Days 3 and 6 of the IFN-γ induction period. As well, weaker expression of SE on DRB1*01 compared to DRB1*0401 may be due to greater affinity of DRB1*01 for CLIP [45,46]. An alternative

mechanism, which was shown for delayed expression of the HLA-DM dependent epitope, 16.23 in a non-conventional APC [47], is that DM and DR may be initially localized in different endosomal compartments.

The lack of DM-dependent epitope, NFLD.D11, on DRB1*0401 molecules was not unexpected, as this epitope was previously shown to require additional but as yet undefined factors for its expression [35]. One of these factors could be a cellular restricted peptide or alternatively it may require a particular protease that is not present in cFLS. Although we did not directly address the issue of protease content, others have shown that synovial fibroblasts express a wide array of proteases [48,49]. Cathepsin S, which is present in conventional APC and required for degradation of Ii [50,51], is strongly up regulated in non-conventional APC by IFN- γ [52]. Thus, the very low levels of CLIP, despite high levels of Ii, in cFLS suggest that Cathepsin S was up regulated in all cFLS.

In summary this study clearly shows that DR+ synovial fibroblasts possess the requisite intracellular components for antigen processing, and based on low CLIP expression, they also have the capacity to efficiently display stable MHC/peptide complexes. However, the composition of these complexes will likely differ from those expressed on conventional APC, possibly due to cell-restricted proteins or processing mechanisms. Although their ability to activate CD4+ T-cells was not addressed in this study, others have shown that class II positive FLS can activate T-cells by presentation of pre-processed peptides or superantigen [21,53]. However, Corrigall *et al.* [54] found that absence of co-stimulatory molecules CD80 or CD86 on DR+ fibroblasts anergized rather

than activated naïve T-cells. Perhaps other accessory and co-stimulatory molecules such as CD40, which was expressed on our cFLS and on fibroblasts in some, but not all, studies [54-56], may compensate for CD80/86. It is likely that different subsets of synovial fibroblasts exist and as such will differ in their ability to present self-antigens and to activate or perpetuate an autoimmune response.

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CHAPTER 3

Cellular Expression of HLA-DM Dependent and Independent HLA-DRB1*04 Epitopes

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Abstract

The major function of HLA-DR molecules is to bind peptides derived from proteins, degraded in the endocytic pathway, and to present them on the cell surface for CD4 T-cell recognition. Peptide binding depends on many factors including the particular DRB alleles expressed by the cell, antigen processing machinery, the proteolytic environment and contribution of chaperone molecules such as Ii, HLA-DM and DO. In this study we investigated expression of allele-specific epitopes on HLA-DRB1*0401 and 0404 molecules that differ by two conserved amino acid substitutions, K71R and G86V, located on the peptide binding site. We show that an antibody-defined, DM-dependent epitope ($D11^{+}0401$) is restricted to DRB1*0401 molecules, expressed by pAPC, notably EBV-transformed BCL. Although DRB1*0401 and DM were strongly up-regulated on various NP-APC, including fibroblast, epithelial and melanoma cells, $D11^{+}0401$ expression was limited to a putative melanoma line, MDA MB 435. A second antibody-defined epitope ($D13^{+}0404$) that is not strictly DM-dependent was limited to DRB1*0404 positive B cells and dendritic cells. Most intriguingly, the same mAb recognized DRB1*0401 molecules on DM negative B cells ($D13^{+}0401$ epitope), and introduction of DM into these cells resulted in loss of $D13^{+}0401$. The limited cellular distribution of these epitopes and their different requirements for DM suggest that alternative antigen processing or trafficking mechanisms are implicated in their generation.

3.1 Introduction

MHC class II molecules are membrane glycoproteins formed from a non-polymorphic α chain and a polymorphic β chain [1-2]. They are expressed constitutively on the surface of professional antigen presenting cells (P-APC) such as B cells, macrophages (MΦ) and dendritic cells (DC) but can be up-regulated on non-professional (NP-APC) by inflammatory mediators such as interferon-γ [3]. These molecules bind short peptides between 13 to 25 amino acids long [4] which are presented to $CD4^+$ T_H cells, for T-cell and immune recognition. Since MHC class II molecules bind a wide array of peptides, produced by efficient, but fairly ubiquitous antigen processing mechanisms, the organism is well equipped to fight infection from a diversity of microbial sources. However, because MHC proteins are highly polymorphic with most substitutions occurring within the peptide binding groove, each MHC allotype has a certain peptide selectivity. The majority of the HLA-DR allele-specific polymorphic residues are located on the surface of the pockets, and this selects for a distinct peptide repertoire amongst different HLA-DR molecules due to the amino acid composition and the electrostatic charge of the individual amino acids. Pocket 1 is made up from DRβ chain amino acids 85, 86, 89 and 90; pocket 4 contains β-chain amino acids 13, 44, 70, 71 and 78; pocket 6 is formed from β chain amino acids 11 and 13; pocket 7 is formed from β chain amino acids 28, 47, 61, 67 and 71, and pocket 9 is formed from β chain amino acids 9 and 57 [5]. Both non-polymorphic and polymorphic residues contribute to peptide binding though, with non-polymorphic residues binding to main chain atoms of

the bound peptide and polymorphic residues within the different pockets binding to the peptide side chains [6].

MHC class II molecules are generated in the endoplasmic reticulum (ER) where they associate with a chaperone molecule known as the invariant chain (Ii) [7-9]. Three MHC αβ dimers bind to three Ii proteins forming a nonomeric complex [1] that travels from the ER through the trans-golgi complex to a series of endocytic compartments and eventually to lysosome-like structures known as MHC class II peptide loading compartments (MIIC), which contain a pH of 3.5-5.5 [10]. Within the MIIC the Ii is slowly broken down in a stepwise manner [11], replaced by peptides that have been generated either from internalized exogenous or endogenous proteins that are degraded within the acidic and protease-rich lysosomal compartments. Although peptide loading of MHC class II molecules may occur in early endosomal compartments, it primarily occurs with MIIC [12-15], where it is facilitated by the co-chaperone molecule HLA-DM, which is transported to the MIIC along with its negative regulator, HLA-DO [16].

Ii is broken down, mainly by the cysteine proteases cathepsin S and L [11] leaving a short fragment called the class II associated invariant chain peptide (CLIP) in the peptide binding groove [17]. DM facilitates removal of CLIP [18-20], along with the stabilization of the "peptide-free" or empty conformation of the MHC class II molecule [21-23] before enhancing the binding of high stability peptides in the groove of the MHC class II molecule and editing which peptides bind to form mature pMHC [24-26] within MIIC [27]. This mature peptide-MHC complex (pMHC) can now travel to the cell surface to interact with the T cell receptor (TCR) on $CD4^+$ cells.

Because of their role in presenting peptides to T cells via the TCR, and since various HLA class II alleles have been associated with autoimmune disease in humans, it has been hypothesized that HLA class II molecules play an important role in the development of autoimmune disease [28, 29]. For example, HLA-DQB1*0302 and HLA-DQB1*0201 are associated with autoimmune type 1 diabetes and Celiac disease and HLA-DR molecules are associated with diseases such as autoimmune hepatitis, multiple sclerosis and rheumatoid arthritis (RA) [30]. All RA-susceptible alleles possess a homologous short stretch of amino acids (LLEQ(K/R)RAA) at positions 67-74 of the β1 chain, and is often referred to as the shared epitope [28]. In Caucasian populations, the major HLA-DR alleles that are associated with increased risk to RA are DRB1*0401 and DRB1*0404, which differ in the shared epitope region, at positions β71 (Lys to Arg) and β86 (Gly to Val). Therefore, amino acids within pockets 1 and 4 of the class II peptide binding cleft are of vital importance in the differentiation between DRB1*0401 and DRB1*0404.

We previously described three mAb-defined, HLA-DRB1*04-specific epitopes. NFLD.D11 recognizes a DM-dependant epitope on DRB1*0401 molecules [31], whereas NFLD.D13 recognizes an epitope on DRB1*0404 molecules [32], and thus, these mAbs are able to distinguish between DRB1*0401 and DRB1*0404. Both epitopes are strongly expressed on normal EBV-transformed B cell lines (BCL) as well as weakly on peripheral blood B cells from RA patients and healthy controls [32]. Intriguingly, NFLD.D13 also recognizes a determinant on HLA-DRB1*0401 molecules in DM negative BCL [33].

We have now extended the characterization of these epitopes to human MΦ and DC, as well as numerous NP-APC in order to learn more about processes that lead to the generation of these epitopes. Using mAbs with known specificities, we also show that the NFLD.D11 and NFLD.D13 recognize determinants located in the peptide binding groove of the class II molecule. In regards to the $D13^{+}0401$ epitope, reconstitution of DM by the gene complementation resulted in the loss of NFLD.D13 binding, confirming the antagonistic effect of this molecule in the generation of the $D13^{+}0401$ epitope.

3.2 Methods

3.2.1 Cell Lines

Human B cell lines (BCL) SAVC (HLA-DRB1*0401) and MT14b (HLA-DRB1*0404), were obtained through the 10th International Histocompatibility Workshop (IHW) [34]; 8.1.6 0401, 9.5.3 0401, 5.2.4 0401 and 5.2.4 0404 were all kind gifts from Dr. N. Patil and Dr. E. Mellins at Stanford University; Daudi, a class I null Burkitt's lymphoma cell line, Daudi Dw4 [31] and Daudi-β2microglobulin (a kind gift from Dr. J.R. Parnes at Standford University Medical Center); BLS-1-Dw4, BLS-1-Dw14 and SJO-Dw4, three class II negative BCL [35]; the class II null B-T hybrid T2-Dw4 was provided by Dr. W.W Kowk at Benaroya Research Institute, Virginia Mason, Seattle, WA; and T2-Dw4DM provided thanks to Dr. P. Cresswell and Dr. L. Denzin [36].

Adherent human breast cancer cell lines MDA MB 435 Dw4 (endogenous DRB1*0405, 13), T47D Dw4 (DRB1*0102) and MCF-7 Dw4 (DRB1*03, 15), all kind gifts from Dr. J. Blum at Indiana School of Medicine were selected in CM supplemented with either 0.5 mg/ml G418 (Gibco BRL) and/or 0.2 mg/ml hygromycin B (Gibco BRL). The human melanoma cells 1359 Mel (DRB1*0401, DRB1*0301), DM331 (DRB1*0401, DRB1*01) and DM13 (DRB1*0404) were kind gifts from Dr. V. Engelhard at the University of Virginia. All cells were maintained in complete IMDM (CM) supplemented with 10% heat inactivated fetal calf serum (FCS), antibiotics and 2mM L-glutamine (all from Gibco BRL, Rockville, ML, USA), in humidified air

containing 5% CO_2 at 37°C, except 8.1.6 0401, 9.5.3 0401, 5.2.4 0401 and 5.2.4 0404, which were maintained in RPMI supplemented as above.

3.2.2 Interferon-γ treatment

Adherent breast cancer and melanoma cell lines were harvested at confluency using 0.25% trypsin (Gibco BRL) in PBS, pH 7.4 and passaged at a ratio of 1:3. the cells were seeded into 25 cm^2 tissue culture flasks (Nalge Nunc), allowed to adhere overnight and left untreated or treated with 500U interferon-γ/ml (Pharmingen, San Diego, CA, USA) for the appropriate time period at 37°C.

3.2.3 Preparation of macrophages and dendritic cells

Whole blood was collected from a DRB1*0401, DRB1*07 healthy donor and separated on a Ficoll-Hypaque gradient. The mononuclear cell layer was collected in HBSS, washed 2X at 400 x g for 10 min and 2X in 10% IMDM (400 x g, 7 min), before being centrifuged at 150 x g for 5 min to remove platelets. The cells were resuspended (at 1 x 10^5 cells/well) in 10% IMDM, pipetted into 6 well tissue culture plates (Nalge Nunc) and incubated in 5% CO_2 at 37°C for 2 hours before washing them with HBSS (4X) to remove non-adherent cells. The remaining cells were incubated with 10% IMDM supplemented with 1000 U/ml human rIL-4 (Sigma) and 1000 U/ml human rGM-CSF (Sigma), which was refreshed on days 2 and 4. For MΦ isolation, the cells were allowed

to adhere to glass 16-well tissue culture chamber slides and either left un-stimulated or stimulated with IFN-γ for 7 days.

On day 6, the immature DC were removed from media containing IL-4 and GM-CSF and treated with 10 :g/ml of TNF-α. These mature DC were harvested from 6-well plates on day 7 and placed in 16-well chamber slides (Nalge Nunc) at 2000 cells/well and centrifuged at 400 x g for 7 min. For surface expression analysis of DC, cells were cultured as above, and kept in cell suspension for cytofluorometry analysis. FITC-GAM IgM (Immunocontact) was used to detect NS IgM, NFLD.D11, and NFLD.D13 while PE-GAM IgG (Jackson ImmunoResearch) was used to detect NS IgG, NFLD.D1, CD1a, CD83 and CD86. Approximately 5000 cells were collected for analysis using FACS, and DC were gated based on size and granularity (Forward vs Side Scatter).

3.2.4 Antibodies

Commercially available mAbs used to differentiate macrophages and blood derived DC included: CD83 (HB15e), CD1a (HI149) (BD PharMingen, Mississauga, Canada), CD86 (BU63) (Cedarlane, Hornby, ON, Canada) and CD68 (EBM11) (DAKO Mississauga, ON, Canada). HLA-DR expression was determined using L243, pan anti-HLA-DR (ATCC). DRB1*04 specific mAbs included NFLD.D1 which recognizes a DRB1*04-specific epitope located on the β_2 domain of all DRB1*04 chains [37]; NFLD.D2 which recognizes a shared epitope that maps to DRB1:70-74:QKRAA/QRRAA sequence [38]; NFLD.D10, which recognizes HLA-DR molecules that express and Q at position 70 of the HLA-DRβ chain; NFLD.D11, which binds to a

HLA-DM dependent Dw4 determinant on DRB1*0401 molecules [31, 39]; NFLD.D13 [39] which binds to DRB1*0404 molecules in the presence of DM, while binding to DRB1*0401 molecules in the absence of DM [33] while NFLD.M15 was used to measure the levels of HLA-B [32]. Antibodies used to identify components of the HLA class II processing pathway included MaP.DM1, anti-human HLA-DM and LN2, which recognizes Ii (BD PharMingen) and cerCLIP (BD PharMingen) which recognizes class II associated invariant chain peptide (CLIP) associated with class II molecules. Isotype-matched non-specific mAbs NS "IgM", NS "IgG1" and NS "IgG2a" [BD Pharmingen] were used in all experiments.

For cytofluorometry, secondary antibodies included goat anti-mouse (GAM) IgG Fc fragment specific and GAM IgM μ chain specific R-Phycoerythrin conjugates (Jackson Immunoresearch). For characterization of MM and DC by confocal microscopy, either a Cy3 labeled GAM IgG1-specific antibody, a FITC labeled GAM-IgG2a specific antibody, or a FITC-labeled GAM-IgM-specific antibody was used (all from ImmunoKontact, Wiesbaden, Germany).

3.2.5 Antibody Blocking Assay

The ability of several class II mAbs to block the expression of the D11 and D13 epitopes on B cell lines was measured by CELISA [31]. SAVC and MT14b were washed in PBS, plated in 96-well plates, treated with the appropriate blocking mAb for 1 hr at RT. The cells were then washed in buffer (3X) which contained 0.5% BSA in PBS, and incubated with either NFLD.D11 for SAVC or NFLD.D13 for MT14b for 1 hr at RT.

The cells were washed again (3X) and incubated with HRP-labeled GAM-IgM for 1 hr at RT. After further washing (3X), the cells were transferred to EIA flat bottomed plates and incubated with OPD substrate. The reaction time was 30 minutes in the dark, which was then stopped using sulfuric acid, and read on a Multiscan Spectrophotometer at 8590.

3.2.6 Cytofluorometry

Cell surface expression of HLA-DR molecules and epitopes was determined by cytofluorometry as previously described [31]. Briefly, cells were washed in PBS containing 0.5% BSA, 0.02% sodium azide (Wash Buffer), and incubated with the appropriate test mAb for 30 minutes at 4°C. The cells were then washed 2X with wash buffer, and incubated with a GAM-PE labeled conjugate (Jackson Immunoresearch) for 30 minutes at 4°C. The cells were washed in wash buffer, fixed in 1% paraformaldehyde (PFA) (Sigma) and analyzed using a FACS Star Plus machine.

3.2.7 Confocal Microscopy

MΦ and DC, grown in 16-well chamber slides, were washed with PBS, fixed in 2% paraformaldehyde for 15 min at 4°C, washed briefly in 10% CM and then permeabilized in 0.1% Tween-20 for 10 min at RT followed by blocking with 15% goat serum in PBS for 1 hour at RT. These cells were then incubated with the first primary mAb for 1 hour at RT. After washing with PBS containing 0.5% BSA (wash buffer) the appropriate GAM conjugate, labeled with either Cy3 (IgG1-specific) or FITC (IgG2a-specific) was then added for 1 hour, washed again in wash buffer followed by PBS. The

slides were mounted with aqueous anti-fade mounting media (DAKO) and analyzed using the confocal microscope (Olympus FluoView™ 300 confocal laser scanning microscope).

Chapter 3.3 Results

3.1 Allele-specific epitopes, D11^{+}0401 and D13^{+}0404, map to peptide binding pockets on the peptide binding groove of DRB1*04 molecules

The monoclonal antibodies, NFLD.D11 and NFLD.D13 were previously shown to be specific for DRB1*0401 (originally called Dw4) and DRB1*0404 (originally called Dw14), respectively [32]. These alleles differ by two conserved substitutions, βK71R and βG86V, which are key residues in pockets 4 and 1 of the peptide-binding groove. Since these residues are not unique to DRB1*0401 and 0404 molecules, we compared the mAb patterns of recognition on naturally occurring variants of DRB1*04 molecules ([31] and Table 1).

Table 1. Key residues in the HLA-DRB1* chain responsible for binding of NFLD.D2, NFLD.D11 and NFLD.D13 mAbs.

DRB1*	Amino Acids at DRB1 Chain Residues							Epitope		
	β37	β57	β67	β70	β71	β74	β86	NFLD.D2	NFLD.D11	NFLD.D13
*0401	Y	D	L	Q	K	A	G	+	+	-
*0413	Y	D	L	Q	K	A	V	+	+	-
*0404	Y	D	L	Q	R	A	V	+	-	+
*0408	Y	D	L	Q	R	A	G	+	-	-
*0405	Y	S	L	Q	R	A	G	+	-	-
*0410	Y	S	L	Q	R	A	V	+	-	-
*0101	S	D	L	Q	R	A	G	+	-	-
*0102	S	D	L	Q	R	A	V	+	-	-
*1402	N	D	L	Q	R	A	G	+	-	-

The extra reactivity of NFLD.D11 with 0413, a dimorphic variant (β86V) of 0401, clearly places 71K in the context of DRB1*04 as the critical residue in the $D11^{+}0401$ epitope. The $D13^{+}0404$ epitope is more complex as it is not present on 0408, a dimorphic variant (β86G) of 0404, thus implicating both β71R and β86V as critical residues. Given the role of β86 in anchoring the core peptide and β71 in peptide selection and T-cell recognition, we surmised that both $D11^{+}0401$ and $D13^{+}0404$ epitopes are similar to alloreactive T-cell defined determinants, Dw4 and Dw14, respectively [40].

To more definitively locate the $D11^{+}0401$ and $D13^{+}0404$ epitopes to the peptide binding grooves, we performed antibody-blocking assays. As shown in Figure 1, L243, a generic DR-specific mAb, frequently used to block T-cell recognition of allodeterminants on class II molecules, completely inhibited binding of NFLD.D11 and NFLD.D13 to B cell lines, SAVC (B1*0401) and MT14 (B1*0404), respectively. Similarly, NFLD.D2, specific for the shared epitope, QKRAA/QRRAA at β-chain positions 71-74, also partly inhibited NFLD.D11 and NFLD.D13 binding. However, the pan-DRB1*04 mAb, NFLD.D1, had no effect, most likely because its epitope is located in the β_2 domain, and binding here may not modulate the conformation of the peptide binding site. Neither anti-HLA-DP (NFLD.M67) nor anti-HLA-DQ (NFLD.M36) mAbs inhibited D11 or D13 (data not shown), further demonstrating that $D11^{+}0401$ and $D13^{+}0404$ epitopes are located exclusively on DR molecules.

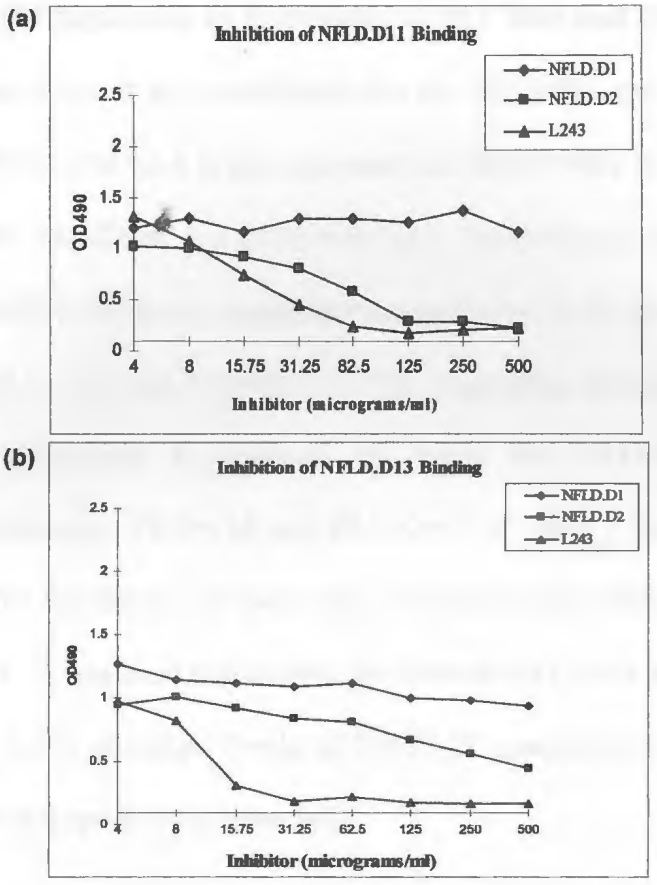

Figure 1. The D11^{+}0401 and D13^{+}0404 epitopes bind to DRB1*04 molecules over the peptide binding groove. Several HLA class II mAbs at titrating concentrations were tested for the ability to block the binding of NFLD.D11 to SAVC (a) and NFLD.D13 to MT14b (b) by CELISA. Briefly, the cells were washed, plated in 96 well plates, treated with the appropriate blocking mAb, before being tested for either D11^{+}0401 or D13^{+}0404 epitope expression. Blocking mAbs included L243 (pan-DR), NFLD.D1 (pan-DR4) and NFLD.D2 (shared epitope mapping to DRB1:70-74:QKRAA/QRRAA).

3.2 Role of Class II Chaperones in Expression of D11$^+$0401 and D13$^+$0404 Epitopes

Prior to this study, it was established that the D11$^+$0401 epitope is dependent on co-expression of HLA-DM as it is not expressed on DRB1*0401 molecules in the DM-null cells, T2-Dw4, BLS.Dw4, and SJO-Dw4 [31]. Interestingly, it was partly restored by DM in T2-Dw4DM, but more completely reconstituted in the gene complementation hybrids, BLS-Dw4 x .174 and SJO-Dw4 x .174, suggesting that additional factors are required for its expression. In contrast, we found that NFLD.D13 binds to the DRB1*0404 transfectants, T2-Dw14 and BLS-Dw14 (Figure 2 and data not shown), although its relative expression on these cells is significantly reduced compared to the 0404$^+$ BCL, MT14. A plausible explanation for reduced D13$^+$0404 and absent D11$^+$0401 in DM-null cells is the abundant levels of DR/CLIP complexes (Fig. 2) and reduced numbers of other DR/peptide complexes [41].

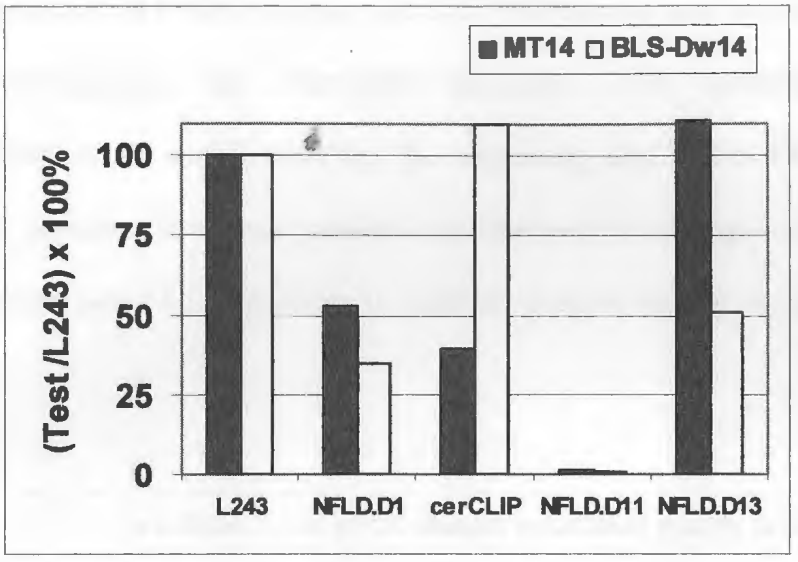

Figure 2. NFLD.D13 binding to DRB1*0401 molecules is inhibited by the presence of HLA-DM. The binding of class II mAbs, including L243 (pan-DR), NFLD.D1 (pan-DR4), NFLD.D11 (DRB1*0401 in DM^+ cells), NFLD.D13 (DRB1*0404 in DM^+ and DRB1*0401 in DM^- cells) and cerCLIP (DR/CLIP complexes) were tested on MT14b, and the DM null BLS Dw14. The results are expressed as a percentage of L243, with isotype negative controls subtracted.

