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Enforcing Dendritic cell Vaccines by manipulating the MHC II Antigen Presentation Pathway

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Ce mémoire intitulé:

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Résumé

Les vaccins à base de cellules dendritiques (DCs) constituent une avenue très populaire en immunothérapie du cancer. Alors que ces cellules peuvent présenter des peptides exogènes ajoutés au milieu, l'efficacité de chargement de ces peptides au le complexe majeur d'histocompatibilité (CMH) de classe II est limitée. En effet, la majorité des molécules du CMH II à la surface des DCs sont très stable et l'échange de peptide spontané est minime. Confinée aux vésicules endosomales, HLA-DM (DM) retire les peptides des molécules du CMH II en plus de leur accorder une conformation réceptive au chargement de peptides.

Il est possible, cependant, de muter le signal de rétention de DM de façon à ce que la protéine s'accumule en surface. Nous avons émis l'hypothèse que ce mutant de DM (DMY) sera aussi fonctionnel à la surface que dans la voie endosomale et qu'il favorisera le chargement de peptides exogènes aux DCs. Nous avons utilisé un vecteur adénoviral pour exprimer DMY dans des DCs et avons montrer que la molécule augmente le chargement de peptides.

L'augmentation du chargement peptidique par DMY est autant qualitatif que quantitatif. DMY améliore la réponse T auxiliaire (T_h) du coté T_h1, ce qui favorise l'immunité anti-cancer. Du côté qualitatif, le chargement de peptides résulte en des complexes peptide-CMHII (pCMH) d'une conformation supérieure (conformère). Ce conformère (Type A) est le préféré pour la vaccination et DMY édite avec succès les complexes pCMH à la surface en éliminant ceux de type B, lesquels sont indésirables.

La fonction de DM est régulée par HLA-DO (DO). Ce dernier inhibe l'habilité de DM à échanger le peptide CLIP (peptide dérivée de la chaîne invariante) en fonction du pH, donc dans les endosomes tardifs. Mes résultats indiquent que la surexpression de DO influence la présentation des superantigènes (SAgs) dépendants de la nature du peptide. Il est probable que DO améliore indirectement la liaison de ces SAgs au pCMH dû à l'accumulation de complexe CLIP-CMH, d'autant plus qu'il neutralise la polarisation T_h2 normalement observée par CLIP.

Ensemble, ces résultats indiquent que DMY est un outil intéressant pour renforcer le chargement de peptides exogènes sur les DCs et ainsi générer des vaccins efficaces. Un effet inattendu de DO sur la présentation de certains SAgs a aussi été observé. Davantage

de recherche est nécessaire afin de résoudre comment DMY et DO influence la polarisation des lymphocytes T auxiliaires. Cela conduira à une meilleure compréhension de la présentation antigénique et de son étroite collaboration avec le système immunitaire.

Abstract

Dendritic cell peptide-based vaccines are the most common immunotherapy approach in cancer therapy. While, in principle, dendritic cells (DCs) could be loaded efficiently by exogenously added tumor peptides, their loading efficacy is severely reduced due to low number of peptide-receptive MHC II on cell surface. Most surface MHC II molecules are either occupied by endogenous peptides or are inactive due to a conformation that is not receptive for free peptides. In MHC II antigen presentation pathway, HLA-DM (DM) in acidic endosomal vesicles removes the self-peptides and grants a peptide receptive conformation to MHC II.

Mutating of an intracellular sorting motif in DM, renders its accumulation on cell surface. We hypothesized that the mutant DM (DMY) is functional on cell surface and can generate peptide receptive MHC II on surface of DCs for exogenous peptide loading. By using an adenoviral vector that expresses DMY, we found that DMY is functional on surface of DCs. DMY supplied peptide receptive MHC II on surface of DCs and improved exogenous peptide loading.

The improvement of peptide loading by DMY is both quantitative and qualitative. DMY improves helper T cell (T_h) response in T_h1 direction that favors anti-cancer immunity. The qualitative improvement of peptide loading extends to loading of superior conformational isomer (conformer) of peptide-MHC complexes. This superior conformer (type A) is the favourite type for vaccination approaches and DMY successfully edits peptide-MHC conformers on cell surface level by eliminating undesirable one (type B).

Function of DM is regulated by HLA-DO (DO) and it is well accepted that in acidic pH of late endosomes, DO inhibits function of DM by preventing removal of class II associated invariant chain peptide (CLIP) from peptide binding groove of MHC II. My results indicate that DO overexpression, changes binding of peptide-dependent superantigens to MHC II molecules. Superantigens (SAgs) are small microbial proteins that bind out side peptide binding groove of MHC II. DO probably enhances binding of peptide-dependent SAgs by forcing the accumulation of CLIP on the cell surface of antigen presenting cells. DO also neutralizes T_h2 polarization by CLIP.

Collectively, these results indicate that DMY is a valuable tool for improvement of exogenous peptide loading in DCs vaccines. An unnoticed effect of DO on SAgs binding

was also recognized. Further investigations are needed to clarify the mechanisms by which, DMY and DO influence T_h polarization. This would provide a better understanding of antigen presentation pathway and its interaction with immune system.

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Dedicated to my wife, mother and father.

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List of abbreviation

Ab	**Antibody**
AEP	**Asparagine endopeptidase**
Ag	**Antigen**
APC	**Antigen presenting cell**
β2m	**β2-microglobulin**
BCR	**B cell receptor**
CLIP	**Class II associated invariant chain Peptide**
Conformer	**Conformational isomer**
DC	**Dendritic cell**
DM	**HLA-DM**
DR	**HLA-DR**
DRiPs	**Defective ribosomal products**
ERAAP	**ER aminopeptidase associated with antigen processing**
ERAD	**ER-associated protein degradation**
ER	**Endoplasmic reticulum**
GC	**Germinal center**
HLA	**Human leukocyte antigen**
IFN	**Interferon**
Ii	**Invariant chain**
IL-	**Interleukin-**
iTreg	**Inducible T regulatory cells**
KO	**Knock out**
LAK	**Lymphokine-activated killer**
mAb	**Monoclobal antibody**
MHC I	**Major histocompatibility complex class I**
MHC II	**Major histocompatibility complex class II**
MIIC	**MHC class II compartment**
NK cells	**Natural killer cells**
NKT cells	**Natural killer T cells**
nTreg	**Natural T regulatory cells**

PDI	**Protein disulphide isomerase**
PLC	**Peptide-loading complex**
SAg	**Super Antigen**
SEA	**Staphylococcus enterotoxin A**
SEB	**Staphylococcus enterotoxin B**
TAP	**Transporter associated with antigen presentation**
TCR	**T cell receptor**
TGN	**Trans-Golgi network**
TILs	**Tumor-infiltrating lymphocytes**
Treg	**T regulatory cells**
TSST-1	**Toxic shock syndrome toxin-1**
vSAg	**Viral Super Antigen**

CHAPTER .1

1.0 Introduction

Cancer is predicted to be an increasingly important cause of morbidity and mortality in the next few decades, in all regions of the world. The challenges of tackling cancer are enormous and – when combined with population ageing – increases in cancer prevalence are inevitable, regardless of current or future actions or levels of investment. The forecasted changes in population demographics in the next two decades mean that even if current global cancer rates remain unchanged, the estimated incidence of 12.7 million new cancer cases in 2008 will rise to 21.4 million by 2030, with nearly two thirds of all cancer diagnoses occurring in low- and middle-income countries.

"*Global status report on noncommunicable diseases 2010 WHO"*

1.1 Cancer Therapy Challenges

The goal of cancer therapy is to destroy residual of tumor that cannot be removed by surgery. However, being of host origin and plasticity of tumor cells limit the full effectiveness of current cancer therapy approaches. Cancer cells share many features with the host cells that cause side effects of effective treatment and limit the therapeutic window. Genetic plasticity of cancer cells usually, allow them to survive traditional radiotherapy and chemotherapy regimens. Because of the capacity of tumor cells to evolve resistance under selective pressures, even when the majority of tumor cells are killed by a cytotoxic drug, a small number of resistant cells can be sufficient to regrowth the tumor. The survived tumor cells do no longer respond to previous successful chemotherapy. Successful treatment of diseases caused by highly mutable entities, such as HIV, requires multiple agents that target different survival mechanisms. Compared to microbes, the genetic space available for the evolution of a cancer cell is much larger due to the greater size of the genome. Thus the effective treatment of tumors is more challenging even when multiple agents are combined. Identification and application of more synergistic therapeutic approaches enhance the chance of blocking these many survival mechanisms and to eradicate the cancer.

Immunotherapy approaches and the awakening of active immunity against tumor have many appeals, but its main advantage among other therapeutic approaches is its flexibility to mutability of tumors. The immune system adapts and modifies its response according to the new traits of tumor and the tumor response to selection pressures. The cancer immunotherapy boosts the immune system to attack tumor directly or indirectly. In direct attack to tumor, we engage the immune system in a way that allows it to eradicate tumor cells just like an infection. In the indirect approach, the focus of the attack is changed from the tumor cell to the environment that supports tumor growth and survival. For example, by depriving tumor of blood supply, antiangiogenic therapies can indirectly kill cancer cells. To evolve resistance to such therapies is difficult because stromal cells are not genetically plastic as tumor cells (Prendergast et al., 2007). However, even these indirect attacks against tumors are prone to subversion through tumor cell evolution. For example tumors by vascular mimicry evade antiangiogenesis therapies (Folberg, Hendrix, & Maniotis, 2000). The immunotherapy has been categorized to passive and active approaches. In *passive immunotherapy*, specific effectors are directly infused and are not induced or expanded within the patient. Lymphokine-activated killer (LAK) cells and Tumor-infiltrating lymphocytes (TILs) are produced from the patient's endogenous T cells, which are extracted and grown in a cell culture system by exposing them to interleukin-2 (IL-2). The proliferated TILs and LAK cells are then returned to the patient's bloodstream (Dudley ME et al. 2003). In *active immunotherapy*, cellular immunity that once failed to develop an effective response to tumor, renfoces by presentation of tumor antigens to host effector cells (Myc et al., 2011; Schuster et al., 2006). Several techniques can be used to stimulate a host response; these may involve giving peptides, DNA, or tumor cells. Peptides and DNA are often given using antigen-presenting cells (dendritic cells) (Myc et al., 2011).

1.2 Antigen presentation

Professional antigen presenting cells (APCs) present peptides to $CD8^+$ and $CD4^+$ T cells in the context of Major histocompatibility complex (MHC) class I and MHC class II molecules, respectively. MHC class I molecules present peptides that originated from intracellular antigens. Peptides originating from exogenous antigens (extracellular) are presented on MHC class II molecules. MHC class II molecules also present Antigen that are intracellular, locating into the endosomal pathway. There are links between these intracellular and extracellular pathways.

1.2.1 MHC Class I Antigen Presentation

All nucleated cells express MHC class I (MHC I) molecules and present protein fragments of cytosolic and nuclear origins at the cell surface. Mature MHC I complexes consist of three noncovalently associated components: a heavy chain (45 kDa), a 12 kDa light chain (β2-macroglobulin) and an antigenic peptide (York et al., 1996). The heavy chain is encoded in the MHC locus on human chromosome 6 and mouse chromosome 17 (York et al., 1996). β2-macroglobulin is a nonglycosylated protein of about 100 amino acids encoded on chromosome 15 in human and chromosome 2 in mice. There are three MHC class I loci that in human are called HLA-A, B, and C and in mice, H-2K, H-2D, and H-2L (York et al., 1996). Light chain is not polymorphic and only one allele is known in human, while there are seven in mice (York et al., 1996). However, the MHC class I heavy chain genes are extremely polymorphic (Parham et al., 1995).

In MHC class I presentation pathway, antigens are degraded by cytosolic and nuclear proteasomes (López et al., 1997; Seifert et al., 2010; Toes et al., 2001). The resulting peptides are translocated into the endoplasmic reticulum (ER) by transporter associated with antigen presentation (TAP) to access MHC I molecules (Reits et al., 2000). In the ER, the MHC I heterodimer is assembled from the polymorphic heavy chain and non-polymorphic light chain. This heterodimer gets stabilized by addition of peptide into its peptide-binding groove. Without peptides, MHC I molecules are stabilized by ER chaperone proteins such as calreticulin, ERp57, protein disulphide isomerase (PDI) and

tapasin. Tapasin interacts with TAP and catalyzes peptide translocation into the ER with peptide delivery to MHC I molecules (Neefjes et al. 2011) (**Figure 1.1**).