3.2.1 Identification of a DM-antagonistic $D13^+0401$ epitope on DM^-, DRB1*0401 mutant cells

Analysis of NFLD.D13 on various DRB1*0401 transfectants revealed that the DM-null transfectants, T2-Dw4, BLS-Dw4 and SJO-Dw4 express $D13^+0401$ epitopes [Figure 3 and data not shown]. This finding was unexpected as NFLD.D13 displayed no cross reactivity with DRB1*0401 PBL B-cells or any homozygous BCL, other than those

which expressed DRB1*0404 ([32]; Table 1). Moreover, unlike the previously described DM-dependent D11^{+}0401 epitope, the D13^{+}0401epitope was abolished in DM-restored cells. Intriguingly, its expression correlated with abundant expression of DRB1*0401/CLIP complexes (Fig. 3), suggesting that bound CLIP could form the epitope. However, it was not possible to reconstitute this epitope on 0401-positive L-cell transfectants using CLIP peptides in standard peptide binding assays (SD, unpublished data).

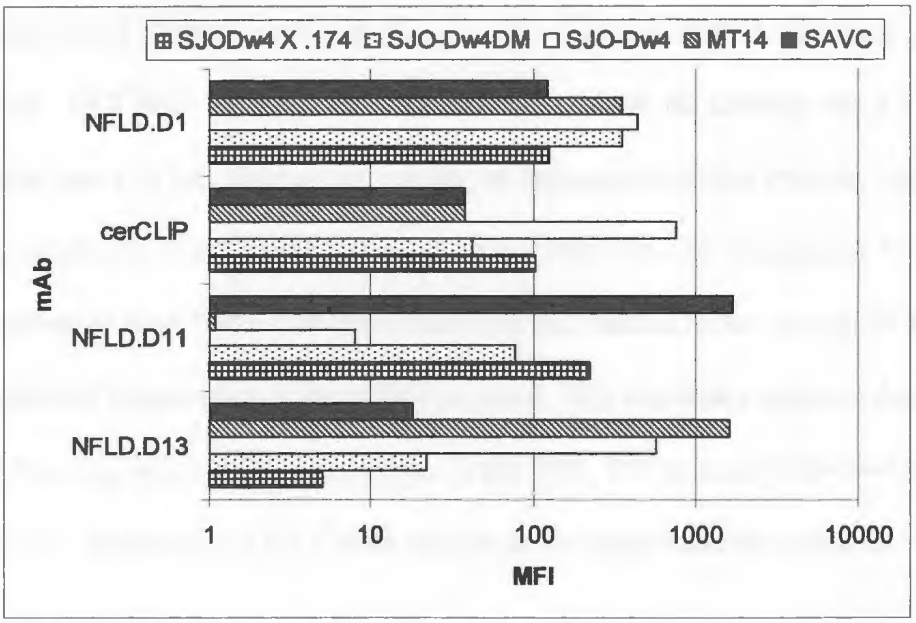

Figure 3. The D13^{+}0401 epitope is lost in the presence of HLA-DM. NFLD.D1, cerCLIP, NFLD.D11 and NFLD.D13 binding to DM null cells (SJO Dw4) and DM^{+} cells (SAVC, MT14b, SJO Dw4DM and SJO Dw4 x .174) were tested by cytofluorometry. Results are expressed as mean fluorescent intensity (MFI) shown with the appropriate isotype negative controls subtracted.

3.2.2 DM-dependent and independent epitopes on DRB1*04 molecules are not affected by DO expression

Since T2, BLS and SJO are both DO^- and DM^- we queried whether DO, a known regulator of DM, especially in B-cells [42, 43], influenced the generation of this epitope. To test this we used the hemizygous BCL 8.1.6 (DM^+, DO^+) and its daughter lines, 9.5.3 (DM^-, DO^+) and 5.2.4 (DM^-, DO^-), all transfected with DRB1*0401. Despite high levels of DRB1*0401 (NFLD.D1 binding), $D11^+$0401 was not expressed on either 9.5.3-0401 or 5.2.4-0401, but was reconstituted on 8.1.6-0401 (Fig. 4). Thus if DO has any role in forming the $D11^+$0401 epitope, it does so in the presence of DM. Similarly, expression of the DM^- $D13^+$0401 epitope on 9.5.3 and 5.2.4, and its absence on 8.1.6 or SAVC, suggests that DO has little or no control on expression of this epitope. Only 5.2.4-0404 was available to analyze expression of D13 (Table 2). By comparing D13 expression, normalized to total DRB1*04 (NFLD.D1) on this mutant to the normal BCL, MT14, it is clear that D13 expression is severely decreased. This correlates with our data presented in Fig 2 showing reduced D13-expression in the DM^-, DO^- mutant, BLS-Dw14. Thus, unlike $D13^+$0401, expression of $D13^+$0404 epitope is suboptimal in DM-negative cells.

Table 2. $D13^+$0404 Epitope is Incompletely Expressed in DM^-DO^- mutant cells.

mAb	5.2.4 0404		MT14b	
	MFI	% NFLD.D1	MFI	%NFLD.D1
L243	2963*	525	3226	203
NFLD.D1	564	-	1582	-
NFLD.D11	0	0	0	0
NFLD.D13	104	18	1270	80
cerCLIP	1827	323	260	16

* Isotype negative controls subtracted and results given in mean fluorescent intensity (MFI).

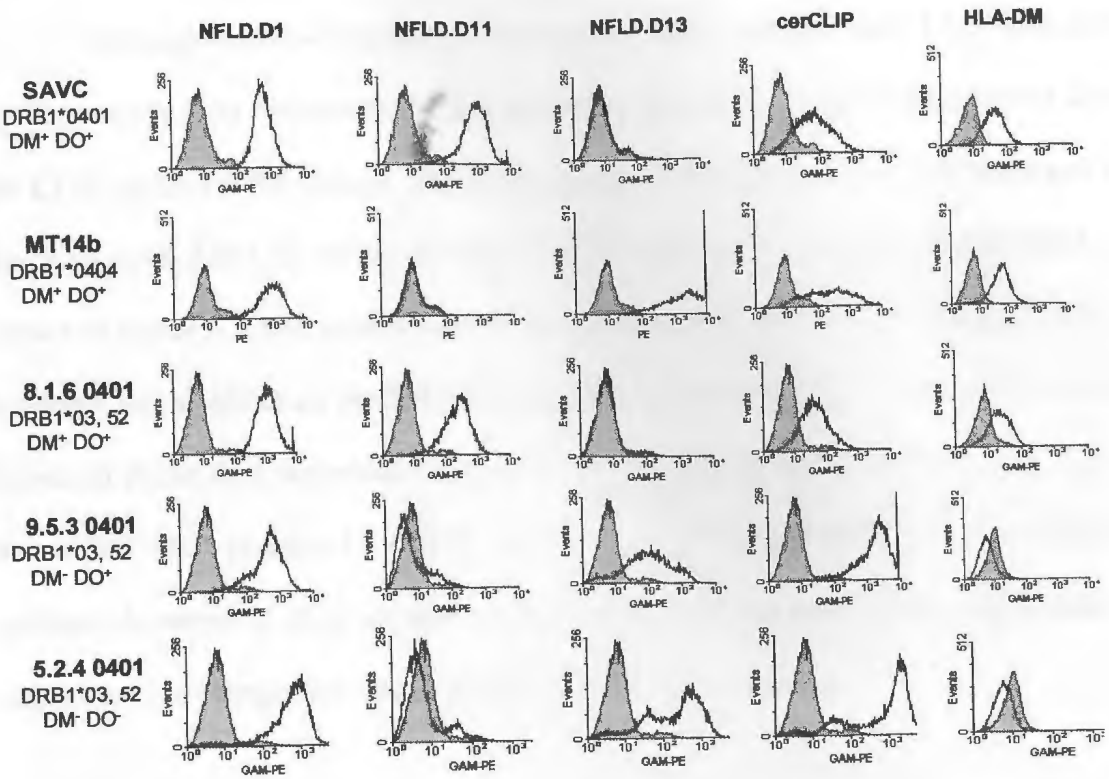

Figure 4. NFLD.D11 and NFLD.D13 binding to normal and antigen processing mutant human B cell lines. Specificity of HLA-DRB1*04 allotypic mAbs NFLD.D11 and NFLD.D13 was measured on the B cell lines SAVC (DRB1*0401), MT14b (DRB1*0404), 8.1.6 0401 (endogenous DRB1*03, *52), and the antigen presentation mutants 9.5.3 0401 (DM^-) and 5.2.4 0401 (DM^- and DO^-) using cell surface cytofluorometry. In addition, total surface HLA-DR4 (NFLD.D1) and DR/CLIP (cerCLIP) along with intracellular DM (MaP.DM1) was also measured on these BCL. A combined cocktail (IgG1, IgG2a and IgM) of isotype negative controls is shown with the grey fill, while the test mAbs are shown by solid blacks lines. Results are representative of three independent experiments.

3.2.3 D13$^+$0401 and D13$^+$0404 epitopes are not blocked by anti-DR/CLIP antibody.

Although previous attempt to reconstitute these epitopes with CLIP in peptide binding assays were not successful, the preceding data were strongly suggestive of a role for CLIP the D13$^+$0401 epitope. Therefore, we tested the ability of cerCLIP mAb and the blocking mAb, L243, to inhibit binding of NFLD.D13 and a control mAb anti-B2M. As shown in figure 5, L243 significantly blocked expression of both D13$^+$ epitopes and, as expected had no effect on the B2M. Surprisingly, pretreatment of the cells with cerCLIP increased rather than decreased D13 expression, especially on 9.5.3-0401. This suggest that if D13$^+$0401 is formed by CLIP complexes, cerCLIP and NFLD.D13 see different epitopes; however, it does not rule out the possibility that cerCLIP binding induces a conformational change that further exposes the D13$^+$0401 epitope.

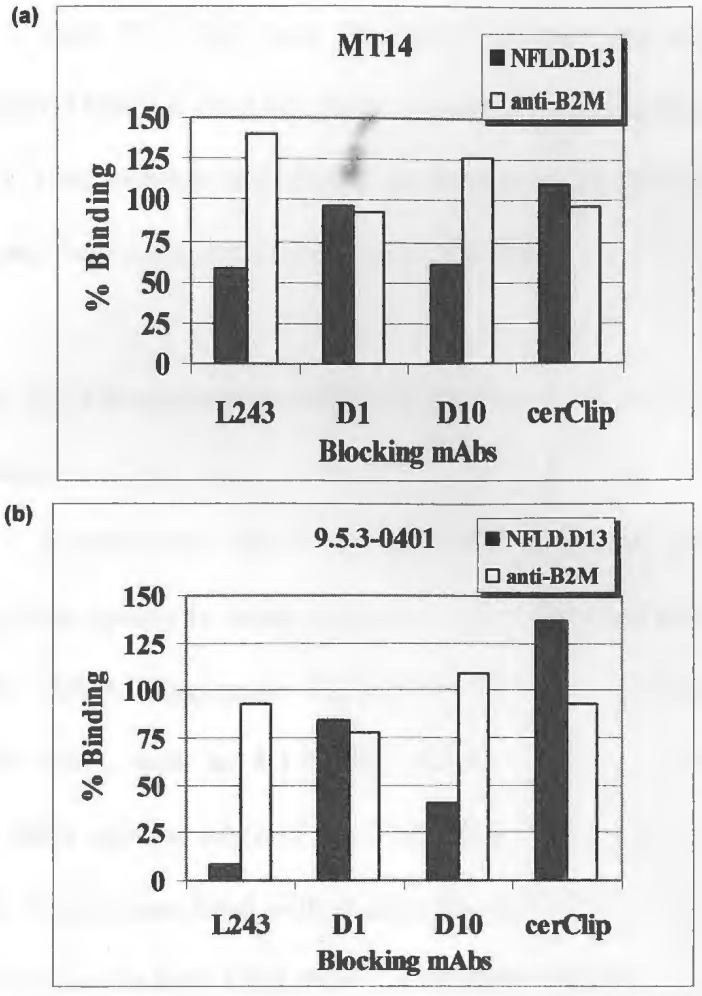

Figure 5. D13$^+$0401 and D13$^+$0404 epitopes are not formed on DR/CLIP complexes in normal and mutant BCL. The ability of class II mAbs to block D13$^+$0404 epitope expression on (a) MT14b and D13$^+$0401 epitope on (b) 9.5.3 0401 was tested by cytofluorometry and shown by black bars. The ability of these mAbs L243 (DR), NFLD.D1 (DR4), NFLD.D10 (Q at position 70 of the HLA-DRβ chain) and cerCLIP (DR/CLIP) to block β2-microglobulin (irrelevant IgM control-open bars) was also tested on each of the cell lines.

3.3 Variable Expression of D11$^+$0401 and D13$^+$0404 Epitopes on pAPC

Both D11$^+$0401 and D13$^+$0404 epitopes are expressed at reduced levels on peripheral blood B-cells but are up regulated by activation in culture (data not shown, and [32]). This, together with their high expression on EBV-transformed BCL, prompted us to study their expression on a variety of pAPC.

3.3.1 All DR-expressing DRB1*0401 lymphoid cells do not express the D11$^+$0401 epitope

Experiments, addressing D11$^+$0401 expression on other types of lymphoid cells were done mainly by using cells that were transfected with DRB1*0401 and if necessary DRA cDNA constructs. Non-DRB1*04 EBV-transformed BCL transfected with DRB1*0401, such as 8.1.6-0401 (shown in Figure 4) and PLH.Dw4 expressed the D11$^+$0401 epitope whereas the T-cell line transfectant, Jurkat-Dw4 did not [31]. This latter finding correlated with studies showing no expression on PHA-activated and DR-positive T-cells from DRB1*0401 individuals (data not shown).

Intriguingly, D11$^+$0401 epitope was present on Raji-Dw4 (unpublished data), but not on Daudi-Dw4 [31], although both are Burkitt's lymphoma derived B cell lines. Daudi differs from Raji and EBV-transformed lines in its complement of EBV genes and CD markers and moreover, is HLA-class I null due to a deletion of Chromosome 15 combined with a mutated β_2 microglobulin (B2M) gene on the remaining chromosome [44]. This, combined with the fact that substantial numbers of DRB1*04 molecules expressed by EBV-transformed BCL contain peptides derived from class I molecules

[45], suggested the possibility that the D11^{+}0401 epitope was formed on DRB1*0401/class I peptide complexes.

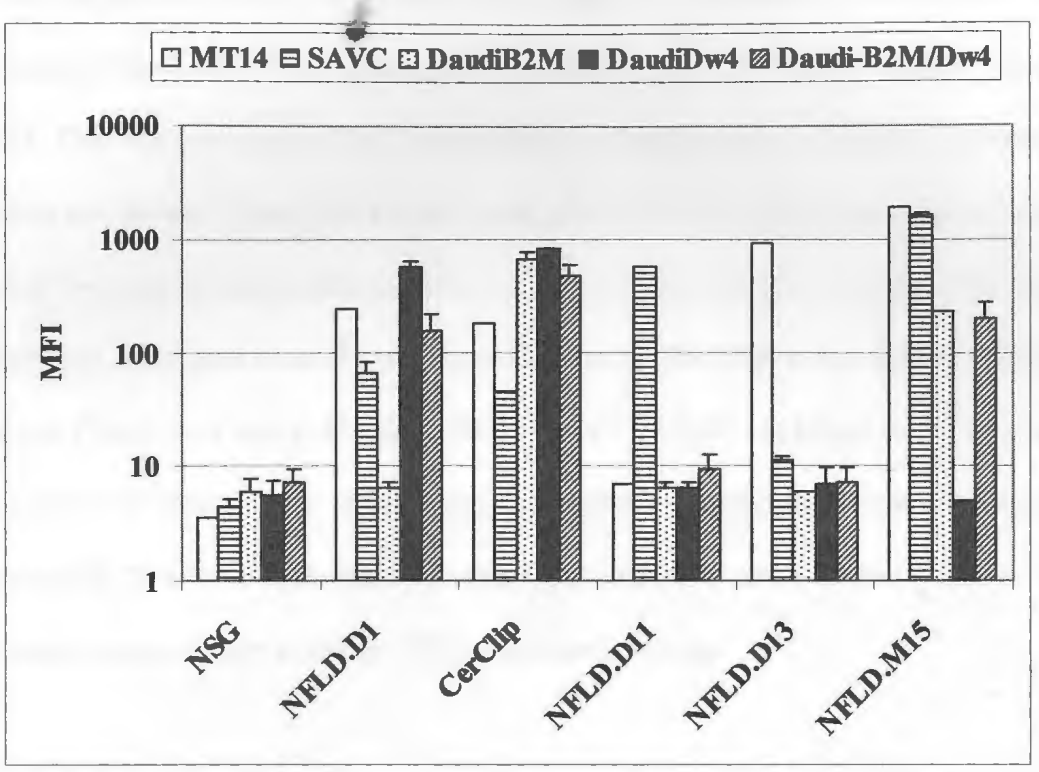

Figure 6. The class I null Burkitt's lymphoma cell line Daudi fails to express the D11^{+}0401 epitope. Cell surface expression of DRB1*04 (NFLD.D1), DR/CLIP (cerCLIP), D11^{+}0401 (NFLD.D11), D13^{+}0404 (NFLD.D13) and HLA-B (NFLD.M15) was measured on SAVC, MT14b, Daudi transfected with; DRB1*0401 (Daudi Dw4), beta-2-microglobulin (Daudi-B2M), and DRB1*0401 and beta-2-microglobulin (Daudi-B2M-Dw4) using cell surface cytofluorometry. Non-specific IgG is given as the negative control for this assay, with error bars measuring standard deviation from the mean of two experiments. Results are expressed as mean fluorescent intensity (MFI).

To test whether lack of D11$^+$0401 on Daudi-Dw4 was related to its HLA class I null status, we used Daudi-B2M, co-transfected with DRB1*0401. As shown in Figure 6, this was not proven correct, as despite HLA class I reconstitution (NFLD.M15$^+$) and expression of the DRB1*0401 transgene (NFLD.D1$^+$), the D11$^+$0401 epitope was not restored. This was not due to DM insufficiency as Daudi contains copious amounts of DM (data not shown). Daudi.Dw4 also lacked the D13$^+$0401 epitope although abundant DR/CLIP were complexes expressed (Fig. 6). Most likely, these CLIP complexes are on endogenously expressed class II complexes as there is little difference between cerCLIP binding to Daudi-Dw4 and to Daudi-B2M. Lack of D11$^+$0401 on Daudi could be due to any number of reasons (see discussion), but further experiments, described below, demonstrating D11$^+$0401 expression on other types of P-APC and a limited subset of NP-APC, would argue against a role for EBV associated proteins.

3.3.2 Investigation of D11$^+$0401 and D13$^+$ epitopes on MΦ and DC

Cytofluorometric analysis of D11$^+$0401 and D13$^+$0404 epitopes on CD14 positive mononuclear cells from DRB1*0401 and DRB1*0404 positive individuals expression on CD14$^+$ monocytes revealed that less than 2% of these cells were positive for either epitope. Using confocal microscopy and/or cytofluorometry, we further investigated whether the epitopes are expressed on cultured MΦ and DC.

3.3.2A Cytoplasmic expression of D11$^+$0401 epitope in activated MΦ and DC

DRB1*0401$^+$ blood derived MΦ and DC were analyzed by immunofluorescence and confocal microscopy with a panel of anti-CD mAbs to check the cell lineage. As

shown in Fig 7A, MΦ, treated or untreated with IFN-γ, correctly expressed the conventional myeloid marker CD68 but not the DC marker, CD83; however, they weakly expressed CD1a after activation with IFN-γ. As expected, all DC expressed the myeloid marker CD68, but TNF-α treated DC additionally up-regulated CD1a and CD83, indicating they are mature DC. Both sets of MΦ and DC strongly expressed HLA-DM and up-regulated HLA-DR after stimulation with either IFN-γ or TNF-α respectively (Fig 7A). DRB1*0401 (NFLD.D1) was expressed at higher levels in the DC compared with the MΦ (Fig 7B) and interestingly, most DRB1*0401 was found intracellularly in MΦ (Fig 7B), whereas, both cytoplasm and membrane staining (Fig 7B) is evident in the DC

The D11^{+}0401 epitope staining pattern differed between the two sets of pAPC. First it was barely expressed in untreated MΦ, which reflected overall reduced DR– expression (see L243 and NFLD.D1) in non-stimulated MΦ (Fig 7B); however, it was significantly up regulated by IFN-γ treatment, with most of the staining appearing intracellular. Although the D11^{+}0401 epitope was expressed in both immature and mature DC (Fig 7B), the staining was weaker and most of it appeared to be intracellular. In a separate experiment done on IDC and DC from the same donor, NFLD.D11 produced a homogenous staining pattern throughout the cytoplasm of MDC, while most of it appeared perinuclear in IDC. Importantly, no non-specific staining was observed with IgM or any other irrelevant immunoglobulins (Fig 7A and 7B), D11 did not bind DC from non-DRB1*04 individuals (data not shown).

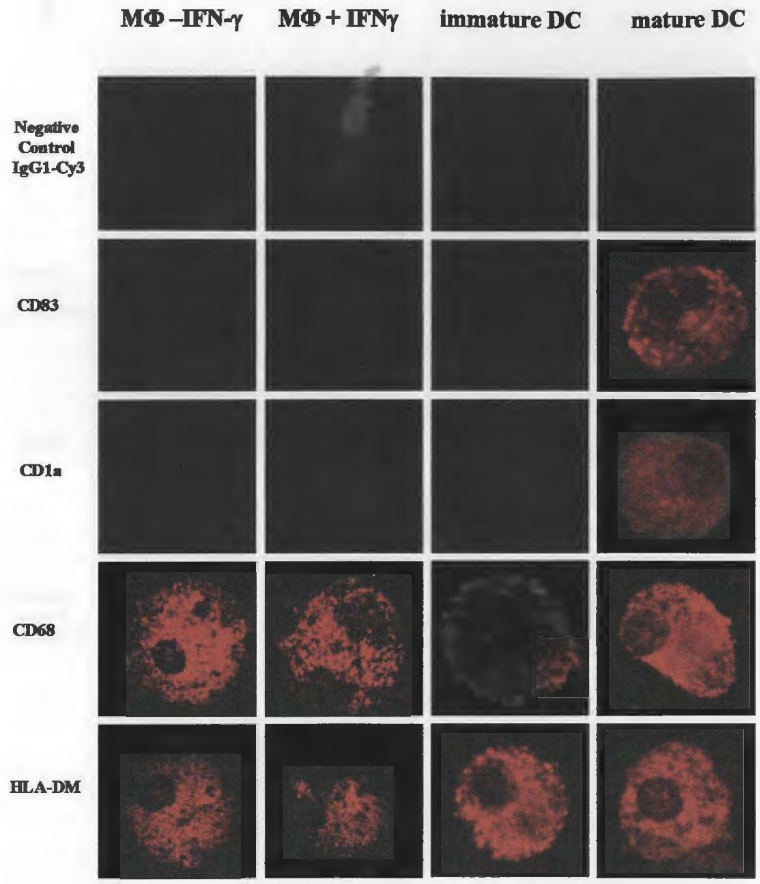

Figure 7A. Characterization of human DRB1*0401^{+} MΦ and DC with differentiation markers. Human MΦ and DC were isolated from a DRB1*0401^{+} healthy donor and assayed for differentiation markers by confocal microscopy to confirm their identity. Inactivated and activated MΦ, derived by treating the cells either with or without IFN-γ and immature and mature DC, derived from cells treated with and without TNF-α, were stained for CD1a (non-classical class II molecule), CD68 (myeloid cell marker), CD83 (marker for mature DC) and DM. The negative control is non-specific mouse IgG1. All pictures were taken at 400x (x2) and are representative examples of 80-100% of the cells tested.

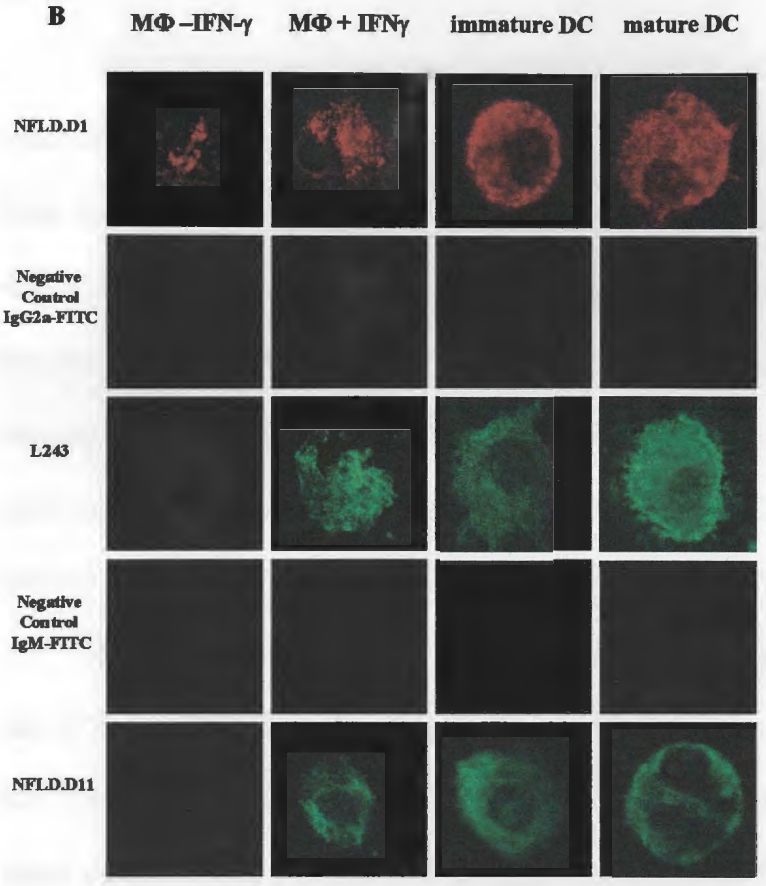

Figure 7B. Analysis of D11^{+}0401 expression on DRB1*0401^{+} MΦ and DC. MΦ treated with and without IFN-γ, and DC treated with and without TNF-α were tested for the D11^{+}0401 epitope using confocal microscopy. Other mAbs used were L243 (pan-DR) and NFLD.D1 (pan-DRB1*04). Isotypes of the test mAbs are IgG2a for L243, IgG1 for NFLD.D1 and IgM for NFLD.D11. Negative controls included non-specific mouse IgG1 followed by GAM-IgG1, Cy3 labeled (Figure 7A), non-specific mouse IgG2a-FITC labeled, and NS IgM-FITC labeled. All pictures were taken at 400x (x2) and are representative examples of 80-100% of the cells tested.

3.3.2B Differential expression of D11$^+$0401 and D13$^+$0404 epitopes on the cell surface of DC

Since the above experiments indicated most (or all) D11$^+$0401 expression was within the cytoplasm, cytofluorometry was performed on unfixed IDC and DC, harvested from the same DRB1*0401 donor. IDC and DC were also prepared from a DRB1* 0404 donor. As shown in Figures 8A, CD83 was not expressed on IDC from both individuals, but was strongly expressed on TNF-α treated cells; both CD86 and HLA-DRB1*04 were also up regulated, indicating a transition from immature to mature DC phenotype. All cells expressed CD1a (Figure 8B), and CD68, but lacked CD14, CD3 and CD19 (data not shown).

In contrast to the confocal analysis shown in Fig 7B, no D11$^+$0401 was evident in any of the combinations (Fig. 8B). However, D13$^+$0404 epitope was weakly expressed on IDC (about 10% of the gated cells) and was strongly up regulated on MDC (30-40% of gated cells). As expected it was co-expressed on DRB1*04-positive in both IDC and MDC from the DRB1*0404 donor; as well, it was present on about 1/2 of the CD1a-positive and 2/3 of the CD83$^+$ cells. The negative control IgM mAb stained less than 1% of the cells and interestingly, the D13$^+$0401 epitope was not expressed on IDC or MDC.

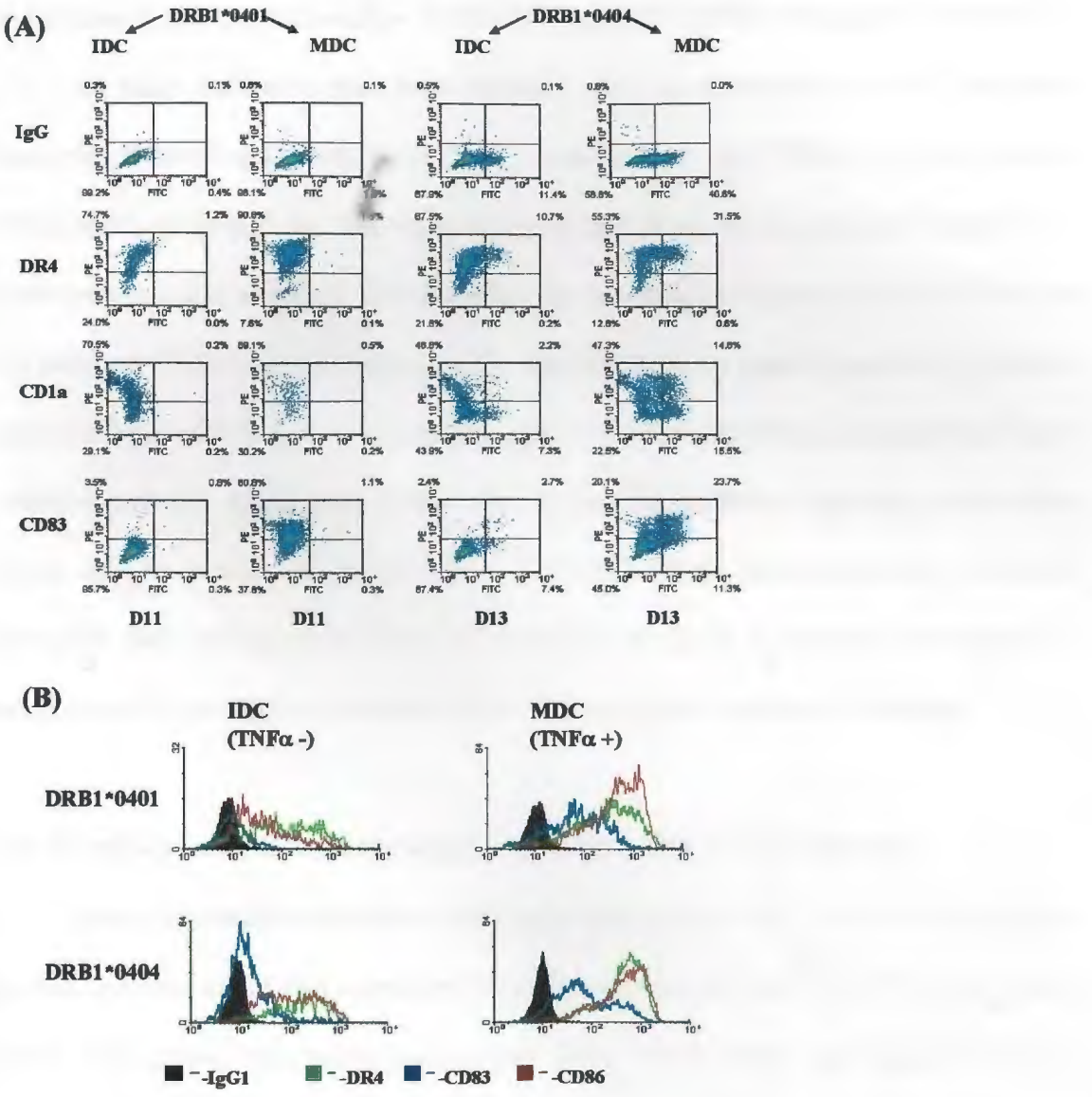

Figure 8. Surface expression of D11+0401 and D13+0404 epitopes on immature and mature DC. (A) DRB1*0401 and DRB1*0404 DC were stimulated with (mature DC) or without (immature DC) 10 :g/ml TNF-α, and assayed for surface DR4, CD1a, CD83 and either D11+0401 (left) or D13+0404 (right) by cytofluorometry. (B) Surface expression of DR4, CD83 and CD86 also shown for DRB1*0401 (top) and DRB1*0404 (bottom) immature and mature DC. In both (A) and (B), IgG is shown as the isotype negative control.

3.4 Cellular-restricted expression of D11$^+$0401 and D13$^+$0404 epitopes on NP-APC

An early indication that these epitopes may be restricted to pAPC was their absence on mouse L-cells, transfected with either DRA or DRB1*0401 or 0404; this was subsequently confirmed for this study (unpublished data). As reported in Chapter 2, a collaboprative effort revealed no cell surface or intracellular expression of D11$^+$0401 or D13$^+$0404 on DRB1*04$^+$ cultured synovial fibroblasts or on class II positive fibroblasts in diseased synovial tissues. Interestingly, class II positive fibroblasts co-expressed class II chaperones and several endosomal and lysosomal markers, suggesting that either cellular specific peptides or proteases might be necessary for their expression. To further investigate their cellular specificity, we extended the study to include carcinoma and melanoma cell lines, which expressed either endogenous or transfected DR genes.

3.4.1 Carcinoma cell lines do not express D11$^+$0401 or D13$^+$0401 epitopes

Since carcinoma cells express little or no endogenous class II or chaperone genes, the cells, whether or not they expressed endogenous or transfected DRB1*04 genes, were treated with IFN-γ. The breast cancer cell lines, T47D Dw4 and MCF-7 Dw4 up regulated DR after IFN-γ treatment, but did not express D11$^+$0401 (Fig 9). The control BCL SAVC expressed copious amounts of DR (L243) and DRB1*04 (NFLD.D1) on its cell surface, as well as abundant D11$^+$0401 epitope. Lack of D11$^+$0401 on the BCCL is not due to DM insufficiency as significant amounts were found in both BCCL, comparable to that found in SAVC (Fig. 9 and data not shown). Similarly, the D11$^+$0401

epitope was not expressed on the human intestinal epithelial carcinoma cell line transfectants HT29 Dw4 treated with IFN-γ (data not shown).

The cell line MDA MB 435 transfected with DRA and DRB1*0401 (MDA MB 435 Dw4, previously described as a BCCL but more recently suggested to be of melanoma origin [46] showed strong up-regulation of total DR, and DRB1*04 (Fig 9), supposedly due to expression of the transfected DRB1*0401 and endogenous DRB1*0405 alleles. As expected the $D11^{+}0401$ epitope was not found on the surface of the un-transfected MDA MB 435, even though there was abundant DM present (data not shown). Surprisingly, both stimulated and un-stimulated MDA MB 435 Dw4 cells expressed the $D11^{+}0401$ epitope, although it was increased in the presence of IFN-γ (Fig 9). Therefore this demonstrated for the first time the expression of the $D11^{+}0401$ epitope on a NP-APC.

The DRB1*0401-positive melanoma cells 1359 Mel and DM331 expressed significant amounts of constitutive DR and DRB1*04 which were further up-regulated by IFN-γ (Fig 9b). Interestingly, stimulation with IFN-γ resulted in slight expression of the $D11^{+}0401$ epitope on both 1359 Mel and DM331 (Fig 9). As seen in Fig 9c, these melanoma cells also expressed abundant DM, DO and Ii, although at levels slightly less than SAVC. This indicates that although DM is required for $D11^{+}0401$ epitope expression, other, currently unknown factors are also required for its expression.

(a)

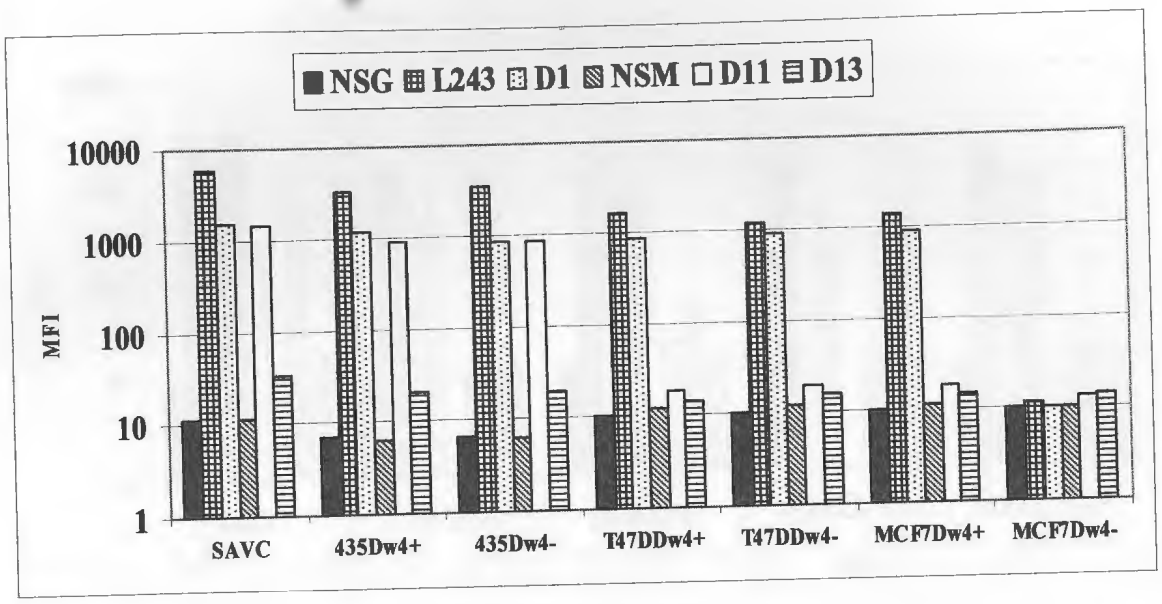

Figure 9. Continued on Next Page.

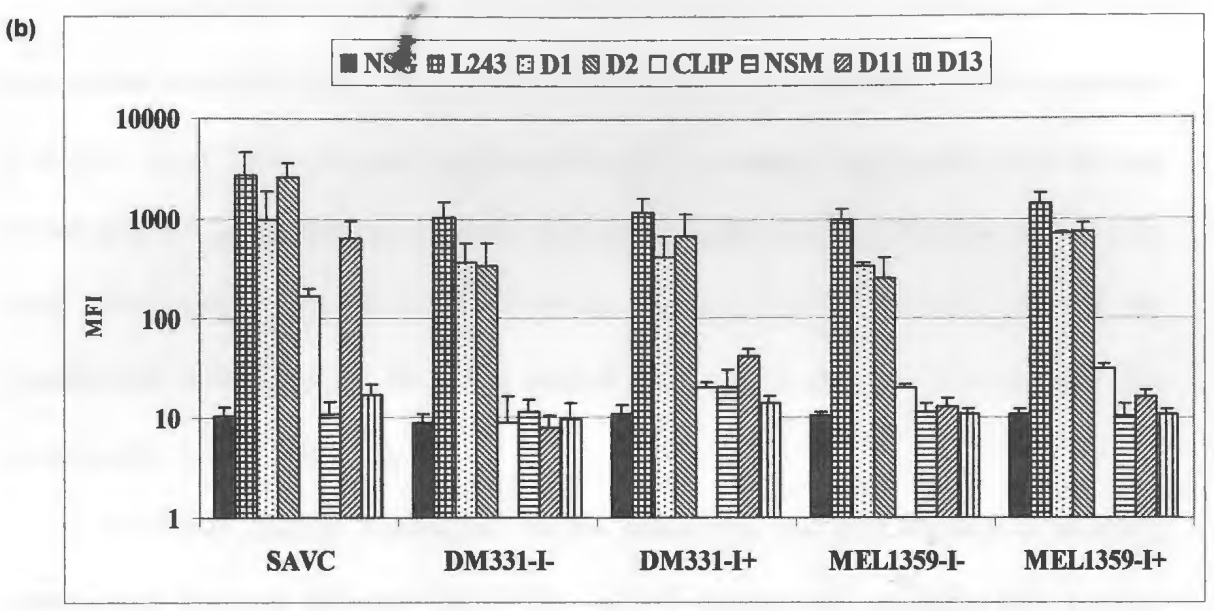

Figure 9. Expression of the D11^{+}0401 epitope on DRB1*0401^{+} human breast cancer and melanoma cell lines. The D11^{+}0401 epitope, DR (L243), DRB1*04 (NFLD.D1) and DR/CLIP (cerCLIP) expression was tested on (a) MDA MB 435 Dw4 (endogenous DRB1*0405, 13), T47D Dw4 (endogenous DRB1*0102), MCF-7 Dw4 (endogenous DRB1*03, 15) and (b) DM331 (endogenous DRB1*0401, DRB1*01) and 1359 Mel (endogenous DRB1*0401, DRB1*0301) by cell surface cytofluorometry. Cells were either left untreated (-) or treated with 500U interferon-γ/ml (+) for 4 days. Intracellular expression of DM, DO and Ii (LN2) was measured by intracellular cytofluorometry (c) in DM331 and 1359 Mel. The DRB1*0401^{+} BCL SAVC was used as a control for class II epitope expression, with results presented as means of two separate experiments.

3.4.2 D11^{+}0401 epitope expression on MDA MB 435 Dw4 shows differential effects of IFN-γ.

To investigate the kinetics of D11^{+}0401 epitope formation, MDA MB 435 Dw4 was treated with IFN-γ for 24 hour increments, ending with 120 hours, or left untreated (Fig 10). Total DR expression on these cells did not change significantly over the test period and was comparable to total DR expression on the control B cell line SAVC (Fig 10a). Similarly, expression of DRB1*04 on untreated MDA MB 435 Dw4 was not significantly different from the IFN-γ treated cells, and in fact was greater than that expressed by SAVC (Fig 10b).

D11^{+}0401 epitope expression on the melanoma cell line showed a different pattern than the total DR and DRB1*04. SAVC consistently expressed this epitope throughout the assay, peaking at 72 hours (Fig 10c). There was a delay in the formation of the D11^{+}0401 epitope on the surface of MDA MB 435 Dw4 treated with IFN-γ, with the lag period being between 24 and 48 hours. After 48 hours, the D11^{+}0401 epitope was expressed, peaking at 72 hours, similar to total DRB1*04 expression. Therefore the maximal presence of the D11^{+}0401 epitope at 72 hours may be explained due to increased DRB1*04 expression, or perhaps due to an increase in an IFN-γ inducible cellular component which is involved in the epitope formation.

On the untreated MDA MD 435 Dw4 cells there was a drop in D11^{+}0401 epitope expression at 48 hours, although it continued to increase until 120 hours (Fig 10c). At this time point, D11^{+}0401 expression was comparable to the treated cells, indicating that the un-stimulated cells were producing a factor that was behaving in a manner similar to

IFN-γ. This factor is not DM, with intracellular DM failing to accumulate within the untreated MDA MB 435 Dw4 cells (Fig 10d). The expression of DM throughout the assay was similar between the melanoma cells and SAVC, indicating that DM is not responsible for the differential kinetics of the D11^{+}0401 epitope in the three groups of cells.

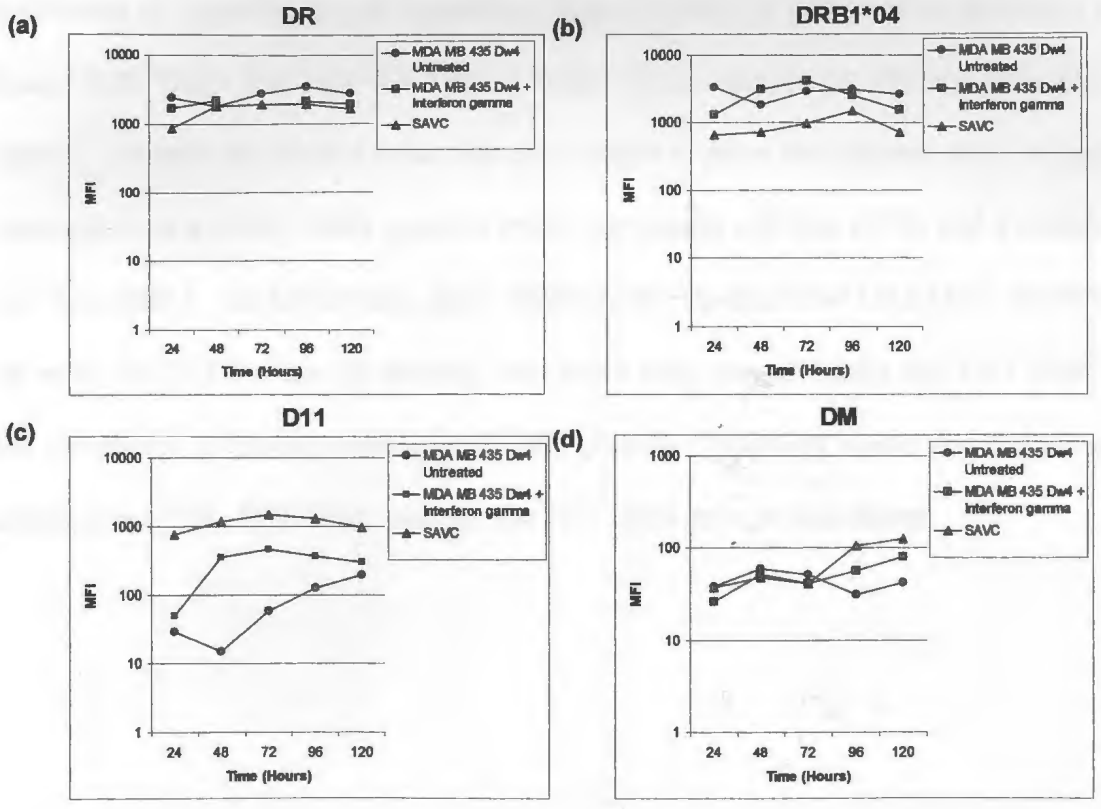

Figure 10. The D11^{+}0401 epitope on MDA MB 435 Dw4 untreated and IFN-γ treated shows differential expression. The cell surface expression of DR (a), DRB1*04 (b), the D11^{+}0401 epitope (c) and intracellular DM (d) was tested on the BCL SAVC (▲), MDA MB 435 Dw4 untreated (●), and treated with 500U interferon-γ/ml (■) by cytofluorometry. Expression was measured at 24, 48, 72, 96 and 120 hours after IFN-γ stimulation. The results are expressed as mean fluorescent intensity (MFI), with the appropriate isotype negative controls subtracted.

3.4.3 The D13^{+}0404 Epitope is not expressed on NP-APC

As previously shown, the D13^{+}0404 epitope is strongly expressed on EBV-transformed B cells and DC. However, it was not expressed on human synovial fibroblast cell lines (T Frost, BSc. Honors Thesis) and mouse fibroblasts transfected with DRA + DRB1*0404 (data not shown). Similarly, immunohistochemical studies performed on synovial tissues containing large numbers of DR positive fibroblast cells from DRB1*0401 and 0404 RA patients lacked these epitopes (S. Oldford, BSc. Honors thesis). To address whether other cell types might express this epitope, we analyzed its expression on a DRB1*0404 positive breast carcinoma cell line BT20 and a melanoma cell line DM13. Unfortunately, since BT20 poorly up-regulated DRB1*04, determined by mAb NFLD.D1 (data not shown), we cannot state unequivocally that D13^{+}0404 was not expressed. However, analysis on DM13 (Figure 11) clearly shows that despite good expression of DR, DRB1*04 and DM, the D13^{+}0404 epitope was absent.

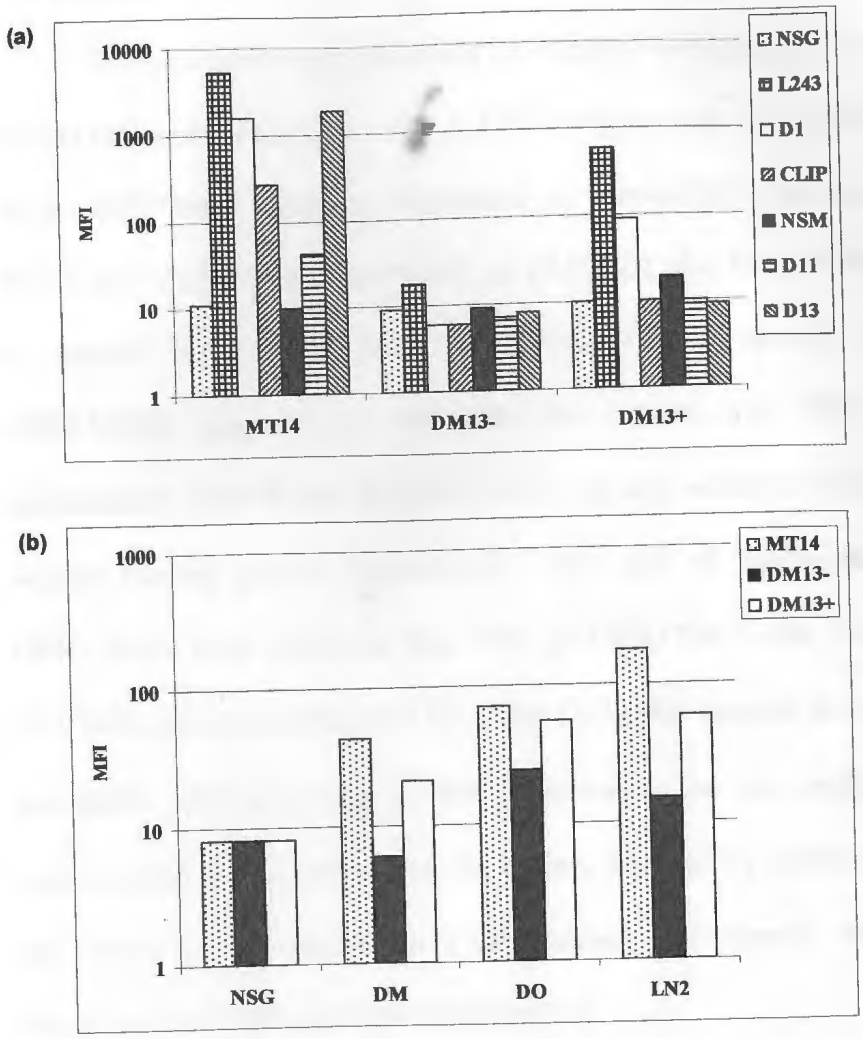

Figure 11. DRB1*0404^{+} melanoma cells do not express the D13^{+}0404 epitope. The expression of total cell surface DR (L243), DRB1*04 (NFLD.D1), DR/CLIP (cerCLIP), and the D11^{+}0401 and D13^{+}0404 epitopes was measured by cytofluorometry on the BCL MT14b and the DRB1*0404^{+} melanoma cell line DM13 untreated (-) and treated with 500U interferon-γ/ml for 4 days (+), as shown in (a). Intracellular DM, DO, and Ii (LN2) was also measured by cytofluorometry as shown in (b). All results are presented as mean fluorescent intensity (MFI), with the appropriate isotype controls given as either NSG or NSM.

3.4 Discussion

We have previously described HLA-DRB1*04-specific epitopes defined by mAbs NFLD.D11 and NFLD.D13. NFLD.D11 recognizes an HLA-DM dependent epitope on HLA-DRB1*0401 molecules expressed on normal BCL, but not on DM^- BCL [31]. NFLD.D13 recognizes DRB1*0404 on DM^+ BCL and DRB1*0401 on DM^- BCL [33]. In normal BCL, NFLD.D11 and NFLD.D13 are specific for DRB1*0401 and DRB1*0404, respectively, two DRB1*04 alleles that differ by two conserved substitutions, βK71R and βG86V, which are key residues in pockets 4 and 1 of the peptide binding groove, respectively. The lack of binding of NFLD.D11 to non-DRB1*0401 BCL indicates that 71K of DRB1*04 is the only critical residue for $D11^+$0401 epitope generation [31]. The $D13^+$0404 epitope is dependent on both β71R and β86V. Given the role of β86 in anchoring the core peptide and β71 in peptide selection and T-cell recognition, we believe that the $D11^+$0401 and $D13^+$0404 epitopes are similar to the alloreactive T-cell defined determinants, which are influenced by recognition of MHC-peptide complexes [31].

The DM-dependent specificity of $D11^+$0401 is similar to a Dw4-specific T cell clone LG.B10, which was also dependent on DM for recognition and proliferation [31]. Interestingly, LG.B10 proliferative responses to Daudi Dw4 was reduced compared to other T cell clones, providing further evidence that NFLD.D11 is recognizing a similar determinants as the T cell clone LG.B10. Supporting this, the absence of the $D11^+$0401 and $D13^+$0404 epitopes on mouse L-cell transfected with either DRB1*0401 or DRB1*0404 also suggests that bound peptide, as well as critical residues on the peptide

binding groove contribute to the generation of these epitopes. This was supported by the fact that D11$^+$0401 and D13$^+$0404 epitopes were both blocked by mAbs that bind over or in the peptide groove, but not by mAbs that bind to the β_2 domain, which confirms that these epitopes are located on the class II peptide binding groove

Both the D11$^+$0401 and D13$^+$0404 epitopes are preferentially expressed on EBV-transformed BCL, reminiscent of the UL-5A1 mAb described by Wölpl et al, 1998 [47]. UL-5A1, which distinguishes DRB1*0101 molecules bound with HLA-A2 derived peptides was found to specifically bind to activated BCL, and not on NP-APC, suggesting that the antigen processing pathway within these cells may be paramount for the generation of this epitope. Likewise, preferential expression of D11$^+$0401 and D13$^+$0404 on EBV-transformed B cells suggests that an activated phenotype, either by the expression of cellular-specific proteins or the mobilization of necessary compartmentalization and antigen processing mechanisms, is important for the expression of the D11$^+$0401 and D13$^+$0404 epitopes. In addition to BCL, expression of the D11$^+$0401 and D13$^+$0404 epitopes on IFN-γ activated MΦ and TNF-α activated HLA-DRB1*0401$^+$ DC supports the notion that an active cell phenotype, perhaps specific to P-APC is required for the formation of these epitopes.