The complex of TAP, tapasin, MHC class I, ERp57 and calreticulin is called the peptide-loading complex (PLC). The PLC ensures efficient peptide loading onto MHC class I molecules. Tapasin may also act as a peptide editor, with a role similar to that of HLA-DM in MHC II presentation (Sadegh-Nasseri et al. 2008), though may load peptides with lower affinity but higher diversity (Zarling et al., 2003). Binding of the peptide to MHC I releases the chaperones and peptide–MHC I complexes leave the ER for presentation at the cell surface to CD8 T cells (Vyas et al., 2008).

Peptides and MHC class I molecules that fail to associate in the ER are returned to the cytosol for degradation (Hughes et al., 1997; Koopmann et al., 2000). A large fraction of MHC I molecules never associates with peptides (Neefjes et al., 1988) and these MHC molecules are ultimately degraded by the ER-associated protein degradation (ERAD) system (Hughes et al., 1997). These misfolded proteins become polyubiquitinylated and transported back into the cytosol for destruction by the 26S proteasome, in ERAD pathway (Jarosch et al., 2002; Tsai et al., 2002).

A substantial fraction (30% to 70%) of cellular proteins is immediately degraded after synthesis (Reits et al., 2000; U. Schubert et al., 2000). These misfolded proteins are the result of defective transcription or translation, alternative reading frame usage, failed assembly into larger protein complexes, the incorporation of wrong amino acids owing to mistakes by aminoacyl-tRNA synthetases or altered ubiquitin modifications and collectively called DRiPs (defective ribosomal products). DRiPs are degraded by the proteasome in the nucleus and cytosol (Dolan et al., 2011; M. Li et al., 2011). Peptides may bind with MHC I molecules directly or they may require further trimming by ER aminopeptidase associated with antigen processing (ERAAP) before they are considered suitable for MHC class I binding in or outside the PLC (Blanchard et al., 2010). Therefore, a large fraction of peptides presented by MHC class I to CD8 T cells is from DRiPs and are non-functional proteins (Seifert et al., 2010).

When MHC I molecules are internalized from plasma membrane into endosomes, they enter the classical MHC II presentation pathway. Subsequent acidification promotes the release of the associated peptides, which can be exchanged for new peptides generated by

the endocytic pathway. This pathway may not be dominant under normal conditions, but it may be more prevalent vacuolar pathway of cross-presentation (Neefjes et al. 2011).

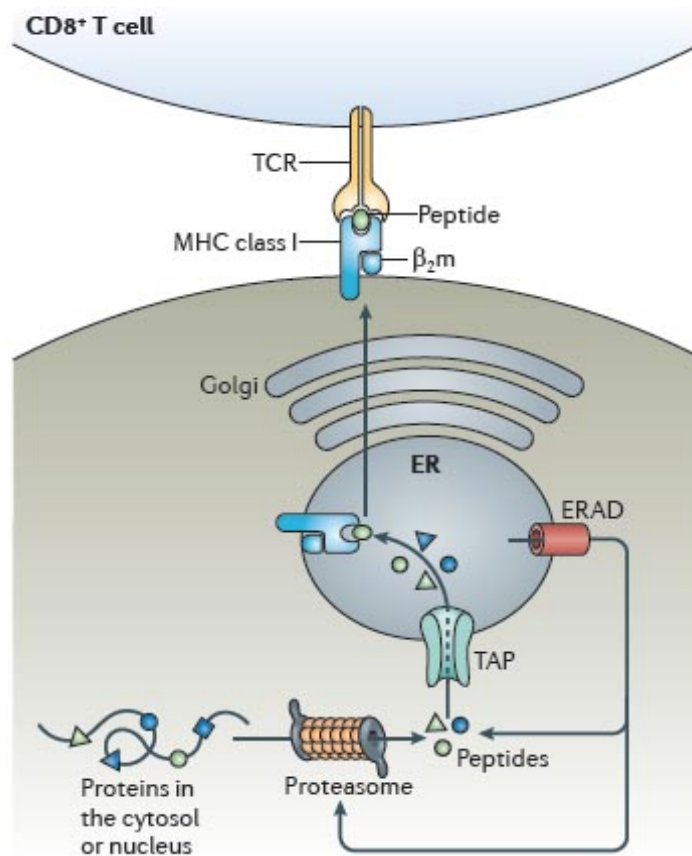


Figure 1.1 - MHC I antigen presentation pathway: The presentation of intracellular antigenic peptides by MHC class I molecules is the result of a series of reactions. First, antigens are degraded by the proteasome. Then, the resulting peptides are translocated via transporter associated with antigen presentation (TAP) into the endoplasmic reticulum (ER) lumen and loaded onto MHC class I molecules. Peptide–MHC class I complexes are released from the ER and transported via the Golgi to the plasma membrane for antigen presentation to $CD8^+$ T cells. - β2m, β2-microglobulin; ERAD, ER-associated protein degradation; TCR, T cell receptor - Adapted from (Neefjes et al. 2011).

1.2.2 Cross-presentation

Cross-presentation is the ability of certain APCs such as DCs to process extracellular antigens for MHC I presentation (Kurts et al., 2010). Cross-presentation plays a central role in the stimulation of $CD8^+$ T cell response against tumor cells and virally infected cells while under homeostatic conditions, it should be strictly controlled to avoid risk of autoimmunity (Compeer et al., 2012). Therefore in homeostatic conditions, cross-presentation is mostly limited to certain subset of DCs, notably $CD8^+$ DCs in mice and their human equivalent $BDCA3^+$ ($CD141^+$) DCs (Bachem et al., 2010; den Haan et al., 2000). Another explanation for subset limitation of cross-presentation is that DCs, with efficient cross-presentation, automatically become targets for CTL killing after endocytosis of viral or tumor debris. DCs that cannot cross-prime can maintain other immune responses that depend on $CD4^+$ T helper cells (T_h), such as antibody production and macrophage stimulation. Indeed, DCs that can not cross-prime will not be killed by CTLs, unless they are virally infected themselves (Kurts C et al. 2010). Non-cross-presenting DCs are superior at activating T_h cells (Burgdorf et al., 2007; Dudziak et al., 2007). However other cell types and even non-professional APCs like endothelial cells and HEK293 cells can cross-present antigens under certain conditions (Bagai et al., 2005; Giodini et al., 2009). Neutrophils, macrophages, mast cells, basophils and γδ T cells can cross-present antigen (Beauvillain et al., 2007; Brandes et al., 2009; Carbone et al., 1998; S. Kim et al., 2009; Kovacsovics-Bankowski et al., 1993; Stelekati et al., 2009). B cells cross-present antigens that have been recognized by their immunoglobulin receptors and can cross-prime CTLs when activated through Toll-like receptors (TLRs) engagement (Heit et al., 2004). Liver sinusoidal endothelial cells (LSECs) cross-present and can efficiently cross-tolerize rather than cross-prime CTLs that are specific for food antigens or commensal bacteria (Limmer et al., 2000).

Internalization of antigen into cross-presenting cells is the first step for cross-presentation. The nature of the antigen, the method of antigen uptake and the type of receptor that are used for uptake are important factors that influence access of antigen to cross-presentation pathway (Wagner et al., 2012). DCs ingest exogenous antigens into endosomes and/or phagosomes and can process these for MHC II presentation to $CD4^+$ T

cells or translocate these antigens into the cytosol to be degraded by proteasomes, as well as amino- and carboxy-terminal peptidases and enter the class I MHC processing pathway (Cresswell et al. 2005; Savina et al. 2007; X. Z. Shen et al. 2011). The produced peptides enter the ER through transporter associated with antigen processing (TAP) molecules to associate with MHC class I molecules, and move up to the cell surface for cross-presentation to specific $CD8^+$ T cells (Cresswell et al. 2005; Savina et al. 2007).

The access of exogenous antigens to MHC I pathway is not fully understood. The molecules responsible for the transfer of antigen across the endosomal membrane into the cytosol are largely unknown. The transfer of exogenous proteins from endosomes to cytosol might has the same protein complex machinery that is responsible for the retrotranslocation of misfolded proteins in the ER (ERAD pathway) (Segura et al., 2011). A mechanistic explanation of the cross-presentation is the indirect observation that the ER membranes may fuse with the nascent phagosome, referred to as the ER-phagosome fusion (Gagnon et al., 2002; Houde et al., 2003). ER-phagosome fusion allows internalized exogenous antigens to transfer to the cytosol, followed by degradation by the proteasome, resulting in the production of antigen peptides presentable by MHC class I molecules (Gagnon et al., 2002; Houde et al., 2003). In that case, the pore structure for translocation of the antigen to the cytosol is the Sec61 complex, which is utilized for retro-translocation of ER-misfolded proteins to the cytosol by the ERAD pathway. In fact, Pseudomonas aeruginosa Exotoxin A, which inhibits retro-translocation of ER-misfolded proteins, substantially blocks cross-presentation (Ackerman et al., 2006). However, it is not clear whether the Sec61 is required for cross-presentation, because it has been reported that the toxin binds AAA ATPase p97, another component of the ERAD machinery, but not the Sec61 complex (Ackerman et al., 2006). AAA ATPase p97 is another component of the ERAD machinery and is required for the export of antigen out of phagosomes and for cross-presentation (Ackerman et al., 2006). Intracellular membrane fusion is controlled by a family of integral membrane proteins, termed soluble N-ethylmaleimidesensitive factor attachment receptor (SNARE) proteins (Südhof & Rothman, 2009). It has been shown that the SNARE protein Sec22b recruits ER-resident proteins to phagosomes that are necessary for phagosome-to-cytosol translocation (Cebrian et al. 2011). Inhibition of Sec22b function by RNAi renders much less efficient antigen translocation to the cytosol in DCs (Cebrian et al. 2011). The Heat shock protein 90 (HSP90) is also an important player in translocation of

exogenous antigen to cytosol and inhibition of HSP90 or knocking out of HSP90α diminished antigen translocation into the cytosol and cross-presentation of the antigen (Imai et al., 2011) (**Figure 1.2**).

In the vacuolar pathway of cross-presentation, the peptide generation may occur independently of proteasomes and in endosomal pathway (Di & Chatterjee 2008; Gommé et al., 1999; L. Shen et al., 2004). This vacuolar (TAP-independent) pathway does not require phagosome-to-cytosol translocation, but depends on endosomal proteases for generation of antigenic peptides (Di & Chatterjee 2008; Grommé et al., 1999). Rock's group has shown that DCs can degrade cell-associated OVA by both the cytosolic proteasome or cathepsin S in the endosomal pathway (L. Shen et al., 2004). Therefore, DCs could use both endosomal and cytosolic compartments to generate peptides for cross-presentation and cross-present antigens in both proteasome-dependent and independent pathways (Di & Chatterjee 2008; Hoeffel et al., 2007; L. Shen et al., 2004).

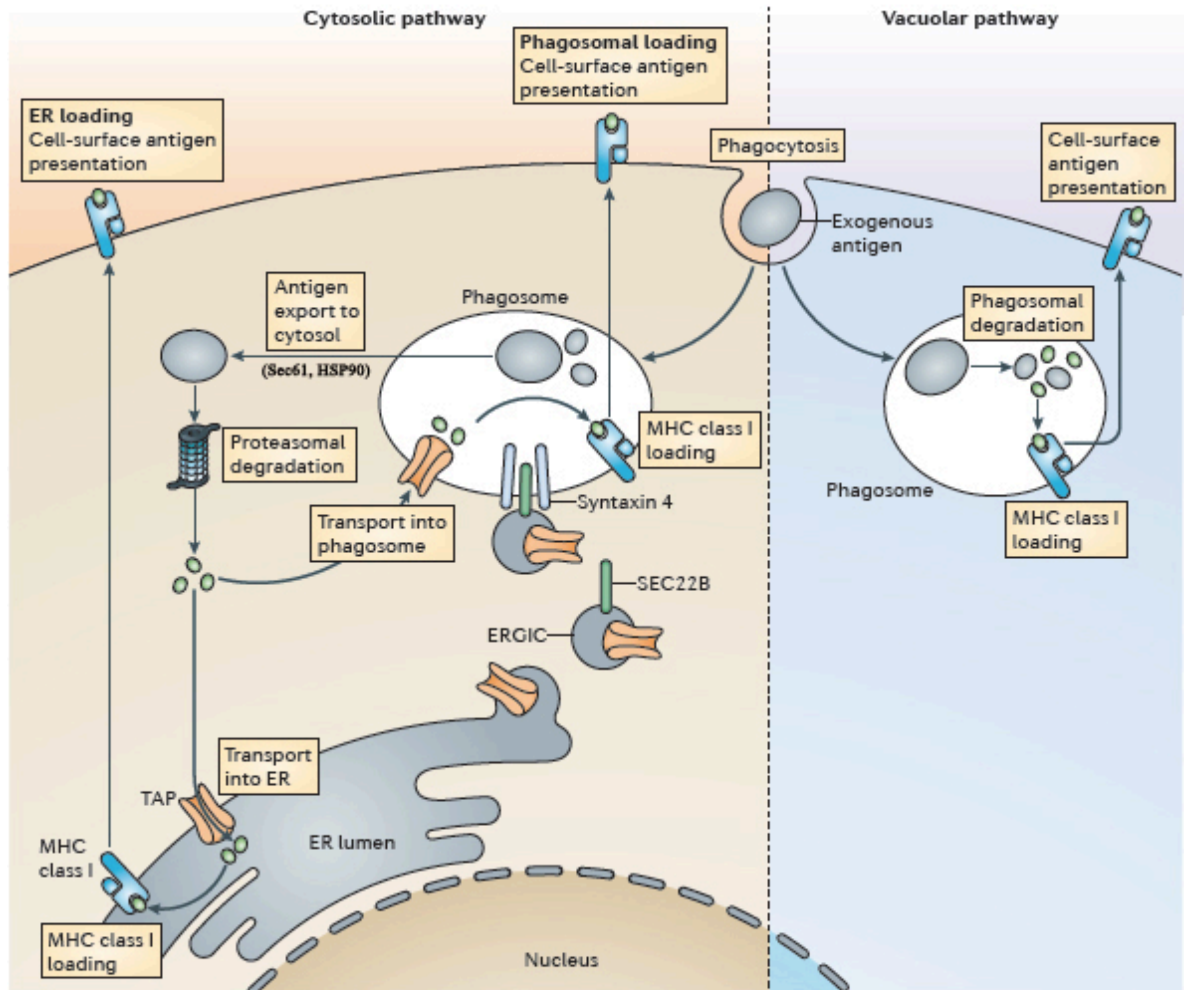


Figure 1.2 – Proposed pathways for cross-presentation in dendritic cells. Exogenous antigens can be exported into the cytosole after phagocytosis and get processed by the proteasome. In the cytosolic pathway, the processed antigens can beloaded on MHC class I molecules in the endoplasmic reticulum (ER) or re-imported into the phagosome to be loaded on MHC class I molecules (the cytosolic pathway with phagosomal loading). The SNARE SEC22B, which localizes in the ER–Golgi intermediate compartment (ERGIC) and interacts with syntaxin 4 on phagosomes, mediates the recruitment of a subset of ER components, including transporter associated with antigen processing (TAP), to phagosomes. In the vacuolar pathway, the exogenous antigens are degraded into peptides in the phagosome, where they are then loaded on MHC class I molecules. – Adapted from (Joffre et al., 2012).

APCs beside direct presentation of their own intracellular antigens or cross-presentation of engulfed materials, can also use a third mechanism to acquire and cross-dress with antigens that have already been processed by other cells (Yewdell et al., 2011)(**Figure 1.3**). *Cross-dressing* is the initiation of a $CD8^+$ T cell response to an MHC I-antigen complex that is not synthesized by the antigen-presenting cell and transferred from another APC or source (Bevan 1976; Melief 2003). Cross-dressed $CD8\alpha^+/CD103^+$ DCs are capable of efficiently activating both naïve and memory $CD8^+$ T cells (Li & Kim et al., 2012). The cross-dressed DCs acquire preformed antigen-MHC I complexes from other cells by the process of trogocytosis or through gap junctions (Wakim et al. 2011; Yewdell et al. 2011).

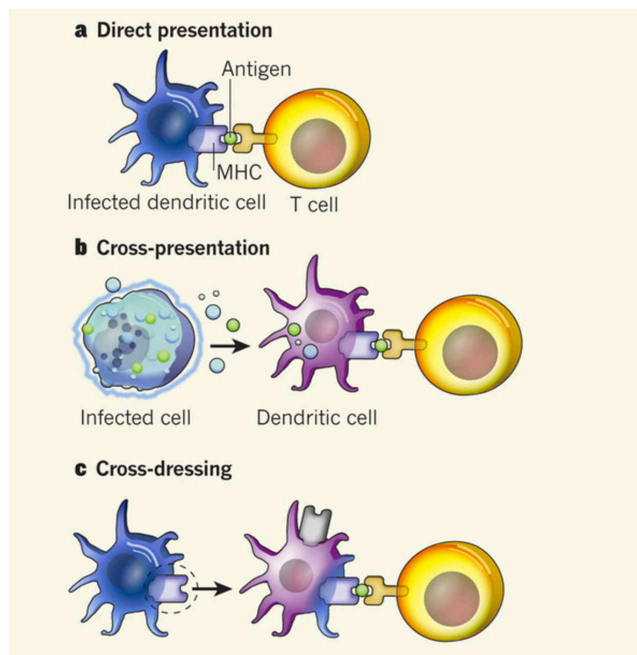


Figure 1.3 - DCs present antigen-MHC I to $CD8^+$ T cells through three mechanisms: (**a**) Direct presentation occurs when an antigen-presenting cell such as a dendritic cell is infected, and displays processed antigenic peptides in complex with MHC on its surface, thereby activating T cells. (**b**) In cross-presentation DCs acquire antigens from infected or malignant cells through endocytosis and phagocytosis, and — with or without some processing — load them onto class I molecules for presentation to T cells. (**c**) In a third pathway, called cross-dressing, DCs acquire preformed MHC class I in complex with antigens from other cells by the process of trogocytosis or through gap junctions. Adapted from (Yewdell et al., 2011).

1.2.3 Autophagy

Autophagy is a homeostatic process that enables eukaryotic cells to deliver cytoplasmic constituents for lysosomal degradation, to recycle nutrients and to survive during starvation. Beside the clearance function, autophagy assists in the restriction and elimination of intracellular pathogens as an innate immune response to viral and microbial infection (Yano T et al., 2011). In addition, by delivering cytoplasmic antigens for loading onto major histocompatibility complex (MHC) class II molecules for $CD4^{+}$ T-cell recognition, autophagy enables the immune surveillance for intracellular antigens and broadens the immunological functions of MHC II presentation (Lünemann et al., 2009). There are at least three distinct pathways of autophagy: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. Microautophagy is characterized by the uptake of cytoplasmic components at the lysosomal membrane via budding into the lysosome, and is the major route of degradation of cytoplasmic constituents (Lünemann et al., 2009).

1.2.4 MHC Class II Antigen presentation

In contrast to MHC class I molecules that are expressed by all nucleated cells, MHC II molecules are mainly expressed by professional APCs. However, MHC II expression can be induced by IFNγ and other stimuli in non-APCs, including mesenchymal stromal cells (Romieu-Mourez et al. 2007), fibroblasts and endothelial cells (Geppert et al., 1986), as well as in epithelial cells and enteric glial cells (Bland, 1988; Koretz et al., 1987).

The immune system in order to fight invaders relies in part on professional APCs to survey the body for the signs of invasion. Professional APCs sample proteins from their environment, proteolytically process them and present a representative repertoire of the protein fragments via MHC II molecules to $CD4^+$ T cells.

1.2.4.1 Structure of MHC II molecules

MHC II molecules are membrane-bound heterodimeric molecules consisting of two polymorphic α and β chains, though the β chain is much more polymorphic than α chain. The 230-residue α chain and the 240-residue β chain are glycosylated, resulting in molecular weights of 33 kD and 28 kD, respectively. A β-pleated sheet floor under the α1 and β1 helices forms the peptide-binding cleft. In contrast to MHC I which peptide-binding groove is closed and accommodates peptides of 8 to 9 amino acid-length, the peptide-binding cleft of MHC II is open and can accommodate a linear peptide of approximately 12-25 amino acids in length. Thus, some of the amino acids from the peptide are located outside of the groove (Brown et al. 1993; L J Stern et al. 1994; Newcomb et al., 1993). The α2 and β2 domains of MHC II are membrane-proximal and CD4 interacts with both α2 and β2 (König et al., 1995). CD4 interacts with MHC II to strengthen the binding of MHC-peptide complex to TCR and promotes the localization of the src family tyrosine kinase p56lck into the receptor complex (König et al., 1995). Peptides are not anchored or fixed into pockets at the N- and C-termini, but rather they bind by pockets throughout the binding groove and through hydrogen bonding interactions, which extend throughout the entire peptide backbone (Brown et al., 1993; Stern et al., 1994). The anchoring amino acids within the peptide vary considerably between MHC alleles (Brown et al., 1993; Dessen et al., 1997; Scott et al., 1998).

1.2.4.2 MHC II expression

The human MHC class II gene cluster is on chromosome 6 and encodes three classical class II molecules (HLA–DP, HLA–DQ and HLA–DR) and two 'non-classical' class II molecules (HLA–DM and HLA–DO) (Horton et al., 2004). Expression of class II genes is tightly regulated and cell-type specific, consistent with their critical role in immunity (Pieters 2000; Reith W et al. 2005). Levels of class II expression also vary according to the developmental stage of antigen-presenting cells. For example, differentiation of B cells into plasma cells as well as maturation of dendritic cells is characterized by the repression of MHC class II gene expression (Ghosh et al., 2001; Pierre et al., 1997). Moreover, activated T cells are known to express MHC class II molecules (Chang et al., 1995). Other cell types such as astrocytes, fibroblasts, epithelial and endothelial cells do not express MHC class II molecules unless they are exposed to specific stimuli, notably interferon-gamma (Dong et al. 1999; Muhlethaler-Mottet et al. 1998; Muhlethaler-Mottet et al. 1997; W. Rohn et al. 1999). Expression of MHC class II molecules is regulated primarily at the level of transcription by a complex process involving highly conserved sequences located in the proximal promoter regions upstream of all classical and non-classical MHC class II genes, which recruit specific binding factors, generating a multiprotein complex, which is known as the MHC class II enhanceosome (Reith W et al. 2005; Mach et al. 1996; Reith et al., 2001; Boss et al., 2003; Harton et al., 2000). At the DNA level, this regulatory unit comprises four sequences (S, X, X2 and Y boxes), the 'SXY module'. The SXY module is present in all classical MHC class II genes as well as in the genes encoding invariant chain (Ii) and non-classical MHC II, HLA-DM and HLA-DO (LeibundGut-Landmann et al., 2004; J. P. Ting et al., 2002). The SXY module is formed by cooperative binding of four factors. The X-box-binding factor regulatory factor X (RFX)- that binds to G-box, the cyclic-AMP-responsive-element-binding protein (CREB) –which binds to X2-box; the Y-box-binding factor nuclear transcription factor Y (NFY); and S-box-binding factor (V Steimle et al. 1993; V Steimle et al. 1995; K Masternak et al. 1998; U M Nagarajan et al. 1999; W Reith et al. 1988; Moreno et al. 1999; Mantovani 1999; Muhlethaler-Mottet et al. 2004). The MHC class II enhanceosome, serves as a 'landing pad' for the class II transactivator (CIITA), which is a non-DNA binding co-activator and the master regulator of MHC II genes expression. CIITA does also coordinate the recruitment of other factors

that are involved in chromatin modification and remodeling. These factors are CREB-binding protein (CBP), p300, p300/CBP-associated factor (PCAF), brahma-related gene 1 (BRG1) and co-activator-associated arginine methyltransferase 1 (CARM1) (Mudhasani & Fontes, 2002; Sisk et al., 2000; Yee et al., 2004; X. S. Zhu et al., 2001) **(Figure 1.4)**. Transcription factors assembly and coordination by CIITA, result in the induction of MHC class II transcription (Ting et al., 2002; Boss et al., 2003; Reith et al., 2001).