Although DM is required for D11$^+$0401 epitope formation, its absence on NP-APC, which express copious DM suggests that other factors are necessary for epitope formation. One possibility could be that the proteolytic environment within activated B cells, MΦ and DC is changed in response to different exogenous stimuli. For example, DC are known to increase proteolysis [48], lysosomal function [49] and presentation of

exogenous antigen [50] after activation. We found that lysosomal and cytoplasmic cysteine proteases are required for the generation of the $D11^{+}0401$ epitope in BCL (Chapter 4), so it is possible that the same proteases are required for the generation of the $D11^{+}0401$ epitope in MΦ and DC. Having said this, the lack of blocking of NFLD.D11 and NFLD.D13 binding to normal and DM^{-} BCL by cerCLIP indicates that these epitopes are not formed on DR/CLIP complexes. This does not disprove however, that other sets of peptides are involved in forming the $D11^{+}0401$, $D13^{+}0404$ and $D13^{+}0401$ epitopes. This could be due to conformational changes induced by DM, as with the 16.23 epitope [51], or through direct recognition of class II-associated peptides. The involvement of specific peptides in the formation of these epitopes is currently under investigation.

Another possibility is that the cellular organization of intracellular compartments or cell surface microdomains such as lipid rafts and tetraspan microdomains may be different between cells that are positive and negative for these epitopes. Related to this, the P-APC-restricted UL-5A1 epitope is present within CDw78-defined tetraspan microdomains on the surface of B cells [52] and DC [53], suggesting that these microdomains may differ between P-APC and NP-APC. Similarly, we have found that the $D11^{+}0401$ and $D13^{+}0404$ epitopes are substantially decreased by tetraspan-disrupting chemicals such as saponin (Chapter 4), indicating that these microdomains may be the additional factor responsible for the presence of these epitopes on P-APC. Related to this, we have observed that CDw78 microdomains are significantly reduced on $D11^{+}0401$ epitope negative cells such as 9.5.3 0401 and T2.Dw4 (data not shown).

Unlike B cells, MΦ and DC, which constitutively express co-stimulatory molecules and MHC class II on their cell surfaces, non-professional APC, must up-regulate these molecules in the presence of inflammatory stimuli. For example, in the presence of IFN-γ, microglial cells [54], intestinal epithelial cells [55], and keratinocytes [56] have all been reported to increase co-stimulatory and HLA class II expression. Interestingly, we found that the $D11^{+}0401$ and $D13^{+}0404$ epitopes were not expressed on human breast carcinoma and melanoma cell lines expressing abundant DRB1*04 and DM. Also, human fibroblasts from patients with rheumatoid arthritis, which express copious amounts of DM (Chapter 2), and murine fibroblasts transfected with DRB1*0401 or DRB1*0404 failed to express the $D11^{+}0401$ and $D13^{+}0404$ epitopes. Although it is possible that the requisite proteases are not up-regulated in these cells, this seems unlikely since published data indicate that various cell lines, including human breast cancer cell lines [57, 58], colon carcinoma cell lines [59], and HT29 [60] express abundant levels of cathepsin B, D and L. Also, a human keratinocyte cell line up-regulates cathepsin S [61] and gastric epithelial cells up-regulate cathepsins B, L, S and D [62] after IFN-γ treatment. Another possibility could be that the source of the putative peptide(s) may not be present in these cells. Further analysis of the proteolytic environment within these cells should answer these questions.

The finding that the melanoma cell line MDA MB 435 Dw4 expressed the $D11^{+}0401$ epitope even without IFN-γ treatment suggests that these cells may have phenotypic features comparable to P-APC. In the presence of IFN-γ there was more surface $D11^{+}0401$, which peaked earlier than the $D11^{+}0401$ epitope on the un-stimulated

cells. Both treatments produced equivalent amounts of DM, confirming that other factors in addition to DM are required. These factors may include proteases, expression of cell specific proteins shared between melanoma cells and P-APC, or similar organization of tetraspan microdomains.

DM negative cells, such as 9.5.3 0401, T2 Dw4, BLS Dw4 and SJO Dw4 express copious amounts of MHC class II/CLIP complexes [63], and the $D13^{+}0401$ epitope. The presence of the $D13^{+}0401$ epitiope on these cells suggests that CLIP is somehow implicated in the formation of this epitope. However, the cerCLIP mAb did not block the expression of the $D13^{+}0401$ epitope, suggesting that CLIP is not involved in the formation of this epitope. Interestingly, we have observed that cells that express $D13^{+}0401$ are also CDw78 microdomain negative, which may explain the presence of this epitope on DM^{-} cells.

Daudi, a Burkitt's lymphoma cell line, which is class I null, and class II positive, including DM and Ii, may provide some insight regarding HLA-DRB1*04 epitopes. For example, Daudi transfected with DRB1*0401 failed to express the $D11^{+}0401$ and $D13^{+}0401$ epitopes. Its high expression of DM likely explains the absence of $D13^{+}0401$, but the absence of $D11^{+}0401$ suggested the possibility of an epitope derived from class I, a result that was not changed by the stable transfection of beta-2-microglobulin into these cells. Related to this, we found that a mAb that recognizes HLA-B caused partial inhibition of $D11^{+}0401$ and $D13^{+}0404$ epitope expression on normal BCL. This inhibition suggests two possible mechanisms for the involvement of class I in the formation of the $D11^{+}0401$ and $D13^{+}0404$ epitopes. One possibility is that $D11^{+}$ and

$D13^+$ DRB1*04 molecules are presenting HLA-B derived peptides in their peptide binding grooves. As discussed above, another possibility is that the HLA class II molecules that form the D11 and D13 epitopes are located within large tetraspan-protein complexes that also include HLA class I. It has already been shown that these protein microdomains are disrupted in the antigen processing mutant cell T2 [53]. It is possible that a similar defect in Daudi is the reason why it is $D11^+$0401 null. Related to this, CD82 has been found associated with HLA class I within normal BCL [64], so it is conceivable that a complex between either of these molecules with DRB1*0401 results in formation of the $D11^+$0401 epitope.

We have shown that mAb-defined $D11^+$0401 and $D13^+$0404 epitopes, which are similar to T cell epitopes, are preferentially expressed on P-APC, suggesting that factors unique to these cells are responsible for generating these epitopes. This may involve the up-regulation of unique proteases or cellular proteins that are broken down within the class II processing pathway differently than in non-professional APC. The involvement of DM and the location of key residues for NFLD.D11 and NFLD.D13 recognition of DRB1*04 molecules strongly suggests that class II-associated peptides are involved in epitope formation. This may be due to direct recognition of MHC-peptide, or through recognition of peptide-dependent conformational determinants on the class II molecules. Alternatively, there is evidence that the DRB1*04 molecules that these mAb are binding are located within tetraspan microdomains. These are active areas of research, and should lead to further knowledge of these DRB1*04 epitopes.

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CHAPTER 4

Differential Modulation of Tetraspan-Associated DRB1*04 Epitopes by Cellular Proteases

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Abstract

The expression of MHC class II/peptide complexes on the cell surface results from a complex intracellular process involving HLA-DM and numerous proteases. We have previously described antibody-defined epitopes on DRB1*0401 and DRB1*0404 molecules which are largely restricted to professional antigen presenting cells. The DRB1*0401-specific epitope, defined by NFLD.D11, is dependent on the presence of DM, whereas the DRB1*0404-specific epitope, defined by NFLD.D13 is less dependent on DM for its expression. Intriguingly, NFLD.D13 also recognizes an epitope on DRB1*0401 molecules in DM-negative mutant B cells. The limited cellular distribution of these epitopes and their different requirements for DM suggest alternative antigen processing or trafficking mechanisms are implicated in their generation. We performed co-localization studies using confocal microscopy for markers for class II antigen processing pathway compartments and protease inhibitors to identify the intacellular mechanisms that generate these epitopes. The $D11^{+}0401$ and $D13^{+}0404$ epitopes both follow the classical endocytic pathway en route to the cell surface in normal B cell lines (BCL) and are implicated in tetraspan protein microdomains on the cell surface. In normal and mutant BCL the DRB1*0401 epitopes recognized by NFLD.D11 and NFLD.D13 are influenced by endosomal and cytoplasmic cysteine proteases, but not aspartyl proteases. Unlike the DRB1*0401 epitopes, the D13-defined DRB1*0404 epitope was not affected by the protease inhibitors tested here.

4.1 Introduction

MHC class II molecules are membrane glycoproteins formed from a non-polymorphic α chain and a polymorphic β chain [1-2] and are expressed constitutively on the surface of professional antigen presenting cells (P-APC) such as B cells, macrophages (MΦ) and dendritic cells (DC). They also can be up-regulated on non-professional (NP-APC) by inflammatory mediators such as interferon-γ (IFN-γ) [3]. MHC class II molecules bind short peptides between 13 to 25 amino acids long [4] with polymorphisms in the peptide binding groove making it possible for the MHC class II molecules to bind a wide array of peptides. The binding capacity of any given peptide to a MHC class II molecule is dependent on the primary sequence of the peptide and on the allelic variation of the residues within the peptide binding groove. For HLA-DR molecules, the peptide is bound within a long cleft formed between two antiparallel helical structures and a floor formed by an eight-stranded β-sheet [4]. The peptides are held in place by hydrogen bonds between the peptide backbone and the residues along the class II molecule itself. Bound peptide residues that project downwards into the peptide binding groove are accommodated by allele-specific pockets found within the class II molecule. It is the class II pocket shape and composition that determines the amino acid make-up of the peptides that can bind to the class II molecule, and these qualities differ greatly amongst HLA-DRB alleles.

MHC class II molecules are generated in the endoplasmic reticulum (ER) and associate with the invariant chain (Ii) [5-7]. Three MHC αβ dimers bind to three Ii proteins forming a nonomeric complex [1] that travels from the ER through the trans

golgi complex to a series of endocytic compartments and eventually to lysosome-like structures known as MHC class II peptide loading compartments (MIIC) [8]. Within these MIIC, Ii is slowly degraded in a stepwise manner leaving a small peptide known as CLIP, an acronym for class II associated invariant chain peptide, in the class II peptide binding groove [9]. HLA-DM facilitates the exchange of CLIP [10-12], for a peptide generated from exogenously-derived proteins or from endogenous proteins that intersect the class II pathway. DM is also important in the stabilization of the "peptide-free" or empty conformation of the MHC class II molecule [13-15] before enhancing the binding of high stability peptides in the groove of the MHC class II molecule [16-18]. This mature peptide-MHC complex (pMHC) moves to the cell surface to interact with the $CD4^{+}T_{H}$ T cell receptor (TCR).

Recent studies suggest that mature MHC/peptide complexes (pMHC) travel to the cell surface as large protein aggregations that include the tetraspanin proteins CD81, CD82 and CD63 as well as DM [19]. Although the function of these aggregates is still unclear, they may stabilize the pMHC or participate in signal transduction by surface receptors [20]. These tetraspan complexes have been shown to exist on the cell surface where they likely concentrate specific pMHC complexes, better enabling T cell activation [21]. This concentration of relevant pMHC on the APC, and the TCR specific for these pMHC on the surface of the T cell results in the formation of the immunological synapse, which also includes a variety of costimulatory, accessory and signaling molecules.

As stated above, proteolytic events play dual roles in the generation of mature pMHC complexes. In addition to digesting endocytosed antigen into small peptides that

bind class II molecules within lysosomal compartments, cellular proteases also gradually destroy Ii. Depending on the cell type, different proteases have been implicated in the breakdown of Ii. For example, cathepsin S is the major protease in B cells and DC [22], whereas cathepsin L is implicated in Ii degradation in mouse thymic epithelial cells [23]. Recently cathepsin S, and not cathepsin L, has been shown to degrade Ii in NP-APC such as epithelial cells [24], suggesting that the cellular processing machinery differs between cell types.

The majority of class II associated peptides results from degradation of proteins in the highly acidic and protease-rich endocytic compartments [25]. The major groups of proteases in these compartments are cathepsins, which can be divided into two major species, cysteine and aspartyl. The dispensability of individual cathepsins has been shown by knock-out mice, which have normal immune cell numbers and as broad peptide arrays as wild type mice [26-27] and the ability of numerous proteases to degrade the same antigen [28-30]. Recent data indicate that class II peptides can be generated from cytoplasmic processing of endogenous antigen, confirming that there is more than one pathway for class II processing [31-32].

We have previously described allele-specific epitopes on DRB1*0401 and DRB1*0404 molecules which are constitutively expressed on EBV-transformed B cell lines (BCL) (Chapter 3). The expression of the DRB1*0401-specific epitope, defined by the mAb NFLD.D11 is dependent on the presence of DM, but requires other unknown cellular factors for its expression [33 and Chapter 3]. In contrast, the DRB1*0404-specific epitope, defined by NFLD.D13 is less dependent on DM for its expression.

Intriguingly, NFLD.D13 also recognizes an epitope on DRB1*0401 molecules in DM negative BCL, which is completely lost when DM expression is restored [34 and Chapter 3]. The limited cellular distribution of these epitopes and their different requirements for DM suggest alternative antigen processing or trafficking mechanisms are implicated in their generation. To identify the intracellular location of $D11^+$ and $D13^+$ DRB1*04 molecules, we performed co-localization experiments using confocal microscopy, where NFLD.D11 and NFLD.D13 were used in combination with mAbs recognizing markers for various intracellular compartments and MHC class II-associated molecules. To address the influence of antigen processing mechanisms, we used brefeldin A and protease inhibitors to identify whether intracellular trafficking of DRB1*04 molecules and proteases were implicated in forming these epitopes.

We found that treatment of the cells with the ER-golgi transport inhibitor brefeldin A reduced expression of all three epitopes. Also, in normal and DM null BCL expression of the $D11^+$ and $D13^+$ DRB1*0401 were influenced by endosomal and cytoplasmic cysteine proteases, and not by aspartyl proteases. The D11 and D13 epitopes in normal BCL appeared to follow similar intracellular trafficking routes, although, unlike the DRB1*0401 epitopes, the D13-DRB1*0404 epitope was not affected by the protease inhibitors tested here.

4.2 Methods

4.2.1 Cell Lines

Human EBV-transformed B cell lines (BCL) SAVC (HLA-DRB1*0401) and MT14b (HLA-DRB1*0404) were obtained through the 10th International Histocompatibility Workshop (IHW) [35] and maintained in complete media (CM) consisting of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat inactivated fetal calf serum (FCS), antibiotics and L-glutamine (all from Gibco BRL, Rockville, ML, USA). BCL 8.1.6 0401 (DRB1*0401, DRB1*0301) and 9.5.3 0401 (DRB1*0401, DRB1*0301) were a kind gift from Dr. N. Patil and Dr. E. Mellins at Stanford University, and kept in complete RPMI media supplemented as above. All BCL were maintained in humidified air containing 5% CO_2 at 37°C.

4.2.2 Antibodies

Total HLA-DR expression was determined with L243 (ATCC), while DRB1*04 specific mAbs included NFLD.D1, specific for an epitope on the β_2 domain of all DRB1*04 chains [36]; NFLD.D11, specific for a HLA-DM dependent Dw4 determinant on DRB1*0401 molecules [33 and 37] and NFLD.D13, which binds DRB1*0404 molecules in the presence of DM and DRB1*0401 molecules in the absence of DM [34]. Antibodies used to identify components of the HLA class II processing pathway included anti-DM, MaP.DM1 (BD PharMingen); cerCLIP (BD PharMingen) specific for MHC class II/CLIP complexes and anti-Ii, LN2 (BD Pharmingen). Other markers included anti-CD71 (M-A712), anti-CD82 (50F11) (BD PharMingen) and anti-CD63 (CLB-180)

(Cedarlane, Hornby, ON, Canada). Isotype-matched non-specific mAbs NS "IgM", NS "IgG1" and NS "IgG2a" [BD Pharmingen] were used in all experiments.

Secondary antibodies included a peroxidase-conjugated $F(ab)_2$ fragment goat anti-mouse (GAM) IgG + IgM (H + L) (Jackson Immunoresearch, West Grove, PN, USA) for CELISA assays and either GAM IgG Fc fragment specific R-Phycoerythrin conjugate or GAM IgM μ chain specific R-Phycoerythrin conjugate (Jackson Immunoresearch) for cytofluorometry. FITC-labeled IgG Fc fragment specific conjugate (Jackson Immunoresearch) and Texas Red dye conjugated IgM μ chain specific (Jackson Immunoresearch) antibodies were used for co-localization experiments. 12nm colloidal gold-AffiniPure GAM IgM, μ-chain specific and 18nm colloidal gold-AffiniPure goat anti-mouse IgG, γ-chain specific (Jackson ImmunoResearch) were used for electron microscopy studies.

4.2.3 Inhibitor Treatments

BCL were treated for 18 hours at 37°C with the following inhibitors: Brefeldin A (BfA) (Sigma) at 0.5ng/ml, Leupeptin (Sigma) at 25μM, Pepstatin A (Sigma) at 25μM, Cathepsin B inhibitor II (CBI) (Calbiochem) at 100μM, Lactacystin (Sigma) at 250nM and Calpeptin (Calbiochem) at 50μM. The concentration of all inhibitors used was predetermined and had no effect on cell viability, as assessed by Trypan Blue exclusion. After incubation, the cells were either fixed with 2% paraformaldehyde (PFA) (Sigma) in PBS, pH 7.4, for 15 minutes at 4°C and washed with cold CM followed by PBS, and

assayed by CELISA (for SAVC and MT14b), or alternatively kept cold and assayed by cytofluorometry (for SAVC, 8.1.6 0401 and 9.5.3 0401).

4.2.4 Saponin and Methyl-β-cyclodextrin Treatments

The existence of DRB1*04 epitopes within lipid rafts or tetraspan microdomains on the cell surface was tested as described in [21]. Briefly, BCL SAVC and MT14b were washed in PBS, fixed in 1% PFA for 15 min at RT and washed again with CM followed by PBS. The cells were then either treated with 0.1% saponin (Sigma) in PBS containing 2% FCS (Gibco BRL) for 20 min at 4°C or with 5mM methyl-β-cyclodextrin (Sigma) diluted in CM for 10 min at 37°C. The presence of various class II epitopes was then assayed by cytofluorometry.

4.2.5 Cytofluorometry

Cell surface expression of HLA-DR molecules and class II epitopes was determined by cytofluorometry as previously described [33]. Briefly, cells were washed in PBS containing 0.5% BSA, 0.02% sodium azide (Wash Buffer), and incubated with the appropriate test mAb for 30 minutes at 4°C. The cells were washed twice with wash buffer, and incubated with a goat anti-mouse PE labeled conjugate (Jackson Immunoresearch) for 30 minutes at 4°C followed by two more washes in wash buffer. The cells were fixed in 1.0% PFA and analyzed using a FACS star plus flow cytometer (BD PharMingen), acquiring 1 x 10^5 events per mAb treatment. For assessment of intracellular antigens, cells were fixed in 2% PFA for 15 min at RT, washed in CM

followed by PBS and permeabilized with 0.2% saponin for 10 min at RT and assayed as described above.

4.2.6 Confocal Microscopy

BCL cytocentrifuge preparations (at 2.5 $x10^4$ cells/prep) were washed with PBS, fixed in 2% PFA for 15 min at 4°C, washed briefly in CM and permeabilized in 0.2% Tween-20 for 10 min at RT followed by blocking with 15% goat serum in PBS for 1 hour at RT. Cell preps were incubated with the first test mAb for 1 hour at RT, removed using wash buffer (PBS containing 0.5% BSA) followed by application of second test mAb for 1 hour. After washing, the appropriate GAM conjugates were applied for 1 hour, followed by washing once in wash buffer and PBS before being mounted with aqueous anti-fade mounting media (DAKO) and viewed under the confocal microscope (Olympus FluoView™ 300 confocal laser scanning microscope).

4.2.7 Immunoelectron Microscopy

2 x 10^6 B cells were washed in PBS before being fixed in 2% EM-grade PFA (Sigma) for 15 mins at 4°C. The cells were then washed in CM followed by PBS and permeabilized with 0.2% saponin in PBS for 15 min at RT. The cells were then incubated with the appropriate test mAbs diluted in PBS containing 0.05% saponin for 1 hr at 4°C, rotating constantly. After 3 washes with PBS containing 0.05% saponin, the cells were incubated with 12nm colloidal gold GAM IgM, μ chain specific, followed by 18nm colloidal gold GAM IgG, γ chain specific for 1 hr at 4°C. The cells were washed

2x in PBS containing 0.05% saponin and pelleted before being fixed with Karnovsky's fixative for 20 min at RT and resuspended in 0.1M sodium cacodylate buffer overnight. The pellets were cut and placed on copper grids before membrane enhancement with uranyl acetate followed by osmium vapor treatment.

4.2.8 Cellular Enzyme-Linked Immunosorbent Assay (CELISA)

2.5 x 10^4 B cells/well were fixed in 2% PFA, washed in CM followed by PBS, and added to a 96 well round-bottom plate containing the appropriate test antibodies. After a 1 hour incubation at RT the cells were washed 3x with wash buffer (0.5% BSA, 0.05% Tween 20 in 1x PBS) and then incubated with the secondary antibody for 1 hour at RT. After 3 washes with buffer, the cells were transferred to EIA flat bottomed plates and incubated with OPD substrate for 30 min in the dark. The reaction was stopped using sulfuric acid, and read on a Multiscan Spectrophotometer using a 490nm filter. All assays were performed in triplicate, with means calculated and backgrounds subtracted using the appropriate isotype negative controls. Means and standard deviation were calculated from two assays in each case and the results expressed as treated/untreated. D11 and D13 epitope expression was also expressed as a percentage of the pan-DRB1*04 mAb NFLD.D1.

4.2.9 Statistical Analysis

To analyze statistical significance of inhibitor treatments, Paired Two Sample for Means, t-Tests were performed, with significance assigned for p-values < 0.05.

4.3 Results

4.3.1 $D11^+$ DRB1*0401 molecules traffic through the endocytic pathway in B cell lines.

To identify the intracellular location of $D11^+$ and $D13^+$ DRB1*04 molecules, we performed co-localization experiments using confocal microscopy. mAbs NFLD.D11 and NFLD.D13 in combination with mAbs recognizing markers for various intracellular compartments and MHC class II-associated molecules were reacted with SAVC (DRB1*0401) and MT14b (DRB1*0404) respectively.

The staining pattern shows abundant co-localization of the $D11^+$0401 and D1 epitopes (Fig 1a), suggesting that the subset of DRB1*04 molecules bearing the $D11^+$0401 epitope reside within the total DRB1*04 population or that $D1^+$ and $D11^+$ DRB1*04 molecules traverse the same intracellular compartments in SAVC. Having said this, there is evidence of discrete staining of both epitopes within SAVC. Also, the presence of the epitopes was more abundant intracellularly compared with on the cell surface, probably because of permeabilization of the plasma membrane by Tween-20. The $D11^+$0401 epitope partially co-localized with HLA-DM, although independent binding of NFLD.D11 and MaP.DM1 was apparent (Fig 1b). This suggests that the $D11^+$ DRB1*0401 molecules exists within DM positive intracellular compartments and diverge from these compartments en route to the cell surface. In contrast, the $D11^+$0401 epitope did not co-localize significantly with Ii (Fig 1c), indicating that either the $D11^+$0401 epitope exists within compartments where the invariant chain has already been degraded,

such as post-golgi compartments, or that there is no association with $D11^+$ DRB1*0401 molecules with DR/Ii complexes within SAVC.

The $D11^+$0401 epitope also partially co-localized with CD63 (Fig 1d) and Lamp-1 (data not shown), which are markers of late endocytic compartments, where DM is typically located [8, 38 and 39]. Complete co-localization of $D11^+$0401 epitope with the tetraspan molecule CD82 (Fig 1e), suggests that $D11^+$ DRB1*04 molecules may reside in large molecular complexes, as reported by Hammond et al, 1998, or may simply exist within the same intracellular compartments as CD82. CD71, which is located in early endosomal compartments and on the plasma membrane did not co-localize with the $D11^+$0401 epitope (Fig 1f) demonstrating that the $D11^+$ DRB1*04 molecules are not formed on recycling class II molecules. Since the $D11^+$ DRB1*0401 molecules co-localized primarily with markers for late endosomes and lysosomes (DM, CD63, CD82, LAMP-1) without co-localizing with CD71 and Ii, this indicates that the $D11^+$0401 epitope forms primarily within MIIC, and may require proteases that are active at this acidic pH [2, 8, and 9].

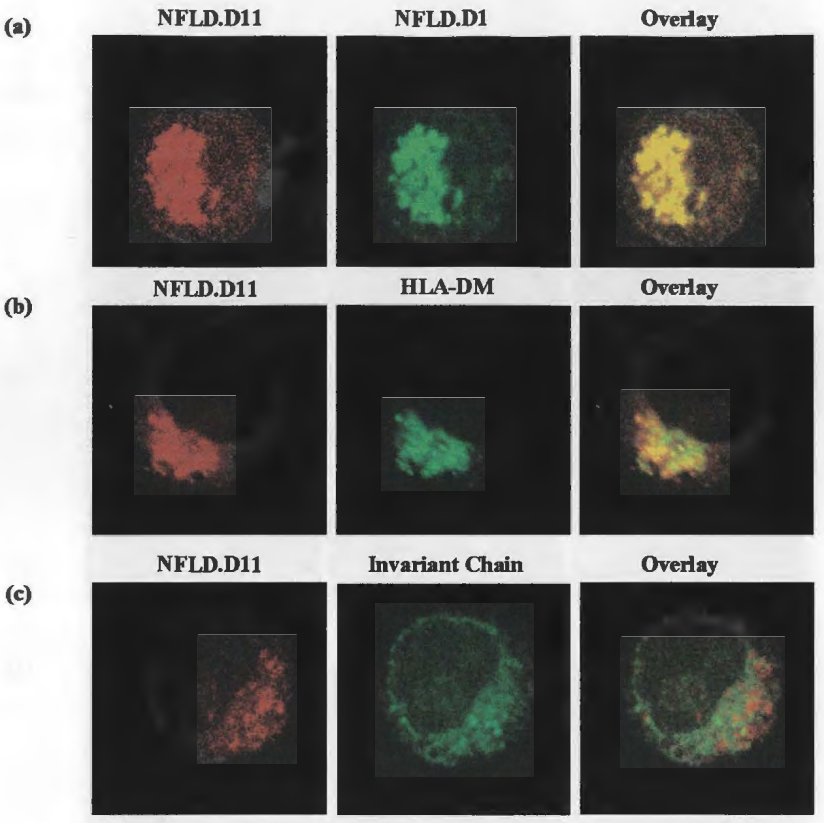


Figure 1. Continued on Next Page.