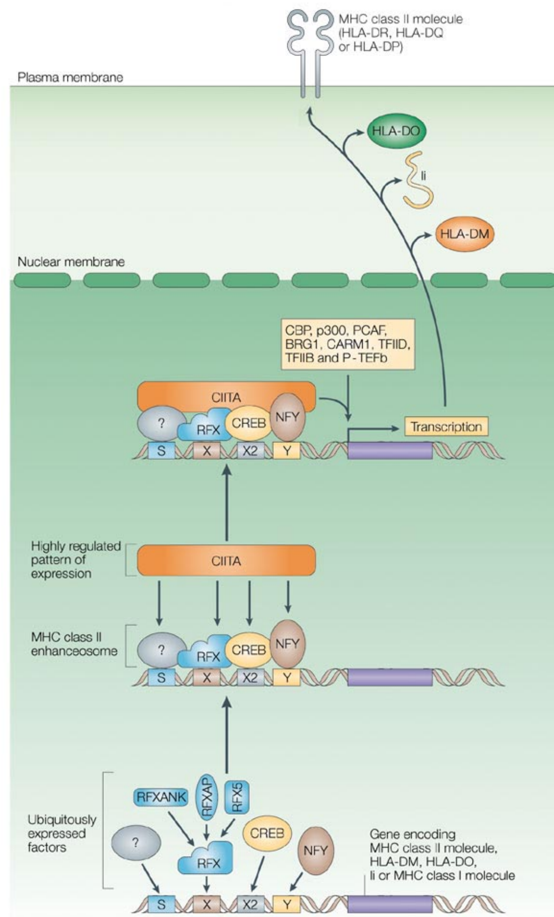


Figure 1.4 - Transcriptional regulation of MHC class II genes: The SXY module that is present in all classical MHC class II genes — as well as in the genes encoding invariant chain (Ii), HLA-DM, HLA-DO and MHC class I molecules — is bound cooperatively by four factors: the heterotrimeric X-box-binding factor regulatory factor X (RFX), which is composed of RFX5, RFX-associated protein (RFXAP) and RFX-associated ankyrin-containing protein (RFXANK); the X2-box-binding factor cyclic-AMP-responsive-element-binding protein (CREB); the Y-box-binding factor nuclear transcription factor Y (NFY); and an as-yet-unidentified S-box-binding factor. The MHC class II enhanceosome is a 'landing pad' for the class II transactivator (CIITA), which is a non-DNA binding co-activator that is recruited by multiple protein–protein interactions with several components of the enhanceosome. CIITA coordinates the recruitment of additional factors that are involved in transcription of MHC II (CBP, p300, PCAF, BRG1,CARM1, TFIID, TFIIB, P-TEFb) - Adapted from (Reith et al. 2005).

Expression of CIITA is controlled through three promoters pI, pIII and pIV (Muhlethaler-MOttet et al., 1997; Pai et al., 2002; Reith et al., 2001). These promoters drive expression of CIITA types I, III and IV, which incorporate different first exon sequences. Different APC types use different CIITA promoters and control these promoters by various mechanisms (Muhlethaler-MOttet et al., 1997). Macrophages use pI and pIV, dendritic cells use pI and B lymphocytes use pIII (Pai et al., 2002). In macrophages, pI and pIV are both responsive to IFNγ (that is, IFNγ induces CIITA, which induces expression of MHC class II genes); in immature DCs, pI drives expression of CIITA type I independently of IFNγ (Pai et al., 2002) **(Figure 1.5)**.

Recently, it has been shown that HLA class III region encoded B-associated transcript 3 (BAT3) regulates MHC II expression through CIITA (Kämper et al., 2012). IFN-γ not only increases CIITA expression but also BAT3 as well. Kämper *et al*. have suggested that BAT3 governs HLA class II expression by chaperoning CIITA to nucleus. Therefore, IFN-γ facilitates nuclear import of BAT3 and CIITA.

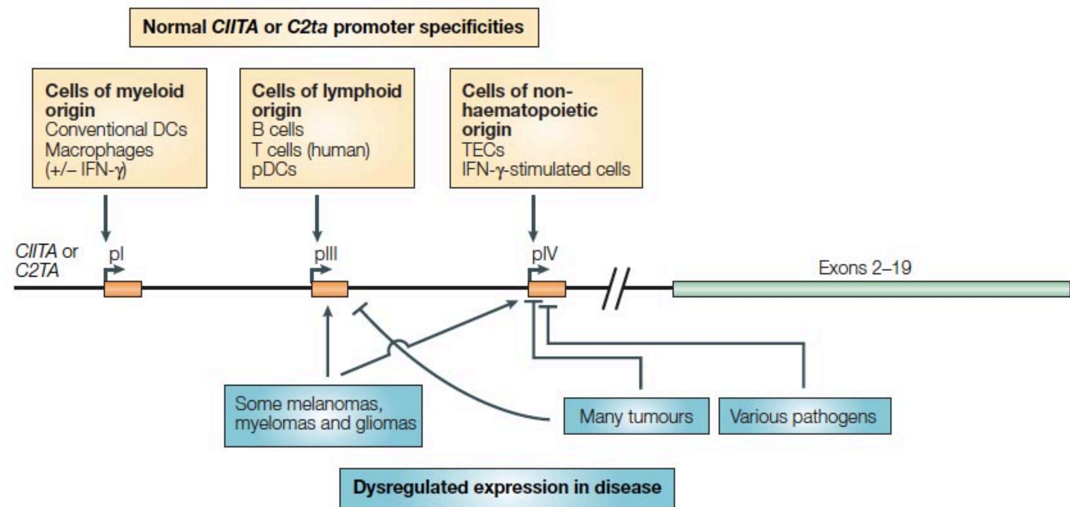
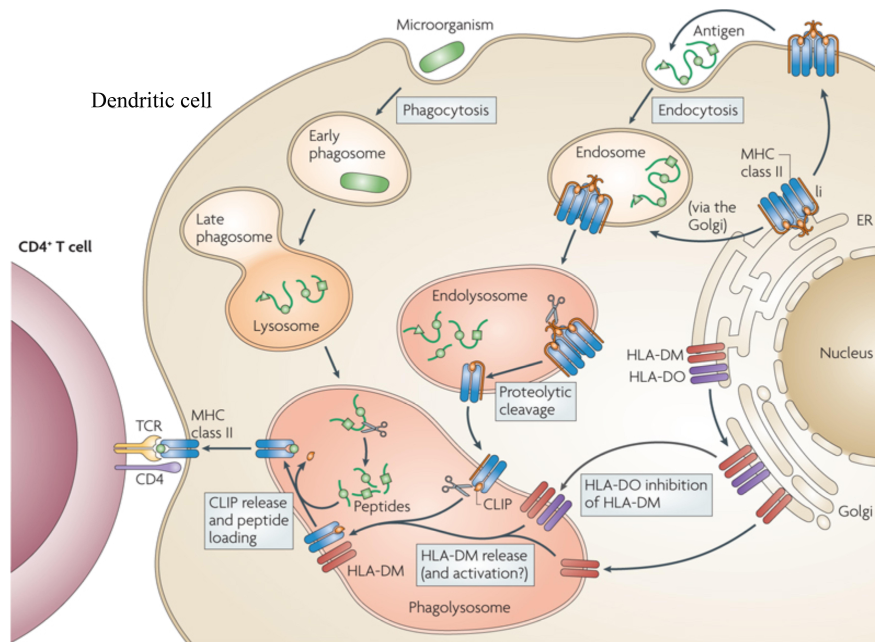


Figure 1.5 - Regulation of CIITA expression: The three regulatory modules (pI, pIII and pIV) of the class II transactivator gene (CIITA in humans and C2ta in mice) have distinct functions. The promoter pI is a myeloid-cell specific promoter that is sufficient to drive CIITA expression by conventional dendritic cells (DCs) and interferon-γ (IFN-γ)-activated macrophages. The promoter pIII is a lymphoid-cell-specific promoter that is essential for CIITA expression by B cells and activated human T cells. It is also required for CIITA expression by plasmacytoid DCs (pDCs). The promoter pIV is essential for driving CIITA expression by thymic epithelial cells (TECs) and for mediating induction by IFN-γ in cells of non-haematopoietic origin, such as endothelial cells, epithelial cells, fibroblasts and astrocytes. - Adapted from (Reith et al. 2005).

1.2.4.3 MHC II trafficking

The newly synthesized α and β chains, sequentially bind to the chaperons calnexin and invariant chain (Ii) (Bikoff et al., 1993; Viville et al., 1993). While calnexin serves for several types of antigen receptors like TCR, BCR and MHC class I, Ii was thought to be a dedicated chaperon for MHC class II molecules (P Cresswell, 1996; Melnick et al., 1995). Further studies revealed that Ii beside chaperoning has other important functions and is involved in inflammatory responses, signalling and B cell differentiation (Borghese et al., 2011). The sequential binding of calnexin and Ii to newly synthesized α and β chains facilitates the assembly of $\alpha\beta$Ii trimers followed by nonameric $(\alpha\beta Ii)_3$ complex formation (Lamb et al., 1992; Roche et al. 1991). Ii stabilizes the $\alpha\beta$ heterodimer and prevents premature binding of an inappropriate peptide found in the ER (Busch et al., 1996; Newcomb et al., 1993). The cytosolic tail of Ii has two sorting signals - di-leucine motif - which direct the nanomeric complex from the trans-Golgi network (TGN) to the endocytic compartments (Bremnes et al., 1994; Odorizzi et al., 1994; Pieters et al., 1993). The sorting motif directs MHC II complexes into the endocytic pathway, either directly from the trans-Golgi network or via rapid internalization from plasma membrane (Bakke et al., 1990; Roche et al., 1993) (**Figure 1.6**). The latter pathway is dominant, occurs via clathrin-mediated endocytosis and requires interactions between dileucine-based signals in the Ii cytoplasmic domain and the clathrin adaptor protein-2 (AP-2) (Dugast et al., 2005; McCormick et al., 2005). The AP-2 complex sorts the $\alpha\beta$-Ii complex into clathrin-coated vesicles, which pinch off from the plasma membrane and fuse with early endosomes (Bonifacino et al., 2004; Dugast et al., 2005; McCormick et al., 2005). The interaction of the adaptors with the GTPase dynamin results in the formation of a ring of dynamin around the neck of the forming clathrin-coated pit, leading to its separation and the formation of clathrin-coated vesicles. These vesicles then uncoat from clathrin and fuse with endocytic compartments (Bonifacino et al., 2004; Dugast et al., 2005; McCormick et al., 2005). While the endocytosis of $\alpha\beta$ /Ii complexes are dependent on the Ii chain dileucine motif and clathrin recruitment, the endocytosis of mature $\alpha\beta$ /peptide (pMHC-II) is regulated by polyubiquitinylation of the β-chain intracytoplasmic tail (Shin et al., 2006; van et al., 2006). Down-regulation of the E3 ubiquitin ligase membrane-associated RING-CH I (MARCH I) is a major biochemical event leading to MHC II surface stabilization during DC activation

(De Gassart et al., 2008; Berger et al., 2009). In addition, it has been shown that internalization and recycling of pMHC-II is dependent on its entrance to elongated tubules that contain the small GTPases Arf6 and Rab35, whereas internalization of Ii-associated MHC-II does not depend on Arf6 and Rab35 (Walseng et al., 2008; Berger et al., 2009).



Harding CV 2010 Nat Rev Immunol.

Figure 1.6 - MHC II antigen presentation pathway : MHC II molecules are assembled in the endoplasmic reticulum (ER) as α-chain–β-chain heterodimers in a nonameric complex containing three heterodimers, each associated with invariant chain (Ii) molecule. The Ii–MHC II complex is transported from the ER through the Golgi to reach late endocytic or lysosomal compartments or may first transit to the plasma membrane and then reach endocytic compartments by endocytosis. Ii is degraded by proteases, leaving an invariant chain-derived peptide, known as class II-associated Ii peptide (CLIP) that remains in the peptide-binding groove, inaccessible to proteases. Human leukocyte antigen (HLA)-DM interacts with MHC class II molecules to catalyze the dissociation of CLIP as well as the exchange of other peptides, resulting in the formation of complexes of MHC class II molecules with high-affinity peptides. HLA-DO can bind HLA-DM and inhibit its function, negatively regulating peptide loading of MHC II in non-acidic endosomes of antigen-presenting cell. Exogenous antigens are internalized by endocytosis or phagocytosis. Proteolysis by lysosomal proteases produces peptides (usually 10–16 amino acids long) that bind MHC class II molecules. Peptide–MHC class II complexes are transported from vacuolar processing compartments to the plasma membrane, where they are presented to CD4+ T cells - Adapted from (Harding et al., 2010).