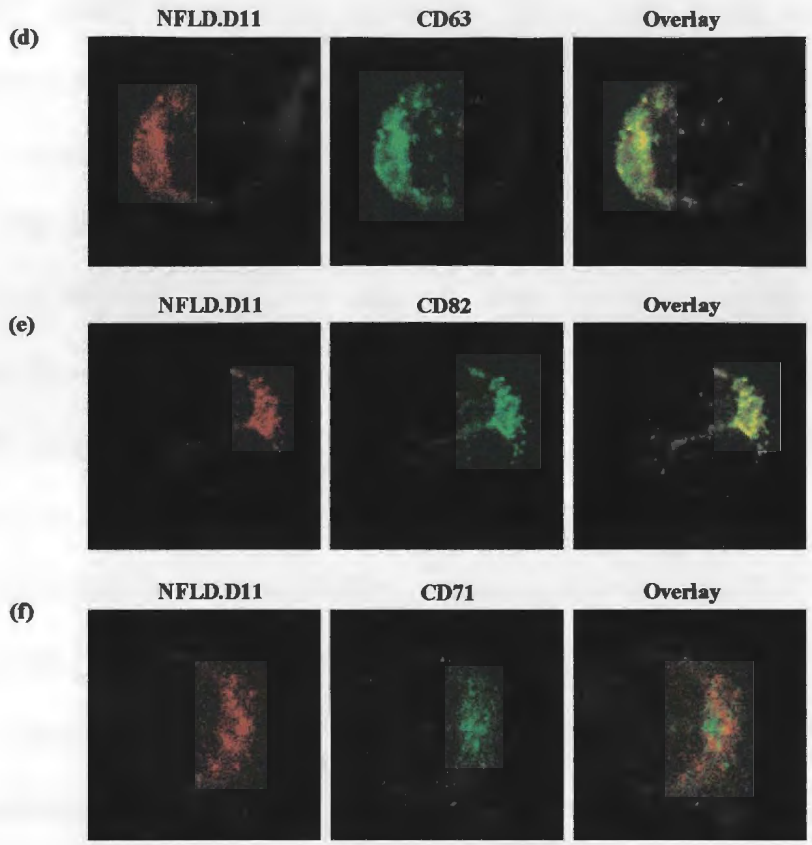


Figure 1. Characterization of the D11^{+}0401 epitope within the class II antigen processing pathway. mAbs that recognized components of the class II antigen processing pathway were used in double labeling studies in conjunction with NFLD.D11. The normal human B cell line SAVC (DRB1*0401 positive) was used in all panels. mAbs included those against (a) HLA-DM, (b) pan-DR4 (NFLD.D1), (c) the invariant chain, (d) CD63, (e) CD82 and (f) CD71. All test mAbs were IgG1, whereas NFLD.D11 is IgM. The conjugates used were GAM IgG FITC labeled and GAM IgM Texas Red labeled. Overlays of the two mAbs are shown on the left, with co-localization seen as yellow to greenish-yellow in color. All pictures were taken at 400x (x2) and are representative of 90-100% of cells viewed.

4.3.2 $D11^+$ DRB1*04 molecules co-localizes with DM, CD62 and CD82 within multilaminar MHC class II compartments in B cell lines.

To further determine the ultrastructural location of the $D11^+$0401 epitope within SAVC, IEM studies were performed. As shown in Fig 2a, the $D11^+$0401 epitope appeared on the cell surface as clusters, often within aggregates which resemble exosomes described by Escola et al, 1998 [40]. The majority of the intracellular $D11^+$0401 epitope positive DRB1*04 molecules were located within multilaminar structures (Fig 2b) similar to MIIC that most resemble lysosomes [8]. Using different size colloidal gold for immunolabeling, it was apparent that the $D11^+$0401 epitope co-localized with DM, CD63 and CD82 within these multilaminar structures (Fig 2c). These findings correlate with those from the confocal analysis, indicating that the interaction of these lysosomal proteins with $D11^+$ DRB1*04 molecules occur within lysosomal compartments along the endocytic pathway.

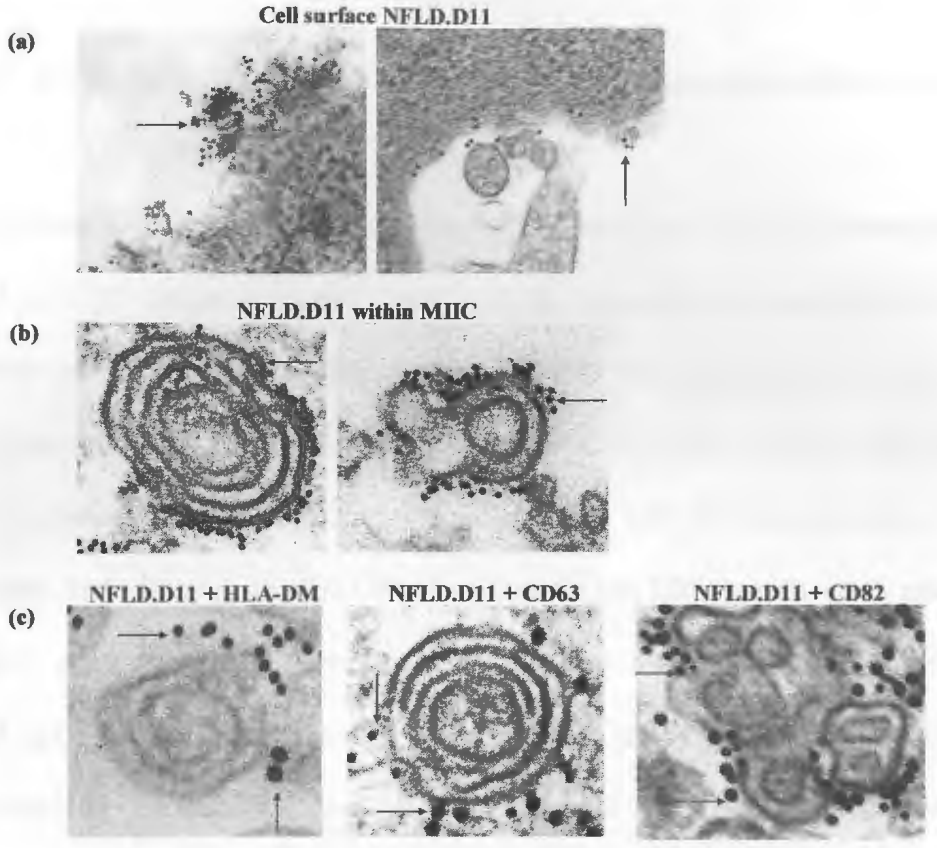


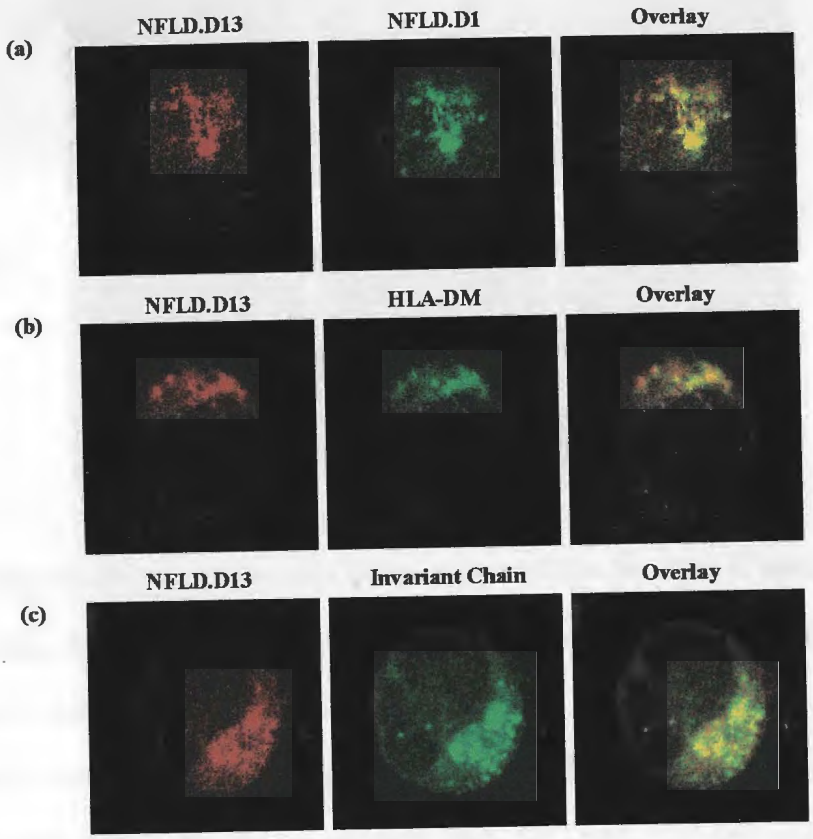
Figure 2. The D11^{+}0401 epitope is located on exosome-like structures on the cell surface and within multilaminar MIIC within the B cell line SAVC. (a) The localization of the D11^{+}0401 epitope (black arrows) on the surface of SAVC was measured by immunoelectron microscopy (IEM), using cells fixed with 2% paraformaldehyde before mAb treatment. Micrographs shown here are at a magnification of 58,000x. (b) and (c) Expression of intracellular D11^{+}0401 epitope within the endocytic pathway of SAVC was also shown using IEM with permeabilized cells (black arrows). NFLD.D11 (12nm gold-black arrows) was used in conjunction with mAbs to either HLA-DM, CD63 or CD82 (18nm gold-red arrows). All micrographs in (b) and (c) are taken at 172,000x.

4.3.3 $D13^+$ DRB1*0404 molecules traffic through the endocytic pathway in B cell lines.

Confocal microscopy studies with the $DRB1*0404^+$ BCL MT14b showed that the $D13^+0404$ and D1 epitopes partially co-localized, indicating that as expected, $D13^+$ DRB1*0404 molecules exist within the total DRB1*04 population (Fig 3a). The $D13^+0404$ epitope co-localized incompletely with DM (Fig 3b), which is not surprising since it is expressed in DM^- BCL and does not require DM for its expression. These results imply that although the $D13^+0404$ epitope is not DM dependent, it still exists within DM^+ compartments. Interestingly, in contrast to the $D11^+0401$ epitope, the $D13^+0404$ epitope co-localized strongly with Ii (Fig 3c), suggesting that Ii is either directly involved in the formation of the $D13^+0404$ epitope, or alternatively, the $D13^+0404$ epitope is formed on DRB1*0404 molecules that traffic through the same intracellular compartments as Ii.

In addition to this, co-localization of the $D13^+0404$ epitope with CD63 (Fig 3d) indicates that the $D13^+$ DRB1*0404 molecules are also present in lysosomes, just as the $D11^+0401$ epitope. The association between the $D13^+0404$ epitope and CD82 (Fig 3e-yellow color), shows that like the $D11^+0401$ epitope, the $D13^+0404$ epitope may interact with this tetraspan molecule within intracellular compartments. The lack of association of the $D13^+0404$ epitope with CD71 (Fig 3f), indicates that this epitope is not present within early endosomes and is not expressed on recycling class II molecules. Taken together, this confocal microscopical analysis suggests that the $D13^+0404$ epitope follows

a similar intracellular trafficking pathway as the D11^{+}0401 epitope. Unfortunately however, although immunoelectron microscopy studies were performed using the NFLD.D13 mAb, IEM staining was poor, and therefore no further information could be collected in regards to its ultrastructural location.



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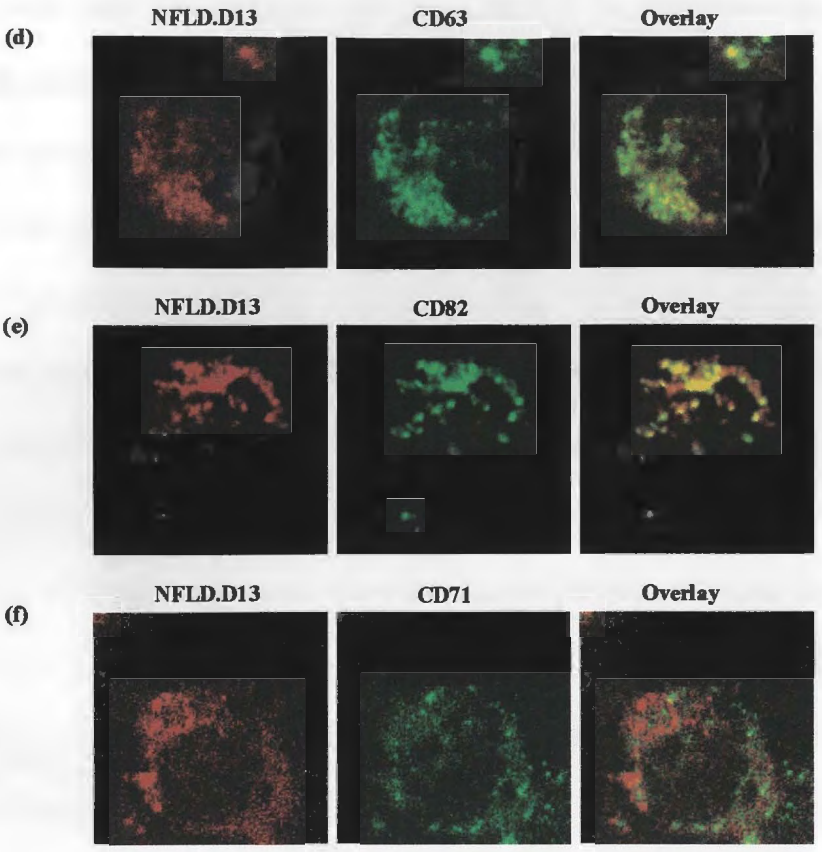

Figure 3. Characterization of the D13^{+}0404 epitope within the class II antigen processing pathway. mAbs that recognized components of the class II antigen processing pathway were used in double labeling studies in conjunction with NFLD.D13. The normal human B cell line MT14b (DRB1*0404 positive) was used in all panels. mAbs included those against (a) HLA-DM, (b) pan-DR4 (NFLD.D1), (c) the invariant chain, (d) CD63, (e) CD82 and (f) CD71. All test mAbs were IgG1, whereas NFLD.D13 is IgM. The conjugates used were GAM IgG FITC labeled and GAM IgM Texas Red labeled. Overlays of the two mAbs are shown on the left, with co-localization seen as yellow to greenish-yellow in color. All pictures were taken at 400x (x2) and are representative of 90-100% of cell viewed.

4.3.4 D11$^+$0401 and D13$^+$0404 epitopes are effected by tetraspan domain, but not lipid raft disrupting chemicals.

The strong co-localization of the D11$^+$0401 and D13$^+$0404 epitopes with the tetraspan molecules CD63 and CD82 suggested that tetraspan microdomains may be involved in the surface expression of these epitopes. To test this, we used the tetraspan microdomain and lipid raft disrupting chemicals saponin and methyl-β-cyclodextrin (MBCD), respectively, as described by Kropshofer et al, 2002 [21]. Although the detergent saponin can be used to permeabilize cellular membranes, it also has been shown to disturb surface tetraspan microdomains of paraformaldehyde-fixed cells when used at low concentrations [21]. As shown in Fig 4a, total DR, measured by L243, and the D11$^+$0401 epitope were dramatically decreased by saponin (D11$^+$0401 MIF of 448.5 in untreated compared with 56.5 in saponin treated), whereas DRB1*04, measured by NFLD.D1 slightly increased. MBCD did not destroy the D11$^+$0401 epitope or significantly reduce total DR expression, although it did slightly decrease the D1 epitope (Fig 4b). Saponin caused a slight increase in binding of NFLD.D13 to SAVC (MIF of 2 in untreated compared with 13.5 in saponin treated). Taken together, this suggests that the DRB1*0401 molecules that form the D11$^+$0401 epitope are present within tetraspan microdomains, but not lipid rafts. Related to this, we did not observe co-localization of the D11$^+$0401 epitope with FITC-labeled cholera toxin subunit B (data not shown) on SAVC, which is reported to be located within lipid raft domains on the surface of human BCL [41].

Similarly, DR and D13^{+}0404 epitopes were also disrupted by saponin, but not MBCD on MT14b (Fig 4c and d). Interestingly, as seen with SAVC, the D1 epitope increased slightly after saponin treatment while decreasing in the presence of MBCD on MT14b (Fig 4c and d). Taken together, this shows that the D13^{+}0404 epitope is also present within tetraspan microdomains, and not lipid rafts on the surface of BCL.

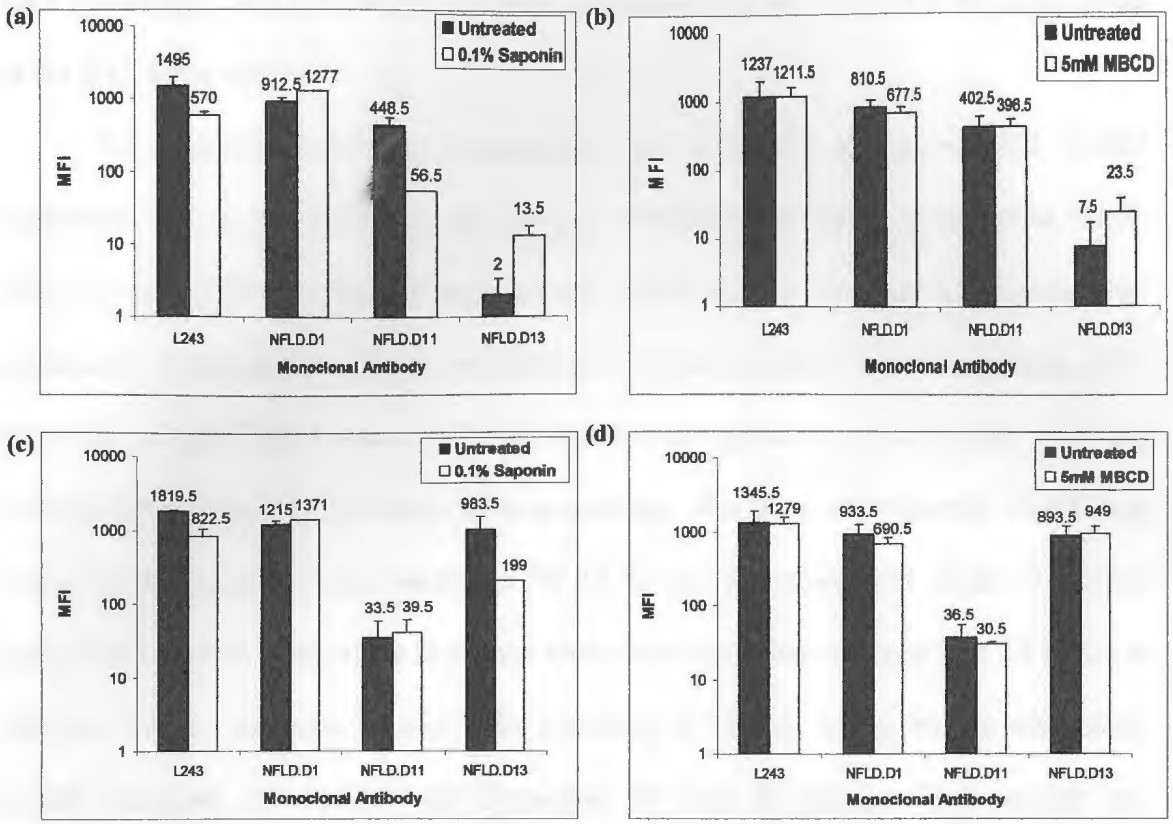

Figure 4. Localization of D11^{+}0401 and D13^{+}0404 epitopes within cell surface microdomains. The normal human BCL (a and b) SAVC (DBR1*0401) and (c and d) MT14b (DRB1*0404) were treated with 0.1% saponin (a and c), 5 mM methyl-β-cyclodextrin (MBCD) (b and d), or left untreated. The expression of total surface HLA-DR (L243), HLA-DR4 (NFLD.D1), and D11^{+}0401 (NFLD.D11) and D13^{+}0404 (NFLD.D13) epitopes were measured by cytofluorometry. The results are expressed as total mean fluorescence intensity (MFI), given above the appropriate bar, for both the untreated (black fills) and the treated cells (no fill), with standard deviation resulting from a combined two experiments.

4.3.5 Endosomal and cytoplasmic cysteine proteases are necessary for the generation of the D11$^+$0401 epitope.

The observations that the expression of the D11$^+$0401 epitope on BCL is DM dependent, but is not expressed by several NP-APC that have up-regulated HLA-DRB1*04 and DM (Chapter 3) suggests that either specific peptides or proteases are required for its generation. Since we could not analyze the peptide repertoire from D11$^+$ molecules as NFLD.D11 is not useful for immunoprecipitation, we elected to study the contribution of particular proteases to its generation. For these experiments, SAVC was treated with several protease inhibitors for 18 hr and the expression of the D11$^+$0401 epitope and various other class II-related molecules were then assayed by CELISA. In addition, SAVC was also treated with Brefeldin A (BfA), which blocks trans-golgi protein transport and inhibits the formation of class II epitopes that require late endosomes and lysosomes [42], making it a useful indicator of the contribution of newly synthesized molecules and the endocytic pathway for D11$^+$0401 epitope formation. Since 0.05% Tween-20 was used in the washing buffer throughout the assay, it is likely that we measured both intracellular as well as surface expression of the various class II molecules.

BfA treatment resulted in decreased levels of total HLA-DRB1*04, -DR and class I (Fig 5a and data not shown) on SAVC, which is not surprising since it blocks ER-golgi protein transport. In contrast, the protease inhibitors used had little effect on the expression of HLA-DRB1*04, -DR and class I molecules (Fig 5a and data not shown)

probably because of the redundancy in generating class I and class II epitopes, where one group of proteases can likely compensate for the lack of another.

We observed decreased D11$^+$0401 expression after BfA treatment (Fig 5b), which partly reflects the overall decrease in DRB1*04; however, D11$^+$0401 loss was greater than D1 loss. This finding suggests that the D11$^+$0401 epitope is formed from a pool of newly synthesized molecules, but also supports the data showing the existence of the D11$^+$0401 epitope within lysosomal compartments. It further suggests that the D11$^+$0401 epitope is not formed on recycling DRB1*04 molecules, which correlates with its lack of association with CD71 in the confocal studies in Figure 1. However, the general cysteine protease inhibitor leupeptin markedly reduced NFLD.D11 binding, whereas pepstatin A, which inhibits aspartyl proteases did not. This indicates that cysteine, but not aspartyl proteases are implicated in generating the D11$^+$0401 epitope (Fig 5b). Specific inhibitors of the endosomal cysteine protease cathepsin B and calpain, a group of cytoplasmic cysteine proteases, also decreased D11$^+$0401 expression on SAVC (Fig 5b). Therefore, both endosomal and cytoplasmic cysteine proteases are involved in the generation of the D11$^+$0401 epitope on DRB1*0401 molecules.

Interestingly, the expression pattern of DR/CLIP complexes somewhat mirrored D11$^+$0401 in the presence of BfA, leupeptin and cathepsin B inhibitor II (CBI) (Fig 5c). In contrast, pepstatin A and lactacystin did not affect CLIP expression (Fig 6c and data not shown) suggesting that the same proteases are likely responsible for the formation of the D11$^+$0401 epitope and the degradation of Ii into CLIP. The possibility that CLIP, or a longer version is responsible for forming the D11$^+$0401 epitope is unlikely since cerCLIP

does not block the binding of NFLD.D11 to SAVC, and the D11$^+$0401 epitope is not present on DM negative cells that express abundant amounts of CLIP [Chapter 2].

Ii/DR complexes were relatively unchanged, although BfA and pepstatin A treatments both caused slight increases in LN2 binding (Fig 5d). Since BfA affects total protein transport, it may have resulted in the accumulation of Ii/DR by blocking the production of proteases and chaperone molecules needed for Ii degradation and CLIP removal.

4.3.6 Combined cysteine protease inhibitor treatments reduce the D11$^+$0401 epitope on BCL additively.

Since individual cysteine inhibitors resulted in partial loss of the D11$^+$0401 epitope, we questioned whether particular protease inhibitor combinations would be additive. As shown in Figure 6, the D11$^+$0401 epitope was significantly decreased by treatment with the combinations of leupeptin + CBI (p value = 0.00137), leupeptin + calpeptin (p value = 0.0191) and calpeptin + CBI (p value = 0.00189). Partial loss of the D11$^+$0401 epitope with treatment of leupeptin + pepstatin A (Fig 6b), provided further evidence that aspartyl proteases are not involved in forming the D11$^+$0401 epitope. In contrast, expression of NFLD.D1, which is not peptide-dependent, was only slightly decreased with these protease inhibitor treatments. Taken together, these results strongly suggest that both endosomal and cytoplasmic cysteine proteases are required for generating the D11$^+$0401 epitope.