Ii gradually gets processed and trimmed by proteases during the transit from endosomes to lysosomes. The trimming proceeds from the luminal C-terminal to the membrane-proximal N-terminal (Watts, 2004). Ii is digested through a sequential cleavage by proteases that first generate Ii-p22 (or LIP) and then Ii-p10 (or SLIP) and finally leaving only CLIP – Class II associated Invariant chain Peptide – in the peptide-binding groove of MHC II (F Castellino et al., 2001; Riese et al., 1996; Roche et al., 1991). The proteases responsible for the digestion of Ii are yet to be fully characterized; however the proteases, which are crucial in the terminal stage of converting Ii-p10 into CLIP, are well defined. It is Cathepsin S that in B cells, myeloid DCs (mDCs or conventional DCs), macrophages, human epithelial cells, murine intestinal epithelial cells, as well as in human $CD4^+$ HLA-DR^+ T cells has crucial role in Ii-p10 digestion to CLIP (Bania et al., 2003; Beers et al., 2005; Costantino et al., 2009; Nakagawa et al., 1999; Riese et al., 1996; Shi et al., 1999; Villadangos et al., 1997). Cathepsin L in murine thymic epithelial cells (Nakagawa et al., 1998), and cathepsin V in cortical human thymic epithelial cells play the pivotal role in the final stage of Ii degradation and cleavage of SLIP to CLIP (Tolosa et al., 2003). The final trimming of Ii that leaves CLIP in the peptide binding groove of MHC II takes place in a late endosomal compartment termed the MHC II compartment (MIIC). CLIP occupies the peptide-binding groove, thereby preventing premature peptide binding (Roche et al., 1990). The same proteolytic enzymes that generate CLIP from Ii also act on the endocytosed antigens and degrading them to peptides. CLIP has to be removed so that the groove of MHC II becomes accessible to antigenic peptides produced from extracellular proteins. In MIIC, HLA-DM facilitates the exchange of CLIP with a suitable peptide derived from proteins degraded in the endosomal pathway (Sloan et al. 1995; Denzin et al., 1995). HLA-DM catalyzes removal of CLIP from peptide binding cleft and keeps the groove open until an appropriate peptide resides in it (Anders et al., 2011). Upon binding of high affinity peptide, HLA-DM releases the peptide-MHC class II complex to go to the cell surface and primes CD4 T cells (Schulze et al., 2012). In B cells and DCs, HLA-DO regulates function of HLA-DM and inhibits its activity in non-acidic pH (Denzin et al. 2005; Fallas et al. 2007).

1.2.4.4 HLA-DM

HLA-DM (H2-DM or previously H2-M in mice) was first described in 1991 as a non-classical MHC II molecule (Cho et al., 1991; Kelly et al., 1991). DM facilitates release of CLIP from peptide-binding groove and its exchange with antigenic peptides (Sloan et al. 1995; Denzin et al., 1995). DM is composed of two trans-membrane α and β subunits, encoded by MHC-linked genes found near TAP and LMP genes in the MHC II region (on chromosome 6 in human and 17 in mice). It is a close homologue of MHC II molecules, with a virtually identical structure (Fremont et al., 1998; Mosyak, et al., 1998) (**Figure 1.7**). The peptide-binding groove is closed and DM can not bind pepides (Denzin et al., 1995; Sherman et al., 1995; Stebbins et al., 1996). DM shows only very limited polymorphism (Mosyak et al., 1998).

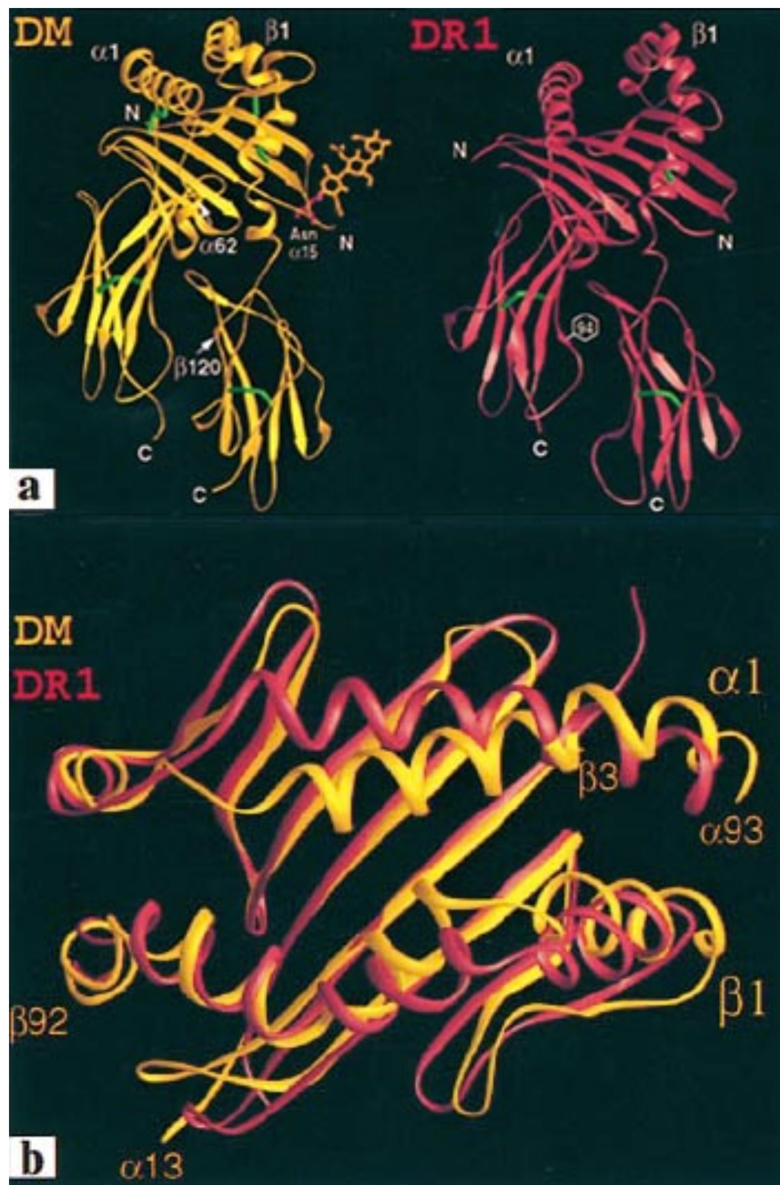


Figure 1.7 - Comparison of the Structures of HLA-DM and HLA-DR1, showing the closed peptide binding groove of HLA-DM: (**a**) DM (yellow), DR1 (red), disulfide bonds (green). (**b**) Superposition of the α1 and β1 domains of HLA-DM (yellow) and HLA-DR1 (red). The two structures are very similar except that the two α helices that flank the peptide-binding groove in the DR molecule are closer together in the DM αβ dimer, which indicates that DM is not a peptide-binding molecule - Adapted from (Mosyak et al., 1998).

1.2.4.4.1 HLA-DM expression and trafficking

HLA-DM (DM) expression is co-regulated with classical MHC II molecules because

they have a common cis-acting elements (SXY module) upstream of their promoters and the same factors that regulate expression of classical MHC II, such as CIITA and IFN-γ and BAT3, regulate DM as well (Mosyak et al. 1998; Kämper et al. 2012; Boss et al., 2003; Reith et al. 2005; Mach et al. 1996b; Reith et al., 2001; Harton et al., 2000).

In contrast to classical MHC II molecules, most DM accumulates in peptide loading compartments – MIIC (Sanderson et al., 1994). Trafficking of DM is independent of Ii however both are clathrin-dependent. While endocytosis of Ii is dependent on dileucine-based motives (Dugast et al., 2005; McCormick et al., 2005), the internalization of DM from the membrane requires a YxxL tyrosine-based endocytosis motif in the cytoplasmic tail of the β chain, allowing interaction with AP-2 (Bonifacino et al., 1999; Marks et al. 1995; Lindstedt et al. 1995; Copier et al. 1996).

It is reported that loss of DM from cell surface involves a cumulative mechanism of DMβ tyrosine-based signaling and ubiquitination of DMα by MARCH1 and MARCH9 (Jahnke et al., 2012). Very low amount of cell surface expression of DM has been reported on B cells and immature DCs (Arndt et al., 2000).

1.2.4.4.2 HLA-DM function

APCs lacking DM due to mutation have a defect in presentation of protein antigens, originally leading to the discovery of DM and awareness of its critical function in antigen processing (Alfonso et al., 2000). In peptide loading compartments, MIIC, where DM resides the optimal pH is 4.5–5.5 with DM:DR ratios of about 1:5 (Kropshofer et al. 1997; Schafer et al. 1996). DM has been considered as a chaperon for MHC II molecules as it binds to peptide-receptive empty MHC II molecules and prevent them from unfolding and subsequent denaturation (Kropshofer et al. 1997; Denzin et al. 1996). DM can also partially replace Ii in the absence of Ii (Serradell et al., 1999). Cells transfected with DR and DM in the absence of Ii expressed mostly stable molecules in their surface, and showed lower accumulation of DR in the endoplasmic reticulum (ER) than cells expressing only DR (Serradell et al., 1999).

DM function as a peptide editor and edits the peptide repertoire presented by MHC II molecules by APCs (Kropshofer et al., 1996; Pathak et al. 2001). The peptide editor function of DM favors the formation of long-lived peptide-MHC II complexes on cell surface of APCs (Kropshofer et al., 1996; Vogt et al. 2002; Lovitch et al. 2003). This offers

more time to $CD4^+$ T cells with cognate TCR to survey APCs and get primed. Eventually, DM could determine the frequency, type and strength of immune response toward certain epitopes (Lazarski et al., 2006; Rinderknecht et al., 2010). The peptide editor function of DM favors presentation of immunodominant peptides (those who elicit a robust CD4 T cell response) over cryptic peptides (those that do not elicit a response when they are contained in an intact complex protein) (Nanda et al. 1997). DM editing is critical in presentation of endogenous peptides and in the context of autoimmune diseases, presentation of the immunodominant insulin-dependent diabetes autoantigen glutamate decarboxylase GAD (273-285) was significantly diminished with increasing expression of DM (Lich et al. 2003).

Using random mutagenesis approach, a mapping study revealed the lateral DM-interacting surface of DR molecules (Doebele et al. 2000) (**Figure 1.8 and Figure 1.9**). This lateral surface includes acidic and hydrophobic DR residues around the region where the N-terminus of the antigenic peptide is located. On the DM side, an acidic face that may be protonated in acidic compartments serves, most likely, as the MHC II interacting surface (Pashine et al., 2003). Protonated carboxy groups may be primary candidates to explain the chaperone function of DM in biochemical terms, as small chemical compounds carrying a H-bond donor group (-OH), such as n-propanol and parachlorphenol, can catalyze peptide exchange. These dissociate CLIP and induce a peptide-receptive state in DR molecules, as they have been called chemical analogues of DM (Marin-Esteban et al. 2004; Call et al. 2009). Thus, an intermolecular hydrogen-bonding network between DM and MHC II molecules may stabilize the peptide-receptive conformer of the MHC II binding groove.

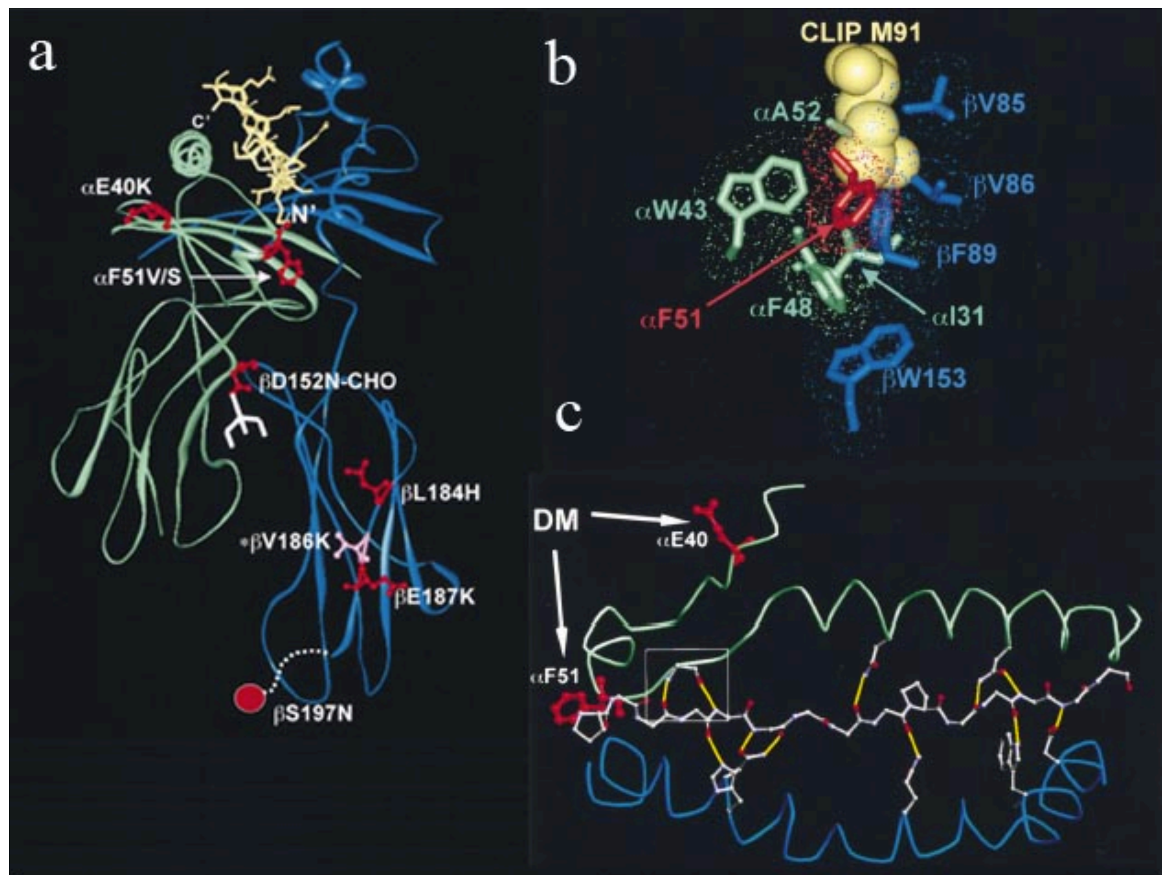


Figure 1.8 - Mapping study that revealed lateral DM-DR interacting surface:

(**a**) Mapping of Mutants onto the DR3/ CLIP crystal Structure. DRα chain is shown in green, DRβ in blue, and CLIP in yellow. The amino (N) and carboxy (C) termini of CLIP are labeled for orientation. Positions in HLA-DR3 that correspond to deleterious mutations are highlighted in red and labeled with the corresponding substitutions. (**b**) View of pocket 1 of the HLA-DR3-CLIP crystal structure and surrounding residues (same orientation as in **a**), showing the cluster of hydrophobic amino acids connecting the P1 pocket of the groove to the surface exposed, DM-interacting residue, DRα F51. Engagement of this residue by DM may transmit a conformational change to the antigen-binding groove. The P1 residue of CLIP (M91) is displayed in yellow, DRα residues are shown in green (except aF51, shown in red), and DRβ residues are shown in blue. (**c**) The DM-interacting residues, DR αF51 and βE40, are positioned close enough to αS53 to allow the hydrogen bonds extending from this residue to the peptide backbone (boxed) to be broken - Adapted from (Doebele et al. 2000).

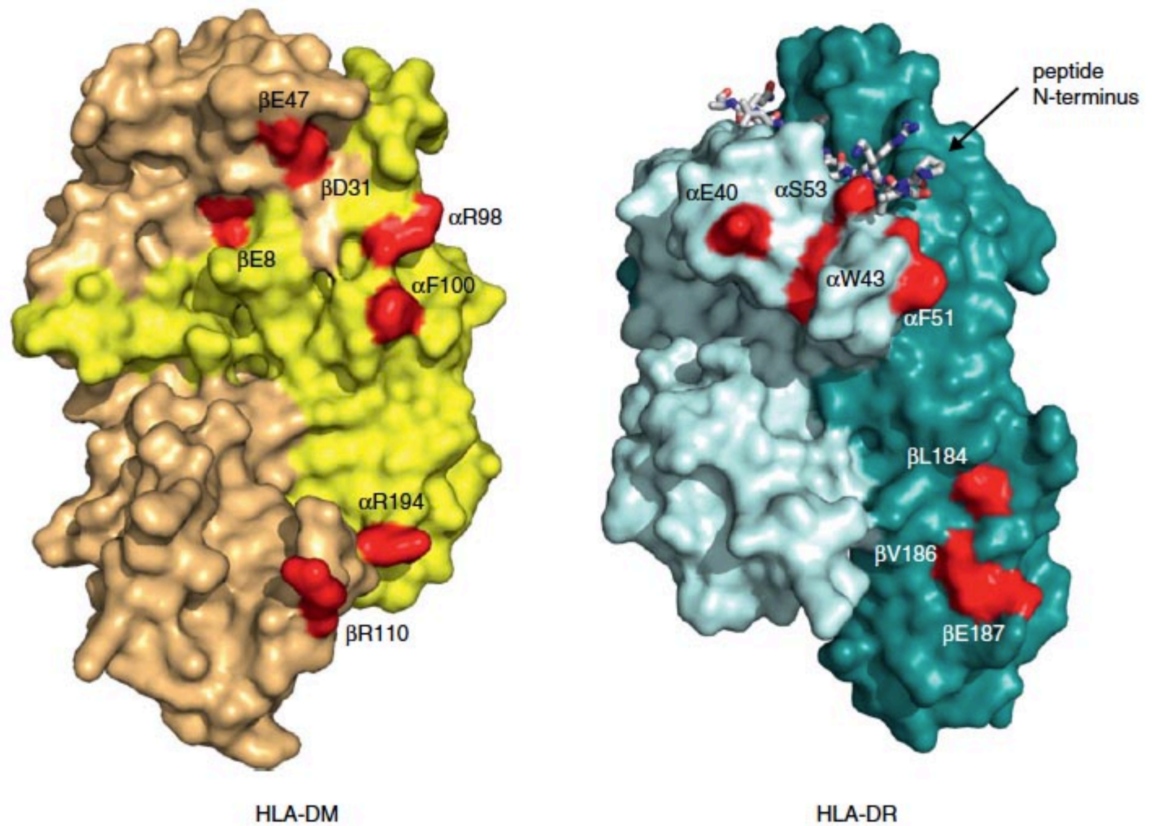


Figure 1.9 - Model of DM-DR-CLIP interaction: Contact residues are colored red on both proteins, based on mutants that substantially reduced susceptibility of DR/peptide complexes to DM or the activity of DM. A functionally important cluster is located in the DRα1 domain close to the peptide N-terminus; a second cluster is present in the membrane proximal DRβ2 domain. DM also shows two clusters of contact residues, located in the membrane-distal α1/β1 domains and the membrane proximal α2/β2 domains. DM chains are colored yellow (DMα) and orange (DMβ), DR chains light blue (DRα) and turquoise (DRβ). Models are based on crystal structures of HLA-DM and HLA-DR3/CLIP - Adapted from (Schulze et al., 2012).

It has been suggested that conformational changes on MHC II molecules imposed by engagement with DM are maintained after DM has dissociated. Initial evidence came from mAb 16.23 that recognizes DR3 loaded with cognate peptide in B cells expressing DM (Sanderson et al., 1994) but which remained negative in DM-deficient B cells (Verreck et

al., 2001). Verreck *et al.* suggested that conformational changes imposed by DM are relevant to T cell recognition, as T cells could discriminate between 16.23-positive and 16.23-negative isoforms of DR3-peptide complexes (Verreck et al., 2001). There are more studies that show immunodominant peptides and their respective MHC II molecule acquire more than one isoform (Schmitt et al. 1998; Rabinowitz et al. 1997). Vireta *et al.* described conformers of an Eα (52-68)-A^b complex that varied in their ability to be negatively selected in mice that express Eα (Viret et al., 2003). Direct evidence for peptide–MHC conformational isomerism has been obtained through NMR spectroscopy in studies by McConnell's group (Schmitt et al., 1999).

The most thoroughly studied conformational isomers originate from hen egg lysozyme peptide HEL(48-61) on I-A^k (Z Pu et al. 2002; Z Pu et al. 2004; Lovitch et al., 2005). Unanue *et al.* have shown that for each given HEL(48-61)- I-A^k complex, there could be multiple conformational isomers with varying stability and that T cells can recognize these different conformers (Z Pu et al., 2002). The more stable conformers are referred to type A and the less stable ones are called type B. The specific T cells responding to each conformer are also named as type A and B, respectively **(Figure 1.10)**. DM can edit these conformers in favor of type A. The importance of these conformers is highlighted in peptide-based vaccine approaches and also in autoimmune diseases (Z Pu et al., 2004). Type A T cells specifically recognize the HEL(48-61)-A^k conformer generated from HEL protein in late endosomal/ lysosomal vesicles in the presence of DM, whereas type B T cells recognize complexes generated from exogenous HEL(48-61) in early endosomes or on the cell surface of APCs in the absence of DM (Z Pu et al., 2004). In MIIC compartments, DM acts directly on the peptide-MHC complexes and abolishes the type B conformers (Z Pu et al., 2004). Therefore, edition of peptide-MHC conformational isomers is another function of DM.

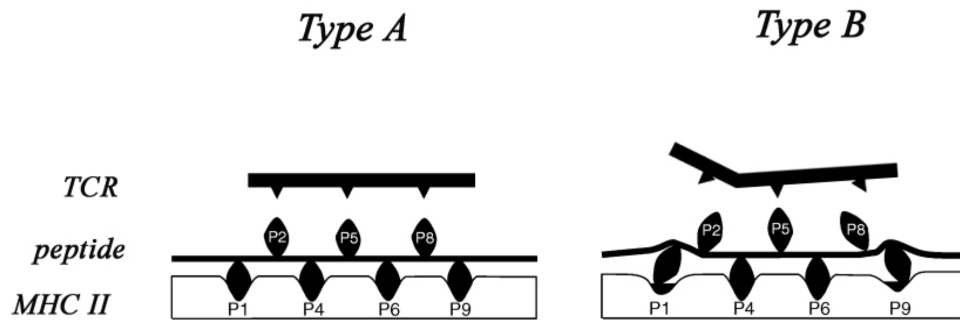


Figure 1.10 Model for Type A and Type B conformers - **(left)** In the type A conformer, all anchor residues fit tightly in the binding groove, orienting the TCR contacts such that only the type A TCR can interact with the complex. **(right)** In the type B conformer, one or more of the pockets in the MHC-binding groove are partially occluded (shown for P1 and P9). This distorts the orientation of the TCR contact residues, such that type A TCR can not recognize them. Therefore, the type B confomers interact with type B TCR that can recognize their different conformation - Adopted from (Lovitch et al., 2005).

This fine editing function of DM is probably linked to its earlier discovered potency to act as a peptide editor (Kropshofer et al. 1997). DM not only exchanges CLIP for cognate peptide but also exchanges low-stability peptides for peptides that display high-stability binding (Kropshofer et al., 1996; van Ham et al. 1996). The presence of appropriate anchor residues at the correct position such as tyrosine at the P1 pocket and a length of >12 residues are features that favor DM resistance (Kropshofer et al., 1996), whereas glycine and proline residues at anchor and non-anchor positions increase susceptibility to DM-mediated release (Raddrizzani et al., 1999).

The catalytic mechanism of DM is under investigation and several models have been suggested. In the 'hit-and-run' mechanism (Narayan et al., 2007), DM interacts transiently and repetitively with MHC class II to induce conformational changes that lead to peptide dissociation. In the 'compare-exchange' mechanism (Ferrante et al., 2008), an unbound peptide makes a transient tetramolecular complex with MHC class II, bound peptide and DM. The peptide with a better fit replaces the pre-bound peptide via a DM-facilitated process.

It is suggested that DM functions through disruption of peptide-MHC hydrogen bonds network (Weber et al., 1996). The network is composed of 12–15 hydrogen bonds involving the MHC main chain (α51–53) and conserved MHC residues (α62, α69, α71, β61, β81, and β82) (Brown et al., 1993). Stratikos *et al.* showed that disruption of hydrogen bonds between the peptide N-terminus and DRα (F51 and S53) resulted in greater susceptibility to HLA-DM (six fold to nine fold) and accelerated peptide release (Stratikos et al., 2004). This finding explains why N-terminal shortening of peptides increases susceptibility to DM (Kropshofer et al., 1996).