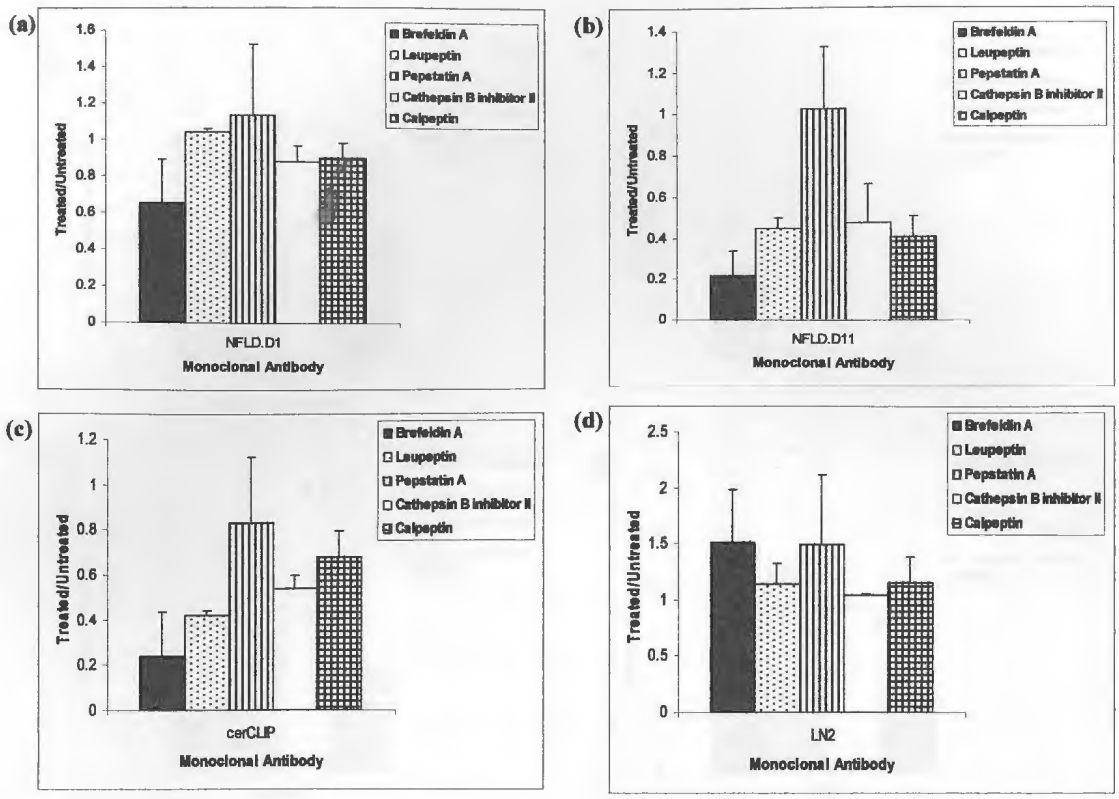

Figure 5. Inhibition of the D11^{+}0401 epitope in the presence of protease inhibitors and brefeldin A. SAVC (DRB1*0401) was incubated with various inhibitors (0.5ng/ml Brefeldin A , 25μM Leupeptin, 25μM Pepstatin A, 100μM Cathepsin B inhibitor II and 50μM Calpeptin) or DMSO alone (vehicle control) for 18 hrs at 37°C. The cells were then fixed and assayed for the expression of class II epitopes by CELISA using the following mAbs; (a) NFLD.D1 (DR4), (b) NFLD.D11 (c) cerCLIP (DR/CLIP complexes) and (d) LN2 (DR/Ii complexes). Triplicate optical densities were averaged, backgrounds subtracted and combined for two separate experiments. Results were then presented as treated/untreated for each of the inhibitors used. Error between experiments was measured using standard deviation of the mean.

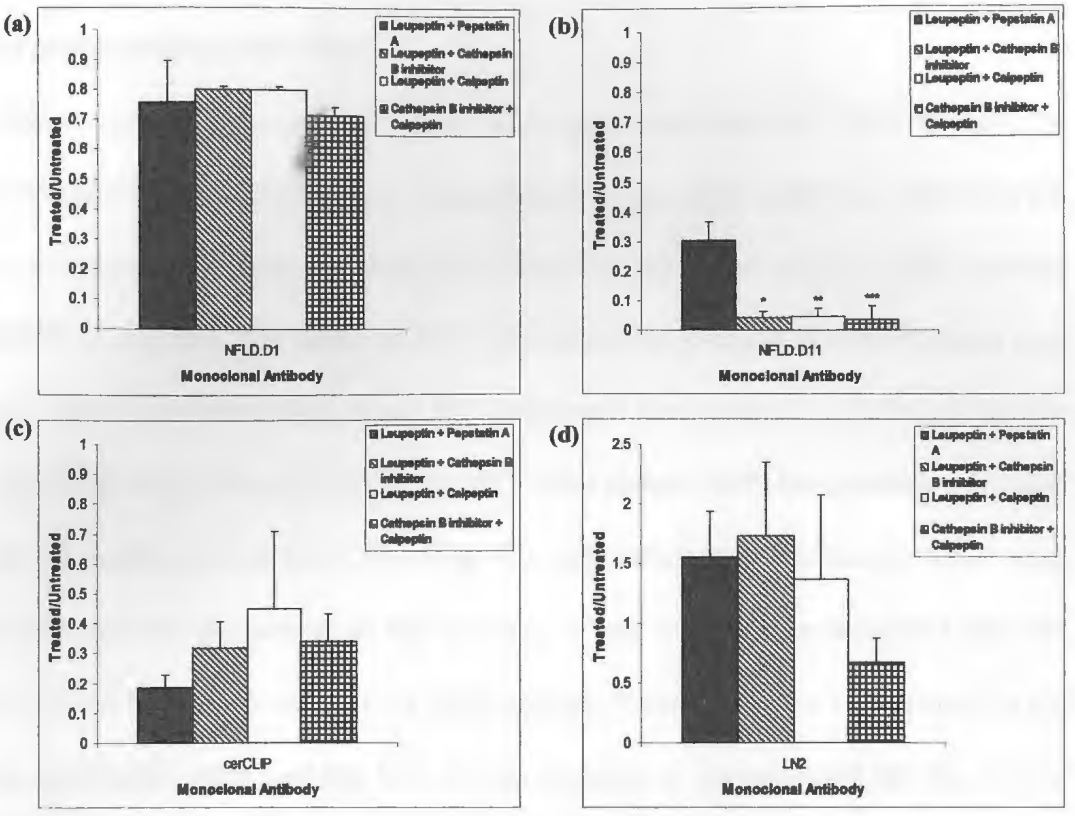

Figure 6. Effect of various combinations of protease inhibitors on the expression of the D11^{+}0401 epitope. SAVC (DRB1*0401) was incubated with combinations of inhibitors (25μM Leupeptin, 25μM Pepstatin A,100μM Cathepsin B inhibitor II and 50μM Calpeptin) or DMSO alone (vehicle control) for 18 hrs at 37°C. The cells were then fixed and assayed for the expression of several epitopes by CELISA using the mAbs; (a) NFLD.D1 (total DR4), (b) NFLD.D11, (c) cerCLIP for (DR/CLIP complexes), (d) LN2 (DR/Iicomplexes). Triplicate optical densities were averaged, backgrounds subtracted and combined for two separate experiments. Results are presented as treated/untreated for each inhibitor. Error between experiments was measured using standard deviation of the mean. Significant p values as assessed by Paired Two Sample for Means t-Tests; * p value = 0.00137, ** p value = 0.0191, *** p value = 0.00189.

4.3.7 BfA treatment reduces the D13^{+}0404 epitope, though inhibition of cysteine and aspartyl proteases has little effect.

Since cysteine proteases are involved in generating the D11^{+}0401 epitope, we queried whether the D13^{+}0404 epitope was affected by the same inhibitors. BfA, but not protease inhibitors reduced total DRB1*04 levels (Fig 7a). Like the D11^{+}0401 epitope, the DRB1*04 molecules that form the D13^{+}0404 epitope also appear to come from a pool of newly synthesized molecules, since BfA decreased the expression of the epitope by about 55% (Fig 7b). The only loss of the D13^{+}0404 epitope with the protease inhibitors was with leupeptin, by less than 20% (Fig 7b), suggesting that cysteine proteases may weakly influence the production of this epitope. If any of the proteases tested here are required for the generation of the D13^{+}0404 epitope, there is likely a redundancy in the cleavage specificity, such that the loss of one protease is compensated for the loss of another.

As with the D11^{+}0401 epitope, the loss of the D13^{+}0404 by the BfA treatment paralleled the decrease in DR/CLIP expression (Fig 7c). This indicates that both these epitopes require lysosomal/late endosomal compartments for their production. Leupeptin and CBI also caused a slight accumulation of Ii complexes in MT14b (Fig 7d), presumably due to inhibition of Ii breakdown.

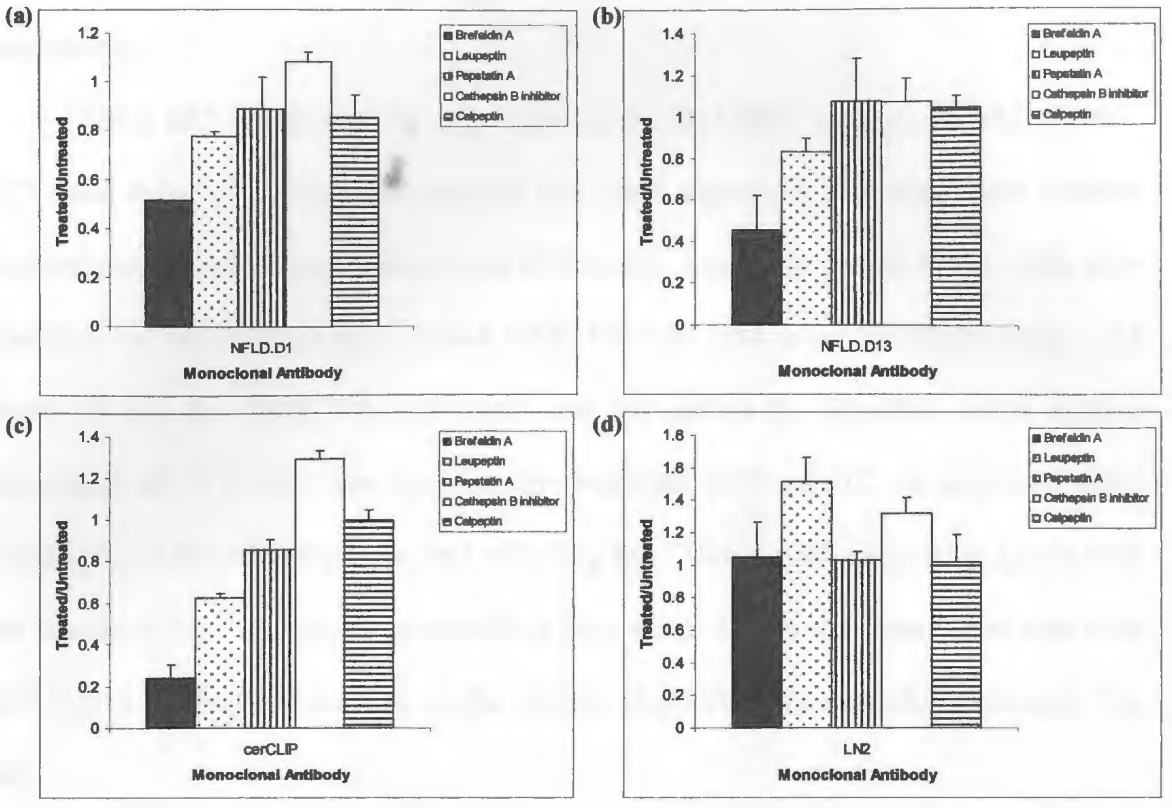

Figure 7. Effect of brefeldin A and proteases inhibitors on the D13^{+}0404 epitope. MT14b (DRB1*0404) was incubated with various inhibitors (0.5ng/ml Brefeldin A, 25μM Leupeptin, 25μM Pepstatin A, 100μM Cathepsin B inhibitor II and 50μM Calpeptin) or DMSO alone (vehicle control) for 18 hrs at 37°C. The cells were then fixed and assayed for the expression of several epitopes by CELISA. The following mAbs were used; (a) NFLD.D1 (DR4), (b) NFLD.D13, (c) cerCLIP (DR/CLIP complexes) and (d) LN2 (DR/invariant chain complexes). Triplicate optical densities were averaged, backgrounds subtracted and combined for two separate experiments. Results were then presented as treated/untreated for each of the inhibitors used. Error between experiments was measured using standard deviation of the mean.

4.3.8 Leupeptin-induced loss of the D11$^+$0401 epitope is not due to decreased DM expression.

Since DM is required for expression of the D11$^+$0401 epitope on DRB1*0401$^+$ BCL, and leupeptin treatment decreased D11$^+$0401 expression, we questioned whether leupeptin treatment reduced intracellular DM levels. Leupeptin treated SAVC cells were analyzed for intracellular and surface DRB1*04 and DM using cytofluorometry. As shown in Fig 8a, DRB1*04 expression was not altered by leupeptin, while surface expression of D11$^+$0401 was significantly decreased (MIF of 602 on untreated cells) compared to 156 on leupeptin treated cells (Fig 8a). This surface expression agrees with the results of total expression presented in Figs 4 and 5. As expected, there was little NFLD.D13 binding and no DM on the surface of SAVC before or after treatment (Fig 8a).

The results of the intracellular cytofluorometry displayed in Figure 8b confirmed that the level of DRB1*04 molecules (NFLD.D1) was unaffected by leupeptin treatment. Strangely, there was little detectable intracellular NFLD.D11 binding, in contrast to the intracellular microscopy results in Figs 1 and 2. Certainly, the differences in permeabilizers (Tween-20 for confocal microscopy, saponin for intracellular flow cytometry) could explain this. It is possible that saponin, at the concentrations used for this experiment, disrupted NFLD.D11 binding. Intriguingly, intracellular binding of NFLD.D13 moderately increased in the presence of leupeptin (MIF = 56 in the untreated compared with 145 in the treated cells), whereas this treatment did not affect the expression of intracellular DM (Fig 8b). Therefore the loss of surface expression of the

D11^{+}0401 epitope by leupeptin treatment is not due to a decrease in total DRB1*0401 or DM levels. Together with the data presented in Figs 5 and 6, this further suggests that cysteine proteases strongly influence the generation of the D11^{+}0401 epitope.

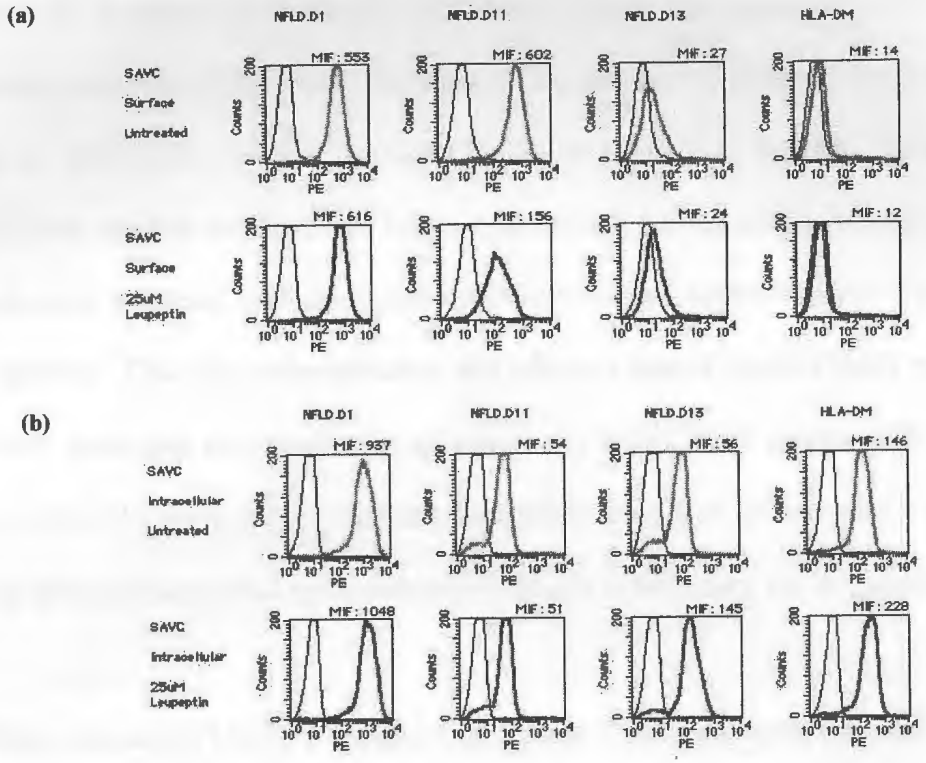

Figure 8. Treatment of the B cell line SAVC with leupeptin does not cause the depletion of intracellular HLA-DM. SAVC was treated for 18 hrs at 37°C with 25μM leupeptin (red histograms) or treated with DMSO in 10% complete media (control- green histograms). The cells were either: (a) kept on ice and assayed by cell surface cytofluorometry, or (b) fixed, permeabilized and assayed for intracellular flow cytometry. mAbs to various molecules included; NFLD.D1 (total DR4), NFLD.D11, NFLD.D13 and MaP.DM1 for total HLA-DM. Appropriate isotype negative controls are shown with a solid black line.

4.3.9 Treatment of SAVC with cysteine protease inhibitors generates the D13^{+}0401 epitope.

Previously we described a D13^{+}0401 epitope on DM negative B cells [34 and Chapter 2]. Here, we found that treatment of the normal BCL SAVC with either leupeptin, CBI or calpain, decreased the D11^{+}0401 epitope, also generated a NFLD.D13-recognizable epitope on DRB1*0401 molecules. In contrast, treatment of these cells with pepstatin A, lactacystin or BfA did not (Fig 9a and data not shown). When these inhibitors were used in combination, BfA + pepstatin A had no effect, whereas various combinations of cytsteine protease inhibitors resulted in an additive effect (Fig 9b and data not shown). Thus, the same inhibitors that caused a loss of the D11^{+}0401 epitope on DRB1*0401 molecules also resulted in an increase in a D13^{+}0401 epitope. The greatest increase in this D13^{+}0401 epitope resulted from the combination of leupeptin + calpeptin indicating that endosomal and cytoplasmic proteolysis is necessary for its generation (Fig 9b).

Since normal NFLD.D13 binding to untreated SAVC was quite low, any increase in binding may be interpreted as artificially large when presented as treated/untreated. To exclude this potential artifact, NFLD.D13 binding was also expressed as a percentage of NFLD.D1, which was not altered by these inhibitors. As shown in Fig 9c, the protease-induced D13^{+}0401 epitope was clearly not present in the untreated cells, but was moderately to strongly expressed in all cells treated with a cysteine protease inhibitor. Maximal expression of 40% of NFLD.D1 was observed with either combination of leupeptin, CBI or calpeptin (Fig 9c).

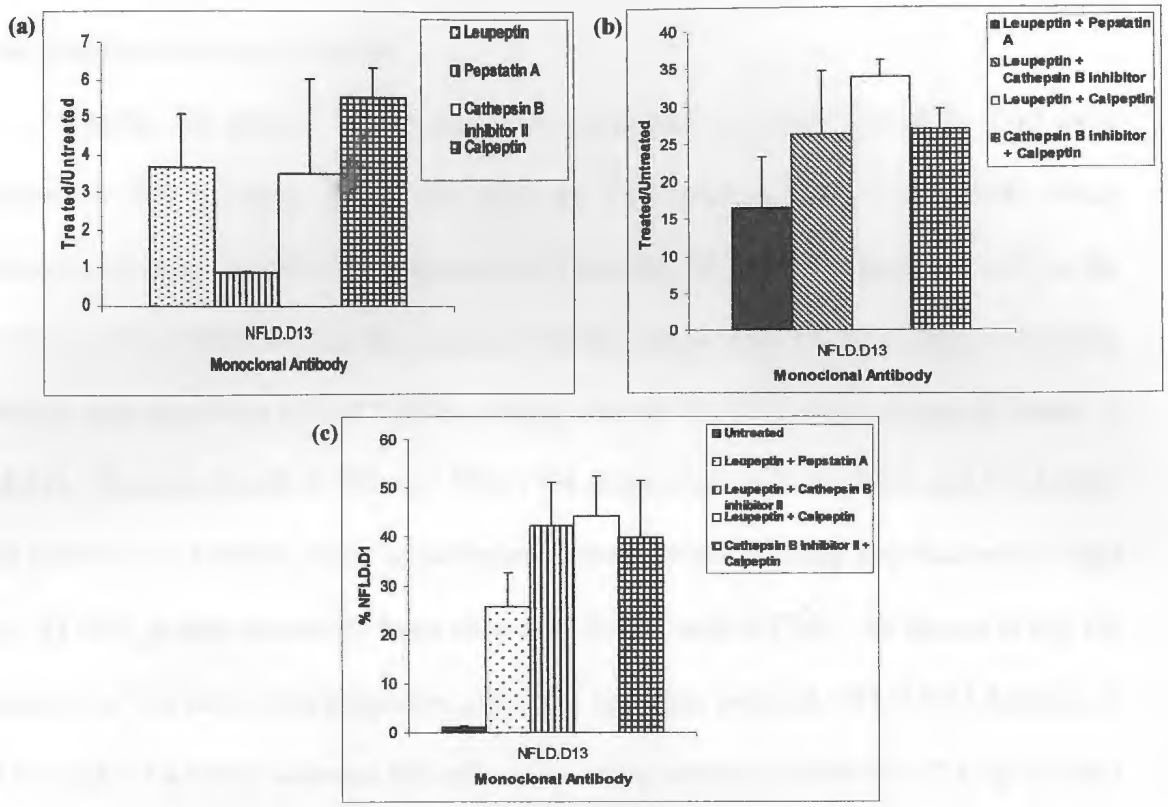

Figure 9. Treatment of the HLA-DM positive B cell line SAVC with cysteine protease inhibitors results in a gain of a D13^{+}0401 epitope. SAVC (DRB1*0401) was incubated with (a) inhibitors (25μM Leupeptin, 25μM Pepstatin A, 100μM Cathepsin B inhibitor II and 50μM Calpeptin), (b) and (c) various combinations of the above protease inhibitors, or DMSO alone (vehicle control) for 18 hrs at 37°C. In all cases the cells were then fixed and assayed for the expression of the D13^{+}0401 epitope using CELISA. Triplicate optical densities were averaged, backgrounds subtracted and combined for two separate experiments. The results in (a) and (b) were then presented as treated/untreated for each of the inhibitors used, and the results in (c) are presented as NFLD.D13 as a percentage of the pan-DR4 mAb NFLD.D1. Error between experiments was measured using standard deviation of the mean.

4.3.10 The DM antagonistic D13$^+$0401 epitope on antigen processing deficient BCL also requires cysteine proteases.

Finally, we queried if proteases also contributed to generation of the D13$^+$0401 epitope on DM negative cells. We used the DM negative BCL 9.5.3 0401, which expresses abundant DR/CLIP complexes [34] and the D13$^+$0401 epitope, as well as the parent cell 8.1.6 0401, which is competent for all components of the antigen processing pathway and expresses the D11$^+$0401 epitope but not the D13$^+$0401 epitope [Chapter 2 and 34]. Because the HLA-DR and DRB1*04 expression on 8.1.6 0401 and 9.5.3 0401 was sensitive to fixation, these experiments were performed using cytofluorometry and not CELISA, as had previously been done with SAVC and MT14b. As shown in Fig 10, treatment of the cells with leupeptin, calpeptin and CBI reduced NFLD.D11 binding to SAVC and 8.1.6 0401, although this effect was more prominent with SAVC (Fig 10a and b). This result confirms the requirement of cysteine proteases for D11$^+$0401 epitope formation on normal BCL.

As seen with the normal BCL, the inhibitors did not cause significant fluctuation in DR and DRB1*04 levels on the surface of 9.5.3 0401, with the exception of BfA, which caused a reduction in the binding of L243 and NFLD.D1 (Fig 10c). BfA also caused a significant reduction in the D13$^+$0401 epitope on 9.5.3 0401, which coincided with a substantial loss of DR/CLIP complexes, DR and DRB1*04 (Fig 10c). Leupeptin, CBI and calpeptin diminished the D13$^+$0401 epitope on 9.5.3 0401 (Fig 10c) whereas pepstatin A (Fig 10c) and lactacystin (data not shown) did not. This shows that, like the DM-dependent D11$^+$0401 epitope, the D13$^+$0401 epitope partially require newly

synthesized molecules in the endocytic pathway, in addition to endosomal and cytoplasmic cysteine proteases. For both of these epitopes, the most common lysosomal cysteine protease, cathepsin B and calpain are prime candidates.

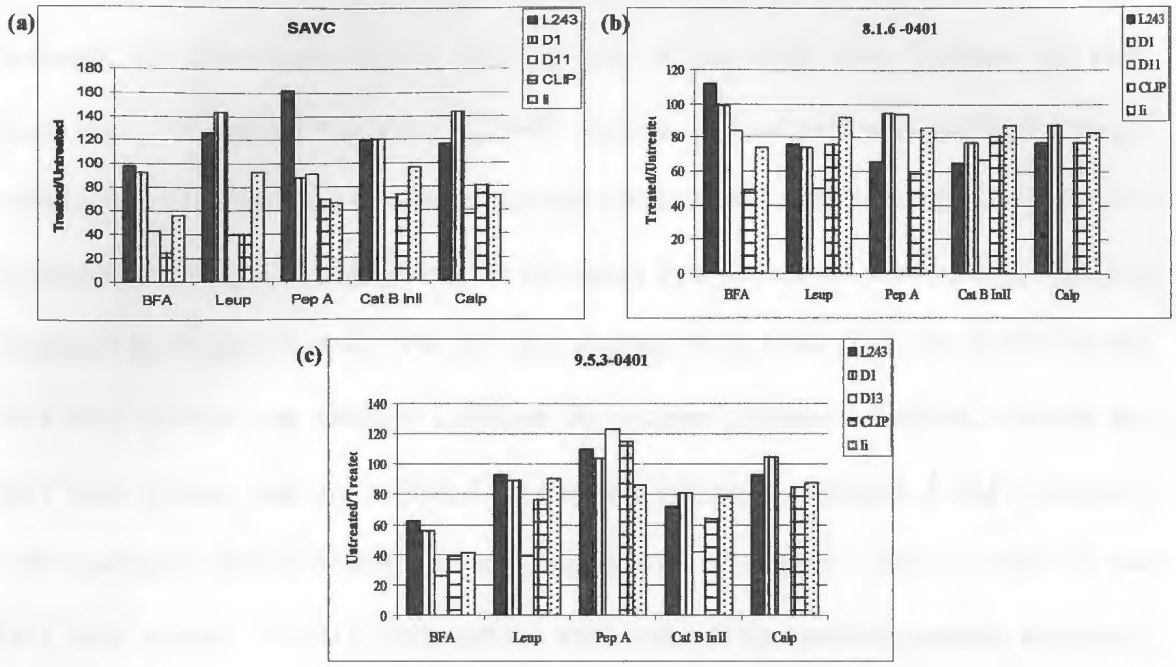

Figure 10. Expression of the D13^{+}0401 epitope on the HLA-DM negative BCL 9.5.3 0401 in the presence of protease inhibitors. The (a) normal BCL SAVC (DRB1*0401), (b) 8.1.6 0401 (DRB1*0401, DRB1*0301) and (c) the HLA-DM negative BCL 9.5.3 0401 (DRB1*0401, DRB1*0301) were incubated with various inhibitors (0.5ng/ml Brefeldin A, 25μM Leupeptin, 25μM Pepstatin A, 100μM Cathepsin B inhibitor II and 50μM Calpeptin) or DMSO alone (vehicle control) for 18 hrs at 37°C. The expression of various MHC class II epitopes were measured by cell surface cytofluorometry, and the results expressed as Treated/Untreated. Results shown are a representative example of three experiments.