Wucherpfennig's group, using Surface Plasmon Resonance (SPR) assay, contributed the most recent finding in peptide-exchange mechanism of DM (Anders et al., 2011). They found that DM makes a stable binding with low-affinity peptide-DR complex. DM dissociates the low-affinity peptide and stays bound to empty DR to keep the groove open and receptive, until a high-affinity peptide fits in the cleft. The high-affinity peptide-DR complex releases DM and becomes DM-resistant (Anders et al., 2011).

1.2.4.5 HLA-DO

HLA-DO (H2-O in mice) is a non-classical MHC II and, similar to DM, is unable to bind peptides (X Chen et al., 2006). HLA-DO (DO) regulates the function of DM and in non-acidic endosomes inhibits the catalytic function of DM (Denzin et al. 1997). Unlike DM that is expressesed in all APCs, the expression of DO is limited to B cells, dendritic cells and subsets of thymic epithelial cells (Hornell et al. 2006; Fallas et al. 2007; Douek et al., 1997; X Chen et al. 2002).

1.2.4.5.1 HLA-DO expression and trafficking

DO is composed of two transmembrane α and β subunits. The DOα and DOβ genes are in the MHC II region of chromosome 6 but TAP and LMP genes separate them. The DOα mRNA is expressed at lower levels as compared to other class II genes, probably because of defective polyadenylation (Trowsdale et al., 1985). However, despite this low level of expression, DOα is co-regulated with other class II molecules and is inducible by IFN-γ (Tonnelle et al., 1985). IFN-γ induces CIITA expression, which in turn activates transcription of the DOα gene in addition to other genes of the MHC II processing pathway,

including MHC II, Ii, and DM (Ponzoni et al., 1993; Taxman et al., 2000; Tonnelle et al., 1985). Thus, comparing DOα and DOβ, it seems that DOβ is the one gene subjected to a tight and differential regulation. It was thought that CIITA does not induce expression of the DOβ gene (Taxman et al., 2000) but later studies showed that CIITA induces expression of DOβ in APCs and epithelial cells (Nagarajan et al., 2002; Khalil et al., 2002). It has been concluded that the affinity of CIITA is weak for the enhanceosome assembled on the promoter of DOβ gene and high expression of CIITA compensates this lack of affinity (Hake et al., 2003; Khalil et al., 2002). Neither IFN-γ nor interleukin- 10 (IL-10) significantly affects transcription of H2-Oα or H2-Oβ gene in the mouse imprtalized B-cell line, although both cytokines enhance expression of H2-DM. IL-4, an important cytokine for B-cell activation and differentiation, increases transcription of both H2-Oα and H2-Oβ genes in addition to H2-M genes (Walter et al., 2000).

In the ER, after its synthesis, DO forms a stable complex with DM. This association with is necessary for efficient egress of DO from the ER, because in the absence of DM, DO is rapidly degraded (Liljedahl et al., 1996, 1998). DO along with DM, is transported to the endosomal/lysosomal compartments (MIIC) (Karlsson et al., 1991; Liljedahl et al., 1996). DO remains engaged in DO:DM complexes in the MIICs and during recycles between MIICs and the cell surface (Karlsson et al. 1991; van L. M. et al. 2001). In human peripheral blood B cells, around 50% of DM is bound to DO (X Chen et al., 2002).

1.2.4.5.2 HLA-DO function

The function of DO is still a matter of controversy. To study its inhibitory effect on DM for CLIP removal, Denzin *et al*. used DM prepared from DO-negative cells and DO-DM complexes isolated from B cell line to catalyze exchange of CLIP in HLA-DR1, DR3 and DR4 complexes. They observed that the DO-DM complex was inactive. Denzin *et al.* also showed a strong surface accumulation of CLIP in MHC class II positive cells transfected with DO (Denzin et al. 1997). Van Ham *et al.* transfected DO-GFP into the DR3-positive melanoma cell line and again showed that DO overexpression was associated with impaired CLIP exchange and its cell surface accumulation (Van Ham et al., 1997). Other studies using DOα-deficient mice, did not confirm this finding and MHC-CLIP accumulation was not observed in DO-proficient mice (Liljedahl et al., 1998; Perraudeau et al., 2000). Perraudeau and co-workers also generated KO mice by targeting the H2-Oα gene. They

obtained no evidence for a defect in CLIP removal while the peptide repertoire was affected and considered H2-O as a modulator of peptide binding (Perraudeau et al., 2000). Kropshofer et al. even observed a decline in DR-CLIP levels upon moderate amounts of DO-transfection (Kropshofer et al. 1998). They have shown that DO promotes loading of DM-dependent peptides and observed that DO-DM complexes, in acidic pH of lysosome, stabilize empty DR more efficiently than DM alone. However, no other study has suggested that DO directly increases DM activity. The down-modulatory activity of DO on antigen presentation is observed only when the model antigen is taken up via pinocytosis (Liljedahl et al., 1996). In contrast, receptor mediated endocytosis via BCR led to indirect enhancement of several epitopes by DO (Liljedahl et al. 1996; Alfonso et al., 2000; Alfonso et al. 2003b). Experiments by purified proteins supported this concept and demonstrated that DO favors peptide loading at pH 4.5–5.0 but reduce loading at pH 6.0–6.5 (Liljedahl et al. 1996; Kropshofer et al. 1998; van Ham et al. 2000). In lysosomes, DO acts as a co-chaperon in at least two ways: first DO prolongs half life of DM and stabilizes it, second DO-DM complexes prevent degradation of empty DR more efficient than DM alone (Kropshofer et al. 1998).

Therefore, based on level of DO overexpression, DO:DM ratio, pH of surrounding media, route of Ag entrance and pathway of endocytosis, DO could exert a spectrum of functions on DM, become an inhibitor or an enhancer and co-chaperon (Denzin et al. 1997; Liljedahl et al. 1996; van Ham et al. 1997; Perraudeau et al. 2000; Alfonso et al. 2003b; Kropshofer et al. 1998; van Ham et al. 2000; Alfonso et al., 2000). We should also consider the possibility of various type of interaction between DO and H2-O with DM and H2-M, respectively. While in human, there is a very good correlation between the level of DO expression and the amount of CLIP (X Chen et al. 2002; Glazier et al. 2002; Chalouni et al. 2003), in mouse, neither H2-O transgenic nor H2-O knockout mice showed difference in the level of CLIP when compared to the wild-type mice (Liljedahl et al. 1998; Perraudeau et al. 2000; Alfonso et al. 2003; Brocke et al. 2003).

The invariant chain derived self-peptide, CLIP, is a principal player in MHC class II antigen processing and presentation (Vogt A and Kropshofer H. 2006). CLIP has a key role in the induction and maintenance of self-tolerance (Chicz et al., 1993; Fung-Leung et al., 1996; T. Hou et al., 2011; Martin et al., 1996; Miyazaki et al., 1996; Surh et al., 1997). Recently, it has been shown that exogenously loaded $CLIP_{81-105}$ regulates helper T cell

response against SAgs (Rohn et al., 2004). N-terminal tail of CLIP, covering residues Leu-81 to Pro-87, is protruding from binding groove of MHC class II molecule and is thought to interact with an effector site outside the binding-cleft (Kropshofer et al. 1995; Kropshofer, Vogt & Hämmerling 1995; Vogt A and Kropshofer H. 2006). This could induce conformational changes in MHC class II molecules that lead to self-release of CLIP (Kropshofer et al. 1995; Kropshofer, Vogt & Hämmerling 1995). DM has evolved to exploit this allosteric site to exchange CLIP with high affinity peptides (Doebele RC et al. 2000; Vogt A and Kropshofer H. 2006). Murine CLIP is shorter than human CLIP and misses that N-terminal allosteric part (Rudensky et al. 1991; Riberdy et al. 1992; Vogt A and Kropshofer H. 2006).

Using Non-obese diabetic-DO (NOD.DO) transgenic mice which express human DO under the control of DC-specific CD11c promoter, the Denzin group shows that the *in vivo* effect of DO is to decrease the presentation of self peptides by DCs to T cells, which limits autoimmune responses without affecting general immune competence (W. Yi et al., 2010). Dampening of autoimmune response was not through central tolerance and pathogenic T cells have not been deleted in NOD.DO mice. Moreover, the number of regulatory T cells (T_{reg}) was similar in NOD.DO and NOD mice. Diabetes development was significantly delayed after transfer of pathogenic T cells from diabetic NOD mice into NOD.DO recipients compared with NOD recipients. Levels of MHC II molecules were similar in NOD and NOD.DO mice but the peptide repertoire that was presented was different, as shown by differences in the binding of a panel of monoclonal antibodies that recognize MHC II in a peptide-dependent manner. They concluded that DO expression shapes the overall MHC II self-peptide repertoire to promote T cell tolerance (W. Yi et al., 2010).

More recently it was shown that H2-O, sets a threshold for B cell entry into germinal centers and its expression reduces the ability of B cells to gain T cell help and participate in the GC reaction (Draghi et al., 2010). They show that in direct competition between Ag specific wild type and H2-O KO B cells, H2-O KO B cells preferentially populate the germinal center (GC) (Draghi et al., 2010). These experiments show that H2-O inhibits Ag presentation primarily in naïve B cells, limiting their ability to receive T-cell help and enter the GC. After passing the check point and upon entry of naïve B cells to GC, H2-O is down-regulated to allow more efficient Ag presentation and more access to T cell help (X Chen et al., 2002; Fallas et al., 2007; Glazier et al., 2002). Therefore, H2-O may establish a

higher threshold of Ag presentation for B cells to start the GC reaction (Draghi et al. 2010).

In light of the inhibitory effect of H2-O on exogenous antigen presentation, it was assumed that H2-O may have a similar effect on presentation of superantigen (SAg) as exogenous Ags. Qi *et al*. have hypothesized that H2-O decrease presentation of SAgs as it does for exogenous Ags (L. Qi et al., 2001). Transfection of H2-O in class II^+ mouse SaI sarcoma cell line, inhibited the binding and presentation of SEA and SEB (L. Qi et al., 2001). However, these have not been done in DO and human cells. Knowing how DO affect the binding of SAgs to MHC II would help to understand immunological function of DO and its possible therapeutic use.

1.3 Immunosurveillance Challenges

The immune response has been classified into either *the innate, antigen-nonspecific response*, or *the adaptive, antigen-specific response*. The immune system by using innate and adaptive effector cells and molecules recognize and destroy cancer cells, a process that is known as *cancer immunosurveillance*. Cancer cells escape innate and adaptive immune responses by immunoselection (that is, selection of non-immunogenic tumor-cell variants, or by immunosubversion (that is, active suppression of the immune response) (Zitvogel et al., 2006).

Leukocytes of the innate immune system circulate through the whole body and serve as immunologic sentinels for detecting general signs of danger using their non-antigen specific receptors. Similarly, B and T lymphocytes survey the body but with their antigen specific receptors. These cells express a comprehensive repertoire of antigen-specific receptors (cell surface immunoglobulin receptors for B cells, and T cell receptors for T cells) that together can recognize millions of distinct antigens (Oltz, 2001). Whereas the B cell antigen receptor directly binds to antigenic determinants present on soluble proteins, carbohydrates, or nucleic acids, the T cell antigen receptor binds most commonly to short fragments of antigens that have been broken down and loaded onto MHC molecules. Thus, B cells can see antigen directly, and respond by differentiating into immunoglobulin-secreting plasma cells which secrete those specific immunoglobulins. In contrast, T cells see processed antigen in the context of self-MHC molecules, thereby providing a basis for self-nonself discrimination (Germain, 1995). Two major subsets of T cells collaborate to mediate an effective immune response, $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. $CD4^+$ helper T cells are activated after binding peptide antigen presented by MHC Class II molecules, and provide cytokine-mediated "help" both to shape the B cell-mediated humoral response and to maximize the quality and durability of the $CD8^+$ T cell response (Bali Pulendran, 2004). $CD8^+$ or cytotoxic T lymphocytes (CTLs) are activated after binding peptides that are derived from endogenously synthesized proteins presented by MHC Class I molecules and lyse their cellular targets by release of cytotoxic granules (Maryanski et al., 1986; Townsend et al., 1986; Zinkernagel et al., 1974). Thus, it is $CD8^+$ effector T cells that are critical for destroying host cells altered by oncogenic

transformation. Due to the cytotoxic property of $CD8^+$ T cells, much attention has been paid to the role of $CD8^+$ T cells in the immunotherapy of cancer. As a result, an increased number of MHC class I-restricted tumor antigens have been identified using tumor-reactive $CD8^+$ T cells derived from peripheral blood mononuclear cells (PMBCs) or tumor-infiltrating lymphocytes (TILs) (Boon et al., 1994; Rosenberg, 1999; Wang et al., 1999). These include important tumor specific antigens like tyrosinase, MART-1, gp100 in melanoma (Brichard et al., 1993; Kawakami et al., 1994) and shared tumor-specific antigens such as MAGE-1 and NY-ESO- 1 that are expressed in a wide variety of tumors such as breast cancer and lung cancer (Jäger et al., 1998; Wang et al., 1998). However, tumor immunotherapy approaches that were solely based on MHC I peptides have been disappointing (Peterson et al., 2003; Monsurrò et al., 2004; Mocellin et al., 2004; Rosenberg et al. 2004; Rosenberg et al. 2005; Rosenberg et al., 1998). One of the main reasons for this weak CTL response is the insufficient $CD4^+$ T cell help (Hung et al. 1998; Toes et al., 1999; Baxevanis et al. 2000; Hunder eta l., 2008). To enhance antitumor immunity and to eradicate cancer cells in patients, it is necessary to recruit both $CD4^+$ and $CD8^+$ T-cell responses (Bos et al., 2010; Toes et al., 1999; Wang, 2001).