4.4 Discussion

In this study, we explored the intracellular pathway in which three HLA-DRB1*04-specific epitopes, defined by the mAbs NFLD.D11 and NFLD.D13, were formed in human BCL. In addition to identifying the subcellular location of these epitopes, we further ascertained which groups of proteases were required for their generation. We found that the $D11^{+}0401$ epitope co-localized with molecules found within late endosomal and lysosomal compartments. Upon further examination, this co-localization occurred in multilamminar structures that resembled conventional MIIC as described by Kleijmeer et al, 1996 [43] and Robbins et al, 1996 [39]. We found that the $D11^{+}0401$ epitope was partially inhibited by cysteine protease inhibitors, whereas the $D13^{+}0404$ epitope was not inhibited by protease inhibitors, although it did co-localize with molecules found within late endosomes and lysosomes. Also, similar to the $D11^{+}0401$ epitope, the $D13^{+}0401$ epitope was inhibited by cysteine protease inhibitors. All three of these DRB1*04 epitopes were inhibited by the ER-golgi transport inhibitor BfA.

Since expression of the $D11^{+}0401$ epitope in human BCL is HLA-DM dependent [33], it was not surprising to observe co-localization of the $D11^{+}0401$ epitope with DM. However, the DM-independent $D13^{+}0404$ epitope partly co-localized with DM, suggesting that both the $D11^{+}0401$ and $D13^{+}0404$ epitopes exists within DM^{+} compartments in BCL. As DM is abundantly expressed within late endosomes and lysosomal compartments, such as MIIC [8, 16, 38, 39, 43], this implies that the $D11^{+}0401$ and $D13^{+}0404$ epitopes either form or traffic through late endosomes and lysosomes

within BCL. This is supported by the lack of co-localization of NFLD.D11 and NFLD.D13 with the transferrin receptor (CD71), a commonly used marker for recycling early endosomes [44, 45].

This being said, the co-localization patterns of the two epitopes with Ii differed, as very little $D11^{+}0401$ co-localized with Ii, compared to significant amounts of $D13^{+}0404$ with Ii. This suggests that Ii may indeed be involved in $D13^{+}0404$ epitope formation, either due to Ii intermediate peptides, or Ii-influenced trafficking of $D13^{+}0404$ molecules. Indeed, other studies suggest that a significant number of class II/Ii complexes may migrate directly to the cell surface [45]. Here, these molecules may either recycle through early endosomes to the plasma membrane, or alternatively, enter the endocytic pathway [45]. Therefore, although molecules that form the $D13^{+}0404$ epitope are found within the endocytic pathway, the co-localization studies with Ii suggest that its intracellular trafficking route is different than the DRB1*04 molecules that form the $D11^{+}0401$ epitope. The microscopical data with the $D11^{+}0401$ epitope leads us to believe that the DRB1*0401 molecules responsible for forming this epitope enter the endocytic pathway and migrate directly to the cell surface via these compartments.

Similarly, 16.23, a mAb which recognizes a DM-dependent epitope on DR3 molecules has also been shown to be present primarily within late endosomal and lysosomal compartments within human BCL [46, 47], and similar to the $D11^{+}0401$ and $D13^{+}0404$ epitopes, 16.23 colocalized with DM, and CD63 within MIIC [46]. Zhong et al, 1997 [48] examined the subcellular location of the epitope recognized by C4H3, specific for $I\text{-}A^{k}$ molecules bound with exogenous HEL peptide 46-61. The C4H3

epitope was found within endosomal organelles expressing the lysosomal marker LAMP-1 within rat basophilic leukemia cells stably transfected with I-A^k and pulsed with whole HEL. [48]. No co-localization was seen with C4H3 and antibodies recognizing the transferrin receptor, suggesting that this mAb-defined epitope, like the $D11^+0401$ and $D13^+0404$ epitopes, does not travel through early endosomes.

CD63, a tetraspan molecule that is present in copious amounts within MIIC, is also reported to be present in CDw78-defined supramolecular complexes, along with class II, CD82, DM and DO in MIIC within BCL [19]. Others have also reported the association of MHC class I, class II, DM and other tetraspanin proteins such as CD9, CD53, CD20, CD81 and CD82 [40, 49, 50]. These widely expressed tetraspanin molecules serve a wide variety of roles, including signal transduction, cell adhesion, motility, activation [51] and may structurally anchor molecules such as HLA-DR in order for proper intracellular and cell surface interaction to occur. These molecular complexes may also serve to concentrate specific MHC/peptide complexes, increasing the chance of TCR interaction. Our findings that $D11^+0401$ and $D13^+0404$ epitopes co-localized with CD63, CD82 and DM, suggests that these epitopes may result from DRB1*04 molecules that exist in such supramolecular tetraspan complexes. Supporting this, we observed that both the $D11^+0401$ and $D13^+0404$ epitopes on the cell surface were disrupted by the detergent saponin, which is reported to disturb tetraspan complexes at low concentrations [21]. Disruption of membrane lipid rafts did not change the expression levels of either of these epitopes. Similarly, the mAb, UL-5A1, which distinguishes DRB1*0101 bound to HLA-A2 derived peptides on activated cells such as EBV-transformed B cells [52], co-

localizes with CDw78 on the surface of BCL and DC [21]. Like the UL-5A1 epitope, the $D11^{+}0401$ and $D13^{+}0404$ epitopes also appear to require additional factors present in activated P-APC, such as tetraspan microdomains for their expression.

The association of $D11^{+}0401$ molecules with CD63, CD82 and DM was found within multilaminar MIIC compartments, which are thought to functionally most resemble lysosomes [53]. Unfortunately, immunoelectron microscopy studies using the NFLD.D13 mAb were unsuccessful, providing no further information into its ultrastructural location. Taken together, our data show that the $D11^{+}0401$ and $D13^{+}0404$ epitopes in normal B cell lines are found associated with the tetraspan proteins CD63 and CD82 within the classical endocytic pathway, where they are most likely transported to the cell surface forming stable pMHC complexes. Our data imply that these two DRB1*04 epitopes do not use the alternative MHC class II pathway, in which class II molecules recycle to and from the cell surface through the early endosomal compartments.

The use of several cell function inhibitors to further dissect the generation of the $D11^{+}0401$ and $D13^{+}0404$ epitopes in normal BCL revealed that both epitopes were partially inhibited by BfA, which blocks the movement of proteins from the ER to the golgi apparatus. This suggests that newly synthesized molecules are required for $D11^{+}0401$ and $D13^{+}0404$ epitope formation, either through the generation of new class II molecules, or through the formation of other molecules that are required for epitope generation, such as proteases and co-chaperone molecules such as DM, DO and Ii. Similarly, Griffin et al, 1997 [45] found differences in the compartments needed for the

processing of I-A^k and I-E^k-specific T cell epitopes from hen egg lysozyme and RNase. One complex, RNase-(42-56): I-A^k, was generated in early endosomal compartments and was inhibited by chloroquine but partially resistant to the effects of BfA. Another pMHC complex, RNase-(90-105): I-E^k, was generated in late endocytic compartments and was resistant to the effects of chloroquine, though blocked by BfA. We found that the D11$^+$0401 and D13$^+$0404 epitopes were not inhibited by chloroquine and bafilomycin A, which block degradation in early endosomes (data not shown), providing further evidence that these class II epitopes were generated in late endosomal/lysosomal compartments within normal BCL.

We speculated that the D11$^+$0401 epitope may require specific peptide(s) for its formation because although the D11$^+$0401 epitope is DM-dependent, other factors must be required for its generation since various NP-APC up-regulate DM, yet fail to express the D11$^+$0401 epitope (Chapters 2 and 3). Thus the D11$^+$0401 epitope is cell type and DM restricted, suggesting that specific peptides may be required for its generation. Other explanations could be that the protease arrays are different between cell types, or that specific protein complexes responsible for the generation of the D11$^+$0401 epitope differ between cell types.

Our finding using protease inhibitors that exclusively impede either a particular protease, or class of protease revealed that the D11$^+$0401 epitope requires both endosomal and cytopasmic cysteine proteases for its generation. Interestingly, although the intracellular trafficking pattern of the D11$^+$0401 and D13$^+$0404 epitopes overlap, the D13$^+$0404 epitope was not dependant on any protease group tested here, even though

leupeptin did cause a slight inhibition. This suggests that there may be redundancy in D13 epitope generation, with several proteases compensating for the loss of function of another. Other explanations could be that one or more proteases, such as cathepsin S or L, are involved in $D13^{+}0404$ epitope formation. Alternatively, specific proteolytic events and specific subsets of peptides are not responsible for the generation of the $D13^{+}0404$ epitope, but NFLD.D13 recognizes a conformational determinant formed by the association of DRB1*0404 with other molecules such as CD82 or CD63.

Recently, much attention has been paid to characterization of alternative class II processing pathways where peptides derived from class I-associated proteolysis, such as through the proteasome, are loaded onto class II molecules. The dependence of $D11^{+}0401$ epitope generation on endosomal and cytoplasmic cysteine proteases is somewhat similar to studies described by Lich et al, 2000 [42], in which alternative processing was involved in the proteolysis of endogenous glutamic acid decarboxylase (GAD). For endogenously expressed GAD, cytoplasmic proteases such as the proteasome and calpain were required for the generation of an immunodominant peptide that binds to DRB1*0401. Alternatively, lysosomal processing, blocked by BfA, was necessary for the processing of exogenous supplied GAD into the same immunodominant peptide.

Inferring from these results, we hypothesize that the source of the putative peptide(s) involved in $D11^{+}0401$ epitope formation may be cytoplasmic, since calpain inhibited NFLD.D11 binding to SAVC. Alternatively, the $D11^{+}0401$ epitope may be

formed from peptides derived from resident proteins within the endocytic pathway since leupeptin, CBI and BfA inhibited D11$^+$0401 epitope expression.

Without knowing whether specific peptides contribute to the D11$^+$0401 epitope, one can only speculate that the source of the antigen is the same for the cytoplasmic and lysosomal generation of D11$^+$0401. It is possible that the D11$^+$0401 epitope could be derived from peptides generated from processing of an endogenous protein within both lysosomes and the cytoplasm, requiring cathepsin B and calpain, respectively. The involvement of cathepsin B, a protease with exopeptidase activity [42], leads to interesting speculation into the antigen source and mechanisms responsible for forming the D11$^+$0401 epitope. Sercarz and Maverakis [54] have recently described two mechanisms how antigens are broken down and bound to class II molecules. The first involves a DM-mediated removal of CLIP for an appropriate sized peptide, while the second mechanism describes how a partially unfolded antigen attaches to the class II molecule, and the ends of the polypeptide are trimmed [54]. For the second mechanism, exopeptidases such as cathepsin B have an important role, and may explain the role of cathepsin B in the generation of the D11$^+$0401 epitope. Additionally, inhibition of the D11$^+$0401 epitope by calpeptin suggests that the source of the putative peptides that form the D11$^+$0401 epitope are derived from the cytoplasm. The mechanism how cytoplasmicly derived peptides enter the lysosomal compartments of the endocytic pathway and whether pre-lysosomal processing followed by lysosomal processing occurs, is still to be resolved. Nimmerjahn and colleagues [55] however, recently provided evidence that this process may primarily occur through autophagy. They showed that

cytosolic proteins sequester to autophagosomes within EBV-transformed BCL, and these autophagosomes are subsequently delivered to the lysosomal compartments where the majority of intracellular class II molecules reside.

Interestingly, the decrease in D11^{+}0401 epitope expression on SAVC treated with cysteine protease inhibitors mirrored the loss of DR/CLIP complexes, suggesting the same proteases are involved in processing Ii. It is most unlikely that CLIP is involved in D11^{+}0401 epitope formation for several reasons; a mAb directed against DR/CLIP complexes did not block the binding of NFLD.D11 to SAVC, CLIP peptides did not reconstitute the D11^{+}0401 epitope and cells that are negative for DM express significant amounts of CLIP, yet fail to express the D11^{+}0401 epitope. This does not rule out that longer versions of the CLIP peptide, LIP and SLIP could somehow be responsible for epitope formation. There is evidence that treatment of cells with leupeptin causes the accumulation of LIP in post-golgi compartments [56], but these Ii intermediates exist at low levels within untreated cells, making them unlikely candidates for D11^{+}0401 epitope formation.

The accumulation of LIP and SLIP within leupeptin-treated cells could however explain the presence of intracellular D13^{+}0401 epitope in SAVC cells treated with the same cysteine protease inhibitors that inhibited D11^{+}0401 epitope formation. The level of intracellular DM was not affected by leupeptin, suggesting the loss of DM does not explain the generation of this D13^{+}0401 epitope on SAVC. Although interesting, this leupeptin-induced DRB1*0401 epitope may prove difficult to study since it is primarily

expressed intracellularly, and is relatively scarce compared with the $D11^{+}0401$ and $D13^{+}0404$ epitopes.

Finally, the effects of protease inhibitors on the $D13^{+}0401$ epitope, which is expressed on DM negative BCL such as 9.5.3 0401 [34] were similar to the $D11^{+}0401$ epitope, in that this epitope was also inhibited by BfA, as well as by endosomal and cytoplasmic cysteine protease inhibitors. This would suggest that the peptides involved in both epitopes could actually be the same despite the opposite influence of DM. Of course, another explanation is that the same proteases are responsible for forming both epitopes, irregardless of the putative peptides. Alternatively, it is possible that the NFLD.D11 and NFLD.D13 mAbs are recognizing conformational epitopes that are influenced by the binding of certain set of peptides and/or the interaction with DM, similar to the epitopes recognized by the mAbs 16.23 [57] and 25-9-17 [58]. Analysis of the profiles of peptides eluted from DRB1*0401 molecules from SAVC, 8.1.6 0401 and 9.5.3 0401 is underway and should yield useful information regarding the identity of these putative peptides.

We have therefore described three DRB1*04-specific epitopes recognized by the mAbs NFLD.D11 and NFLD.D13. The $D11^{+}0401$ and $D13^{+}0404$ epitopes differed in their requirements for cellular proteases, even though the intracellular pathways which form these epitopes in normal BCL appear to be similar. Both endosomal and cytoplasmic cysteine proteases are required for the generation of the $D11^{+}0401$ epitope and interestingly, the same proteases that prevent the appearance of this DM-dependant epitope also caused an intracellular generation of an epitope recognized by NFLD.D13.

Using DM negative cells, we also observed the loss of the D13^{+}0401 epitope in the presence of cysteine protease inhibitors. Further analysis of the peptides eluted from the DRB1*04 molecules isolated from these DM positive and negative B cells is underway and should shed further light on the mechanisms of how these intriguing epitopes are formed.

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5. Thesis Discussion

The main focus of this thesis has been to characterize the generation and expression of epitopes defined by the mAbs NFLD.D11 and NFLD.D13. Both mAbs recognize epitopes on HLA-DRB1*04 molecules that express an amino acid motif, QKRAA/QRRAA, also known as the shared epitope (SE) [1]. This sequence is found on a subset of HLA-DRβ1*04, 01 and 14 molecules at β-chain positions 70-74, and is associated with the development and disease severity of rheumatoid arthritis (RA) in Caucasians [2]. NFLD.D11 and NFLD.D13 were previously shown to bind DRB1*0401 and DRB1*0404 molecules, respectively, on EBV-transformed B cell lines (BCL) [3, 4]. In addition to binding strongly to BCL, NFLD.D11 and NFLD.D13 also bind weakly to peripheral blood B cells from normal controls and RA patients [3].

The formation of the $D11^{+}0401$ epitope is dependent on the presence of the HLA-DM, as it is not expressed on B cells that do not express DM [4, 5]. NFLD.D13 has an intriguing specificity as it is specific for DRB1*0404, but not DRB1*0401 in DM^{+} BCL. However, in DM^{-} B cells, NFLD.D13 recognizes an epitope on HLA-DRB1*0401 molecules [5]. As seen in Table 1 of Chapter 3, DRB1*0401 and DRB1*0404 differ at two amino acid positions, β71 and β86. NFLD.D11 binding absolutely requires a lysine at position β71 but a glycine or valine at position β86 makes little difference. There are four key residues for NFLD.D13 binding to DRB1*0404; a tyrosine at β37, aspartic acid at β57, arginine at β71 and valine at β86. These residues are key contact points for T cell and peptide recognition. For example, β86 is located within pocket 1 of the class II peptide binding groove. Therefore the valine at β86 in DRB1*0404 means that it will be

able to accommodate slightly larger peptide side chains in pocket 1 compared to DRB1*0401. The same can be true for the difference between DRB1*0404 and DRB1*0401 at β71, which along with amino acids 28, 47, 61 and 67 form pocket 7. β57, which is important for NFLD.D13 binding to DRB1*0404, is located in pocket 9 of the peptide binding groove. The location of these key residues within the peptide binding groove strongly suggests that class II-associated peptides are required for $D11^{+}0401$ and $D13^{+}0404$ epitope formation.

That the $D11^{+}0401$ and $D13^{+}0404$ epitopes are primarily expressed on EBV^{+} BCL but not on a wide variety of NP-APC, suggests that an activated cell phenotype is necessary for their formation. This may either be due to proteases that are specifically active within BCL or antigen processing compartments present only in P-APC. The critical role of DM in the formation of these DRB1*04 epitopes, the location of key β chain residues needed for NFLD.D11 and NFLD.D13 binding along with the cellular-restricted expression of these epitopes suggest that a specific set of peptides are involved in their generation. This may be due to direct recognition of the class II peptide/MHC (pMHC) complex by these mAbs, or alternatively, NFLD.D11 and NFLD.D13 may be recognizing conformational determinants on HLA-DRB1*04 molecules similar to the recognition of DR3 by the mAb 16.23 [6]. This putative conformational change in the DRB1*04 molecules may be influenced by the binding of certain peptides to the class II molecule, or by the interaction of the class II molecule with co-chaperone molecules such as DM or MHC class II-associated tetraspan molecules.

5.1 Summary of Major Findings

D11$^+$0401 Epitope

- Strongly expressed on EBV-transformed B cells, IFN-γ-induced MΦ and DC.
- Not expressed on the cell surface of monocytes or DC, but expressed intracellularly on DC.
- Weakly expressed on peripheral blood B cells and not expressed on PHA-stimulated T cells.
- Requires HLA-DM for expression on BCL.
- Not expressed on Daudi Dw4 or Daudi Dw4 DM.
- Not expressed on synovial fibroblasts treated for up to 14 days with IFN-γ, despite strong expression of DR, DM and Ii.
- Not expressed on most carcinoma cell lines that have up-regulated DR and DM.
- Weak to moderate expression on DM$^+$ melanoma cell lines, especially MDA MB 435 Dw4.
- Blocked by mAbs that map to the peptide binding site such as L243, NFLD.D2 and NFLD.D10, but not by NFLD.D1, which binds to the β_2 domain. Also, not blocked by cerCLIP, which binds DR/CLIP complexes, indicating that the D11$^+$DRB1*0401 epitope exists on a different subset of DRB1*0401 molecules.
- Requires a subset of cysteine proteases for its generation.
- Requires newly synthesized molecules for its generation.
- Co-localizes with markers for the endosomal pathway as well as markers for supramolecular complexes, such as CD82.

• Destroyed by tetraspan microdomain-disrupting chemical saponin, but not by methyl-β-cyclodextrin, which disrupts lipid rafts.

D13^{+}0404 Epitope

• Strongly expressed on EBV-transformed B cells, and peripheral blood MΦ.

• Weakly expressed on peripheral blood B cells and negative on PHA-stimulated T cells.

• Does not require HLA-DM for its expression on BCL.

• Not expressed on carcinoma, and fibroblast cell lines that have weakly up-regulated DR and DM.

• Blocked by mAbs that map to the peptide binding site such as L243, NFLD.D2 and NFLD.D10, but not by NFLD.D1, which binds to the β_2 domain. Also, not blocked by cerCLIP, which binds DR/CLIP complexes, indicating that the D13^{+}DRB1*0404 epitope exists on a different subset of DRB1*0404 molecules.

• Does not require proteases for its generation.

• Requires newly synthesized molecules for its generation.

• Co-localizes with markers for the endosomal pathway as well as markers for supramolecular complexes, such as CD82.

• Destroyed by tetraspan microdomain-disrupting chemical saponin, but not by methyl-β-cyclodextrin, which disrupts lipid rafts.

D13$^+$0401 Epitope

- Expressed on HLA-DM negative BCL such as 9.5.3 0401, 5.2.4 0401, SJO Dw4, BLS Dw4 and T2 Dw4.
- Lost after DM reconstitution.
- Blocked by mAbs that map to the peptide binding site such as L243, NFLD.D2 and NFLD.D10, but not by NFLD.D1, which binds to the β_2 domain. Also, not blocked by cerCLIP, which binds DR/CLIP complexes, indicating that the D13$^+$DRB1*0401 epitope exists on a different subset of DRB1*0401 molecules.
- Requires a subset of cysteine proteases for its generation.
- Requires newly synthesized molecules for its generation.
- Expressed on cells that are CDw78$^-$, a marker for tetraspan microdomains.

I will now discuss and examine in detail these main findings as they relate to current immunological research, and explain the basis for future hypotheses with regards to the generation of these mAb-defined eptiopes.

5.1.1 D11$^+$0401 Epitope

The D11$^+$0401 epitope was the most intensely studied in this thesis, and yielded several fascinating insights in regards to the proteases needed for its generation, its intracellular location within BCL and its expression on P-APC and NP-APC. The use of protease inhibitors showed that the cysteine protease inhibitors leupeptin, calpeptin and cathepsin B inhibitor II blocked the formation of this epitope on BCL. In contrast, the

aspartyl protease inhibitor pepstatin A as well as the proteasome inhibitor lactacysin did not have an effect. This indicates that cysteine proteases are critical in generating the peptide repertoire that binds to DRB1*0401 peptide binding cleft to form the $D11^{+}0401$ epitope.

However, without knowing these peptides, it is difficult to conclude that this epitope is peptide-specific with absolute certainty. Certainly, the dependence of this epitope on DM, along with the involvement of cysteine proteases does suggest that the $D11^{+}0401$ epitope could be dependent on a particular set of peptides. Evidence that the $D11^{+}0401$ epitope is on or near the peptide binding groove comes from data showing that NFLD.D11 binding was not blocked by mAb NFLD.D1, which binds to the β2 domain of DRB1*04 molecules. In contrast, mAbs L243, NFLD.D2 and NFLD.D10, which bind to residues in the peptide binding groove, did block NFLD.D11 binding to SAVC. Having said this, this data only provides evidence that the residues that these mAbs recognize are important for NFLD.D11 binding and does not implicate any class II-associated peptide.

Another explanation is that a set of structurally related peptides generated by cysteine proteases, bind to DRB1*0401 molecules and cause a conformational change that is detected by NFLD.D11. Charles Janeway's group showed, using the $I\text{-}A^{b}$-specific mAb, 25-9-17, that peptides can impose subtle structural changes in MHC class II molecules, which can be differentiated by certain mAbs [7]. Conformationally-induced changes may also be peptide-independent as was shown for the 16.23-defined DR3 epitope, which is influenced by the interaction between HLA-DR3 and DM [6]. This epitope, like the $D11^{+}0401$ epitope also disappears in the absence of DM. Although DM

interaction with DRB1*0401 molecules in a peptide-independent manner could possibly generate D11$^+$0401 epitope, we do not think this is the case because it is not present on DRB1*0401$^+$ cells that have up-regulated DM, such as IFN-γ treated synovial fibroblasts (Chapter 2). This suggests that although DM is necessary for D11$^+$0401 expression, other factors are required. These may include P-APC specific proteins or proteases that are differentially up-regulated in NP-APC compared to P-APC.

This differential protein expression may explain why, although there are similarities in the expression patterns of the D11$^+$0401 and 16.23 epitopes, there are also notable differences. Unlike the D11$^+$0401 epitope, the 16.23 epitope was up-regulated on IFN-γ treated fibroblasts after 5 days [8]. We did not observe D11$^+$0401 expression on various fibroblast cell lines as well as other NP-APC treated with IFN-γ, even when the cells were treated for 14 days, although these cells expressed copious amounts of HLA-DR and DM. This expression pattern of the D11$^+$0401 epitope on various cell types, which will be further discussed later, suggests that although a similar DR-DM interaction may be forming the D11$^+$0401 epitope, other cell-specific factors are also required for its expression.

5.1.1.2 Proteolytic Events Involved in D11$^+$0401 Epitope Formation

The requirement of both endosomal and cytoplasmic cysteine proteases for D11$^+$0401 epitope expression may help explain the additional factors required for D11$^+$0401 epitope expression in addition to providing further insights into how certain MHC class II epitopes are formed. Others have shown that cytoplasmic processing is a

prerequisite for the generation of particular class II epitopes [9, 10]. More recently a proposed mechanism of how peptides derived from cytosolic antigens can enter the lytic compartments and bind to class II molecules has been described [11]. Lich et al, 2000 showed that alternative processing was involved in the proteolysis of endogenous glutamate decarboxylase (GAD). For endogenously expressed GAD, cytoplasmic proteolysis by the proteasome and calpain were required for the generation of an immunodominant peptide that binds to DRB1*0401. However, lysosomal processing, blocked by brefeldin A, was necessary for the processing of exogenous supplied GAD into the same immunodominant peptide. Similarly, Mukherjee et al, 2001 showed that processing by the proteasome was required for the class II presentation of peptides derived from cytoplasmically expressed OVA and conalbumin [10]. The same authors also showed that class II presentation of an endogenous I-E∀ peptide was also inhibited by inhibitors of cytosolic proteolysis.

These results infer that the source of the putative peptide(s) involved in $D11^{+}0401$ epitope formation may be cytoplasmically-derived, since calpain inhibited NFLD.D11 binding to SAVC, or due to peptides derived from resident proteins within the endocytic pathway since cathepsin B inhibitor II and brefeldin A also inhibited $D11^{+}0401$ epitope expression. Involvement of the exopeptidase cathepsin B leads to interesting speculation into possible mechanisms that form the $D11^{+}0401$ epitope. Sercarz and Maverakis [12] have recently described two mechanisms for how antigens are broken down and bound to class II molecules. The first involves a DM-mediated removal of CLIP for an appropriate sized peptide, while the other describes how a partially unfolded antigen attaches to the

class II molecule, and is trimmed into peptides while bound [12]. For the second mechanism, exopeptidases such as cathepsin B have an important role, and may explain the role of cathepsin B in the generation of the $D11^{+}0401$ epitope.

Of course, it is possible that the $D11^{+}0401$ epitope could be derived from peptides generated from processing of an endogenous protein within both the lysosomes and cytoplasm. The mechanism by which cytoplasmicly derived peptides enter the lysosomal compartments of the endocytic pathway is still to be fully resolved, although Nimmerjahn et al, 2003 recently provided evidence that this process primarily occurs through autophagy. They showed that cytosolic proteins sequester to autophagosomes within EBV-transformed BCL, and these autophagosomes are subsequently delivered to the lysosomal compartments where the majority of intracellular class II molecules reside. Therefore it may be useful to test whether autophagy is related to the calpain inhibition of $D11^{+}0401$ epitope expression on SAVC. One could imagine that calpain pre-processes a putative antigen in the cytoplasm, where it is then transported into the lysosomes by autophagy, and further processed by lysosomal cysteine proteases such as cathepsin B. To determine whether autophagy is important for $D11^{+}0401$ epitope expression, cells could be treated with wortmannin, a known inhibitor of autophagy [11].

The differential expression of proteases required for $D11^{+}0401$ epitope expression could explain why it is not expressed in most DM^{+} NP-APC such as synovial fibroblasts (Chapter 2). Having said this, cultured fibroblast-like synoviocytes from RA patients have been shown to express and secrete copious amounts of cathepsin B, S and L after stimulation with inflammatory cytokines such as IFN-γ [13, 14]. The seemingly

ubiquitous expression of these cysteine proteases within NP-APC suggests that the protein source of the putative D11$^+$0401 peptides may differ between P-APC and NP-APC. Therefore, although DM and cysteine proteases are required for D11$^+$0401 epitope formation, another cell type-specific factor is likely also necessary.

5.1.1.3 Cellular Expression of the D11$^+$0401 Epitope

Since the D11$^+$0401 epitope was strongly expressed on B cells, MΦ, DC and not on a wide variety of NP-APC tested, this suggests that a factor specific to P-APC is influencing its production. Together with the protease inhibitor results, differential expression of certain proteases between D11$^+$0401$^+$ and D11$^+$0401$^-$ cells is a possibility and requires further testing. In regards to the D11$^+$0401 epitope expression on myeloid cells, DC are known to increase proteolysis [15] and lysosomal function [16] after activation with inflammatory stimuli such as LPS and TNF-α. However, because the D11$^+$0401 epitope was expressed equally on DC before and after stimulation, this suggests that a ubiquitous protein/pathway within these DC is responsible for forming this epitope.

Another explanation for differential expression of the D11$^+$0401 epitope could be intracellular compartmentalization and cellular organization differences between P-APC and NP-APC. For example, the putative melanoma cell line MDA MB 435 Dw4 expressed significant amounts of D11$^+$0401 epitope before and after IFN-γ stimulation. We do not think this is simply because of high DM levels, as several other DM$^+$ NP-APC failed to express D11$^+$0401; more likely, there is a shared factor or mechanism causing

the expression of the D11$^+$0401 epitope in MDA MB 435 Dw4, B cells, MΦ and DC. Further studies on MDA MB 435 Dw4 such as co-localization studies with markers of intracellular compartments or by the treatment with protease inhibitors should further define the factors responsible for generating the D11$^+$0401 epitope on BCL compared to MDA MB 435 Dw4.

Our studies described in Chapter 4 clearly show that the D11$^+$0401 epitope co-localizes with DM as well as the tetraspan molecules CD63 and CD82. Since the intracellular distribution of CD63 is restricted to lysosomes, its co-localization with NFLD.D11 suggests that the D11$^+$0401 epitope exists within the lysosomal/endosomal pathway. Furthermore, as shown by immunoelectron microscopy, D11$^+$0401 colocalized with CD63, CD82 and DM was located within multilaminar MIIC compartments, which are thought to functionally most resemble lysosomes [17]. Since the D11$^+$0401 epitope was also sensitive to brefeldin A treatment, which inhibits ER-Golgi as well as transport of molecules to lysosomal compartments [11], this suggests molecules bearing the D11$^+$0401 epitope traffics through the classical MHC class II pathway to traffic through BCL and is generated in post-Golgi compartments.

CD63, which is present in copious amounts in MIIC and lysosomes, is also present in large complexes that include MHC class I, class II, DM, DO and other tetraspanin proteins such as CD9, CD53, CD20, CD81 and CD82 [18-20]. Related to this, Hammond et al, 1998 [21] also found an enrichment of supramolecular complexes involving HLA class I, class II, CD63, CD82, DM and DO in MIIC within B cell lines. These widely expressed tetraspanin molecules serve a wide variety of roles, including

signal transduction, cell adhesion, motility, activation and may structurally anchor molecules such as HLA-DR in order for proper intracellular and cell surface interactions to occur [22]. These tetraspan molecular complexes have also been suggested to concentrate specific MHC/peptide complexes, increasing the chance of TCR-MHC interaction [23].

Our finding that the D11^{+}0401 epitope co-localized with CD63, CD82 and DM also raises the possibility that the D11^{+}0401 epitope requires DRB1*04 molecules existing within such supramolecular tetraspan complexes. Since HLA class I molecules are also reported to be located within these microdomains, further indirect evidence that the D11^{+}0401 epitope is located within tetraspan microdomains is partial blocking of NFLD.D11 binding to BCL with the HLA-B specific mAb NFLD.M15 (data not shown). Related to this, CD82 has been found associated with HLA class I within normal BCL [24], so it is conceivable that a complex between either of these molecules with DRB1*0401 results in an epitope recognized by NFLD.D11.

Supporting this, we observed that cell surface NFLD.D11 binding was disrupted by the detergent saponin, which, when used at low concentrations is reported to disturb tetraspan complexes [25]. Many groups, including Roche and colleagues have also suggested that class II molecules located within lipid raft domains are important for the presentation of peptides when antigen is at limiting concentrations, in addition to concentrating relevant pMHC complexes into the central immunological synapse interface, better enabling T cell activation [26, 27]. We did not observe a loss in NFLD.D11 binding to BCL in the presence of the lipid raft disrupting chemical methyl-

β-cyclodextrin, suggesting that the D11$^+$0401 epitope likely does not require lipid raft microdomains for its expression. A similar mAb, designated UL-5A1, distinguishes DRB1*0101 bound to HLA-A2 derived peptides only on activated cells such as EBV-transformed B cells [28]. This suggests that an activated phenotype, either by the expression of cellular-specific proteins or the mobilization of necessary compartmentalization and antigen processing mechanisms is also important for UL-5A1 epitope expression. The UL-5A1 epitope is reported to co-localize with CDw78, a marker for tetraspan microdomains, on the surface of BCL [23] and DC [25]. Like the UL-5A1 epitope, the D11$^+$0401 and D13$^+$0404 epitopes also require a factor present in activated P-APC, such as tetraspan microdomains for their expression.

Taken together, we conclude that the D11$^+$0401 epitope in BCL requires DM, cysteine proteases, and is located within the classical MHC class II pathway, in particular within late endosomes and lysosomes. Here, the D11$^+$0401 epitope may be located within tetraspan protein aggregates that include CD63 and CD82. As will be discussed later, immunoprecipitation of D11$^+$DRB1*04 molecules has proven technically difficult, thus, a potentially useful experiment would have been to immunoprecipitate CD82 or CD63-positive complexes using mild detergents that do not destroy the tetraspan microdomains, such as *n*-octylglucoside [25], and test for the presence of the D11$^+$0401 epitope. Comparison of these aggregates between a D11$^+$0401 epitope positive cell line such as SAVC or MDA MB 435 DW4 and a D11$^+$0401 epitope negative cell line such as T2.0401, or 9.5.3 0401, which do not express CDw78, should answer whether such tetraspan microdomains are required for D11$^+$0401 expression.

5.1.2 D13$^+$0404 Epitope

The second epitope, D13$^+$0404 is somewhat similar to the D11$^+$0401 epitope, in that its expression is restricted. For example, it is more strongly expressed on BCL than peripheral blood B cells [3], but unlike D11$^+$0401, it is more strongly expressed on resting and activated MΦ (data not shown). Because of the lower frequency of DRB1*0404 compared to DRB1*0401 in the general population, fewer cell lines were available for testing the D13$^+$0404 epitope. From those DRB1*0404$^+$ cells tested, we found that like the D11$^+$0401 epitope, the D13$^+$0404 epitope appears to be preferentially expressed on P-APC. Thus, this pattern of expression suggests that a cell-specific mechanism is responsible for the generation of the D13$^+$0404 epitope.

Although, like the D11$^+$0401 epitope, the D13$^+$0404 epitope co-localized with molecules found within late endosomes and lysosomes such as CD82, LAMP-1 and CD63, but not with CD71, a marker for early endosomes, its strong co-localization with Ii suggests that Ii may be involved in D13$^+$0404 epitope formation, either due to Ii intermediate peptides, or through Ii-influenced trafficking of D13$^+$ DRB1*04 molecules. Indeed, data suggests that a significant number of class II/Ii complexes migrate directly to the cell surface [29]. Here these molecules may either recycle through early endosomes to the plasma membrane, or alternatively, enter the endocytic pathway from the cell surface directly to the MIIC [29]. Therefore, although the D13$^+$0404 is found within the endocytic pathway, the co-localization studies with Ii suggest that its intracellular trafficking route may be different from the molecules that form the D11$^+$0401 epitope.

The partial loss in D13$^+$0404 expression in the presence of brefeldin A indicates that this epitope is formed in post-golgi compartments, and provides further evidence that NFLD.D13 recognizes DRB1*0404 molecules within the classical MHC class II pathway. Unfortunately, the NFLD.D13 mAb could not be used in the immunoelectron microscopy assays and therefore we can not conclude anything about the ultrastructural location of this epitope with BCL.

Searching for other factors that influence the generation of the D13$^+$0404 epitope, we found that unlike the D11$^+$0401 epitope, none of the protease inhibitors used in this study significantly affected the expression of the D13$^+$0404 epitope. Our finding that the D13$^+$0404 epitope was not significantly altered by inhibiting proteases such as cathepsin B and calpain, suggests, along with it being less DM-dependent, that the D13$^+$0404 epitope is probably peptide-independent and is conformationally determined. When compared to DRB1*0401, DRB1*0404 differs in two residues; positions β71 and β86. DRB1*0401 has lysine and glycine, whereas DRB1*0404 has arginine and valine at positions β71 and β86, respectively. The arginine at position β71, in particular is a likely key residues responsible for the ability of NFLD.D13 to specifically recognize DRB1*0404 since it has an extra methyl group compared with lysine, which points up and out, and is thought to contact the TCR as well as the bound peptide [1, 2]. Having said this, this does not explain the absence of the D13$^+$0404 epitope on NP-APC.

Since the D13$^+$0404 epitope co-localized with the CD63 and CD82 mAbs within MT14b, another possibility is that these tetraspan molecules are somehow influencing the binding of NFLD.D13 to DRB1*0404. One can imagine that the D13$^+$0404 epitope

could be formed by DRB1*0404 molecules located within tetraspan microdomains in a manner similarly suggested for the D11^{+}0401 epitope. This prospect is supported by data in which the D13^{+}0404 epitope on the surface of MT14b was dramatically disrupted by the tetraspan microdomain disrupting chemical saponin, but not by methyl-β-cyclodextrin, which disrupts lipid rafts by dispersing cholesterol aggregations on the cell surface. If the D13^{+}0404 epitope requires DRB1*0404 molecules expressed in supramolecular complexes, this may explain its absence on NP-APC such as synovial fibroblasts. For example, although synovial fibroblasts express CD63, CD82 and DM, it is possible that they do not express other proteins seemingly required for proper tetraspan microdomain formation, such as CD20, CD40, CD9 or CD81 [23].

5.1.3 D13^{+}0401 Epitope

The third epitope (D13^{+}0401) under investigation in this thesis is present on DM negative BCL and disappears after DM expression is restored [4]. Since the D13^{+}0401 epitope is inhibited by DM, this suggests that certain peptides, present in the DRB1*0401 peptide binding groove, somehow influence the binding of NFLD.D13, either through direct interaction of the mAb with the peptide/MHC complex or through a conformational change in these molecules. Since DM negative cells express abundant amounts of CLIP in the class II peptide-binding groove, CLIP was considered a prime candidate to either form or influence the expression of the D13^{+}0401 epitope. However, evidence against this included failure to reconstitute the epitopes with CLIP in peptide binding assays and failure of cerCLIP to block the D13^{+}0401 epitope. Alternatively, the

absence of DM could be creating a conformational change in the DRB1*0401 molecules that is detected by NFLD.D13. This could be explained by the previously discussed, DR3-reactive mAb 16.23, which is not expressed in DM negative BCL, and thus is similar to the D11^{+}0401 epitope. It has been shown that DM alters the conformation of the DR3 molecules in a peptide-independent manner such that 16.23 binding is obliterated in its absence [6].

Although we have not tested it here, it is possible that the opposite situation is occurring with the D13^{+}0401 epitope, where the absence of DM induces a conformational change in the DRB1*0401 molecules that is recognized by NFLD.D13. After the introduction of DM into these D13^{+}0401 epitope negative cells, this required conformation would be lost and result in the loss of NFLD.D13 binding. This putative conformational change may or may not involve specific peptides in the DRB1*0401 peptide binding groove. To test whether peptides might be involved, we used NFLD.D10 to purified DRB1*04-derived peptides from the mutant BCL 9.5.3 0401, which is DM negative and D13^{+}0401 [5], and peptides from the parent BCL, 8.1.6 0401, which expresses the D11^{+}0401 epitope and not the D13^{+}0401 epitope. It is expected that differential display of these peptides (ongoing work that is being carried out in collaboration with Drs Englehard and Hunt at the University of Virginia) will yield major peptide-population differences since these cells are genetically identical with the exception of a deletion on chromosome 6, which includes DMB [30]. Using peptide binding algorithms, we will select and synthesize the most promising peptides for reconstitution in peptide binding assays. It is expected that peptide fractions that differ

between 8.1.6 0401 and 9.5.3 0401 could then be used in more functional assays such as epitope reconstitution assays.

Unfortunately, confocal microscopy, immunoelectron microscopy and CELISA based experiments that were performed on normal BCL could not be done on 9.5.3 0401. L243 (HLA-DR), NFLD.D1 (HLA-DRB1*04), as well as NFLD.D13 ($D13^{+}0401$) binding was effected by the fixatives acetone and paraformaldehyde when these experiments were repeated with 9.5.3 0401 (data not shown). Therefore the data on the $D13^{+}0401$ epitope presented in this thesis is restricted to cell surface expression. I feel that it would have been valuable to compare the intracellular generation of the $D13^{+}0401$ epitope by confocal microscopy with the $D11^{+}0401$ epitope in DM positive BCL, especially with the knowledge that both epitopes are influenced by the same proteases.

5.2 Usefulness of $D11^{+}0401$ and $D13^{+}0404$ Epitopes in the Field of Immunological Research.

Although the basic mechanisms and molecules involved in MHC class II antigen processing and presentation are well known, some questions still remain unresolved. First, recruitment of peptide-loaded class II molecules to the cell surface, whether it is through specific tetraspan or lipid microdomains still remains a mystery. Several lines of evidence point towards both structures being involved in MHC class II antigen processing and presentation [23, 25-27]. The differences in internal compartmentalization between P-APC and NP-APC and how this relates to the presentation of class II/peptide complexes is another active field of research. For

example, recently melanocytes have been reported to contain unique melanosomes that resemble conventional MHC class II antigen processing compartments and function in the storage, synthesis and secretion of melanins [31, 32]. Many of these melanins are tumor-associated antigens and therefore the generation and functioning of these lysosome-like compartments within melanocytes as well as similar compartments within other non-conventional APC is of great importance for tumor immunology and autoimmunity. If the compartmentalization within MDA MB 435 Dw4 is shown to be more like that of P-APC, this could explain the presence of the $D11^{+}0401$ epitope on these cells. Similarly, differences in compartmentalization within other NP-APC would explain the lack of the $D11^{+}0401$ epitope.

Although we have known for years through biochemical studies that MHC class II molecules contain peptides derived from endogenous proteins within their binding clefts [33-36], the process of how these peptides are generated within the cell is only recently being resolved. These peptides may be derived from either lysosomal and/or cytoplasmic proteolysis, and recent data strongly suggests that proteasomal proteolysis can generate certain MHC class II-associated peptides [9, 10]. Therefore, the proteolytic events leading to the generation of mature class II molecules are another active field of research. Although many of the individual proteases involved in class II processing have been defined [37-39], the exact interplay between proteases in the development of mature class II/peptide complexes, and how this may differ between cell types still needs further unravelling.

5.3 Future Studies

(1) The interesting finding that endosomal and cytoplasmic proteolysis by cysteine proteases is important for optimal expression of the D11^{+}0401 epitope within BCL, these proteolytic events could be further defined. Inhibiting cathepsin L had little effect on the D11^{+}0401 epitope (data not shown). However, the effects of an abundant lysosomal cysteine protease cathepsin S, was not rigorously examined. For this, the inhibitor LHVS or the more recently developed specific inhibitor of cathepsin S, Clik60 [40] would be useful.

(2) The strong expression of the D11^{+}0401 epitope within the melanoma cell line MDA MB 435 DW4, but not within other NP-APC, is tantalizing. It would be insightful to compare its intracellular location as well as the proteolytic events that form the D11^{+}0401 epitope in MDA MB 435 DW4 to normal BCL. Related to this, I believe that the cellular restriction of the D11^{+}0401 epitope makes it a useful tool in investigating the antigen processing differences between P-APC and NP-APC, which may be due to compartmentalization and association with tetraspan molecules as well as proteolytic activity within the cell. Of course, the possibility that cellular-specific peptides bound to the DRB1*0401 peptide binding groove are necessary for this epitope needs to be resolved.

(3) To resolve this issue of peptide dependence, it would be ideal to purify D11^{+}0401 epitope positive DRB1*0401 molecules by immunoprecipitation and acid elute the peptides from the peptide binding groove. The ability of these peptides to reconstitute

the D11$^+$0401 epitope in cells that do not normally express this epitope may answer if it is peptide-dependent. Unfortunately, the NFLD.D11 mAb has not proven to be useful for such biochemical assays in the past. In fact, analysis of BCL lysates by western blots using the NFLD.D11 mAb yields a band the size of the HLA-DR β chain. Knowing this, we have purified DRB1*04 molecules from SAVC using NFLD.D10, which requires a Q at position 70 of the DRβ chain and blocks NFLD.D11 binding to SAVC.

It is expected that the pool of DRB1*04 molecules that form the D11$^+$0401 epitope will lie within this NFLD.D10-defined DRB1*04 population. Although not presented in the context of this thesis, I have purified DRB1*0401-associated peptides from several BCL in order to address the issue of peptide dependence of these epitopes. Analysis of peptides derived from 8.1.6 0401 (D11$^+$0401 epitope positive) and 9.5.3 0401 (D13$^+$0401 epitope positive) is underway within the lab and hopefully should answer whether or not the D11$^+$0401 epitope is peptide dependent. If this proves to be the case, comparing whether these putative peptides are generated within BCL, MDA MB 435 and NP-APC that do not express the D11$^+$0401 epitope could provide valuable information about differences in the generation of class II pMHC complexes between cell types. Alternatively, if the D11$^+$0401 and D13$^+$0404 epitopes do prove to contain nested sets of peptides, it would be interesting to analyze their expression in the thymus and other lymphoid organs.

(4) From the cumulative data on the D13$^+$0404 epitope, I feel that it is important to investigate factors responsible for its P-APC specificity. This will hopefully lead to

insights into the factors responsible for its generation and also could hold valuable information into the differences in DRB1*04 epitope expression between P-APC and NP-APC. For the $D13^{+}0404$ epitope, these differences may be due to the organization of tetraspan protein complexes and therefore this needs further attention. As proposed for the $D11^{+}0401$ epitope, immunoprecipitation of CD82 or CD63 in the presence of chemicals that either disrupt or preserve tetraspan microdomains followed by immunoblotting with NFLD.D13 may answer whether or not the $D13^{+}0404$ epitope is found on DRB1*0404 molecules within these protein complexes.

5.4 Concluding Statements

In summary, I feel that studies with the mAbs NFLD.D11 and NFLD.D13 have yielded interesting information into numerous immunological processes. The cell type-restricted nature of these epitopes has been useful in detecting differences in HLA-DRB1*04 complexes in cells that would not be detected using pan-DRB1*04 mAbs such as NFLD.D1. For the $D11^{+}0401$ and $D13^{+}0404$ epitopes, their expression on BCL and in the case of $D11^{+}0401$, MΦ and DC, also makes them useful tools for examining antigen processing and presentation differences between P-APC and NP-APC. For example, their absence on synovial fibroblasts indicates that the peptide repertoire on these cells is different from BCL even though they have a similar class II pathway with respect to chaperones. Similarly, other MHC class II specific mAbs have been used to dissect the basic mechanisms of antigen processing and presentation [41-43]. Another mAb, Y-Ae, which recognizes $I\text{-}A^{b}$ molecules complexed with a major self-peptide, $E_{\alpha 52\text{-}68}$ [44, 45]

has been used to address a variety of questions, including the expression of this self peptide in the thymus and peripheral lymphoid organs [41, 42, 46, 47]. Also, the role of self peptides in positive and negative selection of T cells has also been investigated using Y-Ae [48, 49], and mAb, designated G35 [50]. Of course the advantage of working with these mAbs is that they recognize murine MHC class II molecules, which makes the design and ethics of the appropriate experiments more reasonable. Also, the epitopes recognized by these mAbs are known to require specific peptides, which we do not know is the case for the $D11^{+}0401$ and $D13^{+}0404$ epitopes.

Since NFLD.D11 and NFLD.D13 are located on the same DRB1*04 molecules that carry the SE, which is associated with severe RA, these mAbs might be useful to compare the levels of expression of DRB1*04 on P-APC in healthy controls and patients presenting with RA. This may lead to some insight into the cells and HLA-DRB1*04 molecules that are responsible for disease progression. Similarly, studies with the MK16 mAb, which is specific for HLA-DR2 bound with the immunodominant myelin basic protein (MBP) peptide 85-99 have been used to study the presence of this pMHC complex in multiple sclerosis lesions, as well as identifying the predominant APC responsible for presenting this immundominant pMHC complex [51].

The potential involvement of tetraspan microdomains in the generation of these DRB1*04 epitopes makes them useful tools for studying how these microdomains function compared to lipid raft microdomains. Little is known about how these tetraspan microdomains traffic, are organized on the cell surface, and how they function to concentrate pMHC complexes, so if the DRB1*04 molecules that form the $D11^{+}0401$ and

D13^{+}0404 epitopes are tetrapan-associated, the NFLD.D11 and NFLD.D13 mAbs could be useful for answering these questions. Furthermore, the tetraspan environment on the surface of different cell types could explain why these epitopes are absent on synovial fibroblasts.

The involvement of endosomal and cytoplasmic cysteine proteases in the formation of the D11^{+}0401 and D13^{+}0401 epitopes also provides information into how cytoplasmic proteases function in the generation of mature class II pMHC complexes. From a clinical perspective, the role of lysosomal cathepsins in human cancers has recently been under investigation. This may be due to over expression of certain cathepsins which may either lead to breakdown of the surrounding tissues [52], or through the inappropriate breakdown of antigens, which could play an active role in the T cell dependent anti-tumor immune response through MHC class II molecules. Therefore the role of lysosomal cysteine proteases in the formation of the D11^{+}0401 and D13^{+}0401 epitopes may be important in our general knowledge of the role of these proteases in MHC class II maturation and functioning.

The involvement of DM and the inhibition of these epitopes by protease inhibitors only indirectly implies that peptides are involved in the expression of these epitopes. Comparison of the DRB1*04-associated peptides derived from 8.1.6 0401 (D11^{+}0401 epitope positive) and 9.5.3 0401 (D13^{+}0404 epitope positive) in addition to testing if these peptides will be able to reconstitute these epitopes on other cells is likely to answer the question of peptide dependence. A potential problem to this approach could be the nature of the class II peptides, which are of variable lengths. Lippolis et al, 2002 [53]

purified peptides from DRB1*0401 molecules and subjected them to mass spectrometry analysis. They found that of the 700 peptides analyzed, there appeared to be an abundance of nested sets of peptides sharing the same core sequence. This core sequence can be flanked by numerous and different amino acids, resulting in peptides with various lengths and charges. Because of this, peptides with the same core sequence may not elute in the same fractions making it difficult to obtain enough peptides to reconstitute the class II epitope.

Alternatively, the NFLD.D11 and NFLD.D13 mAbs may be recognizing conformational epitopes on DRB1*04 molecules that are influenced by tetraspan complexes, or DM as is the case with the 16.23 epitope. Another possibility could be that cysteine proteases are normally generating groups of peptides that once bound to the DRB1*0401 molecule, changes its conformation such that a subset of DRB1*04 molecules are recognized by either NFLD.D11 or NFLD.D13, in a similar manner to how 25-9-17 recognizes a conformational epitope on I-A^b molecules [7]. This putative conformation could also be influenced by the presence of DM. This, of course is mere speculation, but these open-ended questions may spark future research involving the NFLD.D11 and NFLD.D13 recognized HLA-DRB1*04 epitopes.

Whether or not these epitopes are generated by specific peptides in the class II peptide binding grooves, through direct pMHC complex recognition or conformational changes induced by these peptides, or through protein-protein interaction between class II molecules and tetraspan molecules, these mAbs will still prove useful for studying basic immunological processes.

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