$CD4^+$ T cells play a pivotal role in tumor immunity. They orchestrate antibody production by B cells against tumors. $CD4^+$ T cells have a crucial role in cell-mediated immunity, that is the major arm of tumor immunity and our main focus. For example, they initiate, enforce, expand and maintain CTLs against tumors. The important role of $CD4^+$ T cells in cell-mediated immunity against tumor will be discussed in next section.

1.3.1 Importance of $CD4^+$ T cells in tumor immunity

1.3.1.1 Dendritic cell licensing and clonal expansion of CTLs

Presentation of peptides by immature DCs to cognate naïve $CD8^+$ T cells (signal 1) could lead to anergy or deletion of T cells due to absence of second signal (co-stimulatory molecule) (Janeway et al., 1996; Matzinger, 1994; D. L. Mueller et al., 1995; Van Gool et al., 1999). DCs become mature and activated in response to engagement of toll like receptors and upregulate co-stimulatory and MHC class II molecules (Aderem & Ulevitch, 2000; De Smedt et al., 1996; Kamath et al., 2000; Medzhitov, 2001). Stimulation of naive $CD8^+$ T cells with these mature DCs that deliver both signal 1 (Ag) and signal 2 (co-stimulation) can lead to strong in vitro proliferation and *in vivo* clonal expansion, but the cells fail to become fully activated and develop effector function unless they receive signal 3 (cytokines) from a licensed DC (Curtsinger et al., 2003; Hamilton-Williams et al., 2005; Smith et al., 2004). Furthermore, cells that initially expand in the absence of signal 3 are rendered tolerant, in that they fail to develop effector function upon rechallenge with a stimulus that is normally fully activating for naive cells. Thus, $CD8^+$ T cells can undergo substantial clonal expansion in response to Ag and co-stimulation, but have profoundly different fates depending on whether or not they receive the third signal from a licensed DC (Curtsinger et al., 2003; Smith et al., 2004).

To get licensed, DCs first by expression of peptide and B7 (signal 1 and 2) activate $CD4^+$ T cells that in turn secrete IL-2 and express CD40L to interact with CD40 on DCs. This DC-$CD4^+$ interaction activates DCs which increase expression of co-stimulatory molecules and secretion of cytokines like IL-12 (signal 3). The licenced DCs fully activate $CD8^+$ T cells through co-stimulatory molecules interaction (like CD40-CD40L and OX40–OX40L) and cytokines (like IL-12, IL-15 and IL-21) (Curtsinger et al., 2003; Grewal & Flavell, 1998; Hernández et al., 2002; Schmidt et al., 1999; Schmidt et al., 2002) **(Figure 1.11).** Signaling via IL-12 supports the proliferation and development of cytolytic activity in $CD8^+$ T cells in vitro (Agarwal et al., 2009; Schmidt et al., 2002; Xiao et al., 2009). IL-2 and IL-21 signaling drive the upregulation of B lymphocyte-induced maturation protein 1

(Blimp-1/ Prdm1), a transcription factor known to induce CD8^{+} T cell differentiation and effector function (Gong & Malek, 2007; Kwon et al., 2009).

Interaction of CD4^{+} T cells and DCs through CD40L–CD40 can also protect DCs from CTL-mediated death (Mueller et al., 2006), perhaps leading to more-efficient CD8^{+} T cell priming.

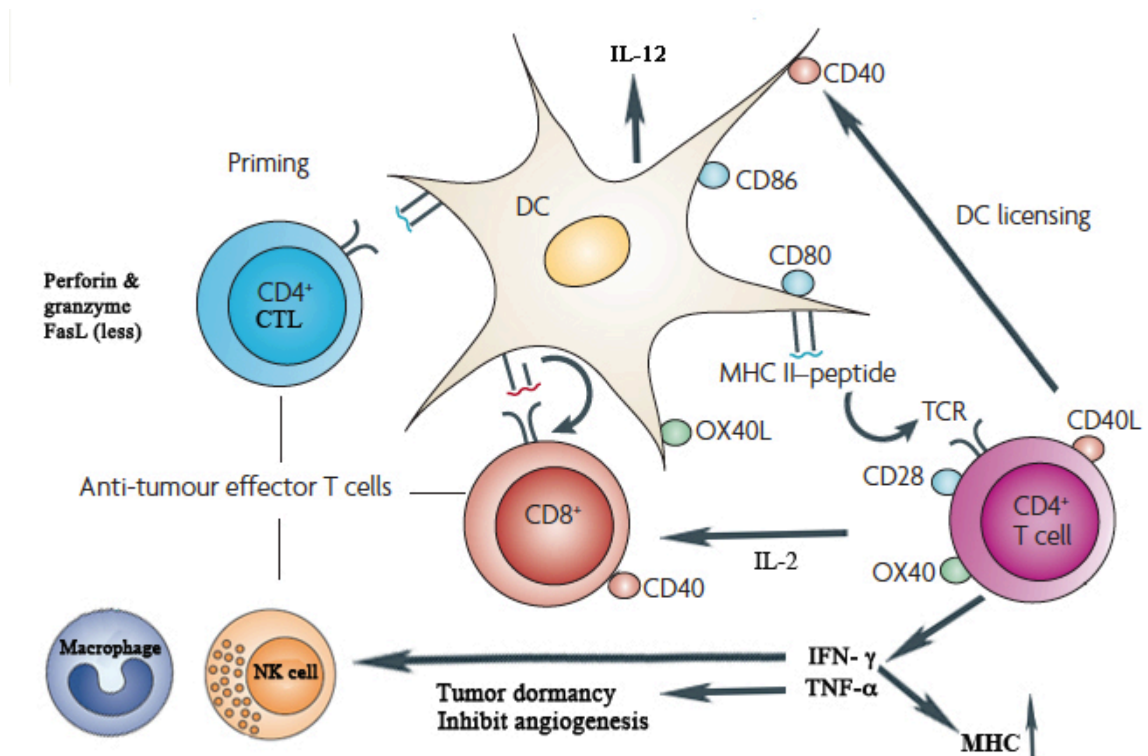


Figure 1.11 - Importance of CD4+ T cells in tumor-immunity (description in text) - Dendritic cells (DC), Cytotoxic T lymphocyte (CTL), Natural killer (NK) cells.

It has been shown that the DCs requirement can be bypass by direct delivery of the helper signal from the CD4 T cells to the CD8 T cells (Bourgeois et al., 2002). The frequency of CD4^{+} and CD8^{+} T cells specific for a single Ag is quite low, a mechanism for bringing these rare cells together in time and/or space is required. The obvious platform for such signal transfer is the DC, whether the CD4^{+} and CD8^{+} T cells simultaneously engage with the same DC (the three-cell cluster model) or the antigen activated CD4^{+} T cell leaves

the scene after activating DC (Lanzavecchia, 1998). The licensed-DC then delivers help to $CD8^+$ T cell (Lanzavecchia, 1998). The three-cell cluster model is consistent with the early hypothesis that $CD4^+$ and $CD8^+$ T cells need to recognize their specific antigens simultaneously on the same DC, so that local paracrine action of cytokines such as IL-2 secreted by DC-bound $CD4^+$ T cells can support clonal expansion by the colocalized $CD8^+$ T cells (Keene et al., 1982). A more recent variation of this paracrine three-cell cluster model suggests that antigen-primed $CD4^+$ T cells can directly stimulate $CD8^+$ T cells via CD40L when the two lymphocytes are in close proximity on an APC (Bourgeois et al., 2002). The three-cluster model and the probability of meeting three rare cells - an antigen-bearing DC coming from an infected site, an antigen-specific $CD4^+$ T cell, and an antigen-specific $CD8^+$ T cell - in the same place at the same time is very low (Guerder et al., 1989; Ridge et al., 1998). The intuitive feeling that such clusters could not be efficiently generated led to a new model in which antigen-stimulated $CD4^+$ cells activate DCs via CD40L-CD40 interaction, and the resultant "licensed DCs" become fully competent to activate naïve $CD8^+$ T cells, even in the absence of an associated $CD4^+$ T cell (Bennett et al., 1998; Grewal et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). In one version of this model, the $CD4^+$ T cell moves from DC to DC, activates them and one of these activated DCs provide help to a $CD8^+$ T cell which bears the appropriate TCR (Ridge et al., 1998). This moving around was proposed to amplify T_h function by creating many licensed DCs for each antigen-activated $CD4^+$ T cell.

1.3.1.2 Recruitment of naïve CTL to lymph node

The precursor frequency of naïve $CD4^+$ and $CD8^+$ T cell specific for any given antigen is quite low and the likelihood of meeting a DC that co-present the same antigen is rare. They need an active mechanism that facilitates their cell–cell associations. $CD4^+$ T cells not only play a role in activating dendritic cells but also produce the chemokines CCL3 and CCL4, which promote the migration of $CD8^+$ T cells toward these activated dendritic cells

(Castellino et al., 2006; Castellino et al., 2007). In fact, Both $CD4^+$ T cells and DCs were found to produce CCL3 and CCL4 when activated. DCs licensing and CD40 ligation induces the DCs to secrete inflammatory cytokines and chemokines, including CCL3 and CCL4. CLL3 and CCL4 attract naïve $CD8^+$ cells to licensed DCs and/or antigen specific clusters of DCs and CD4+ T cells (Castellino et al., 2006). If the recruited naive $CD8^+$ T cells do not find cognate antigen on the DC, their association will last only a few minutes, after which they will leave and engage other DCs in a search for a suitable TCR ligand. If instead the DC does present the relevant antigen, the $CD8^+$ T cell will stop and adhere for a prolonged time, estimated to be up to several hours (Bousso et al., 2003). The effect of chemokine (CCL3/4) production by the CD4+ T cell– DC pair will thus be to enhance the rare likelihood of meeting antigen-specific CD8+ T cells with co-presenting DCs. In addition, once the recruited $CD8^+$ T cell finds its cognate antigen on licensed DC, it can also begin to secrete CCL3 and CCL4 (Castellino & Germain, 2006). The combined secretion from the $CD8^+$ T cells and the DC will sustain the chemokine gradient toward these licensed DCs, even if the antigen-stimulated $CD4^+$ T cell has already dissociated.

$CD4^+$ T cells also can stimulate the trafficking of $CD8^+$ T cells to sites of inflammation by the secretion of IFNγ as well as the chemokines CXCL9 and CXCL10 (Nakanishi et al., 2009). Indeed, IFN-γ, which Th1 cells are a major producer of, is known to activate high endothelial venules and enhance attachment and transendothelial migration of lymphocytes by programing the homing of $CD8^+$ T cells and changes in their integrins and homing receptors expression (Hendriks et al., 1989; Kraal et al., 1994; May et al., 1992). Nakanishi *et al.* have shown that $CD4^+$ T cells serve as gate-keepers of CTL entry into the inflamed tissues through the secretion of IFN-γ and turning on the expression of chemokines CXCL9 and CXCL10, which enable the $CXCR3^+$ effector CTL population to migrate from the peripheral blood into the inflamed areas (Nakanishi et al., 2009). $CD4^+$ T cells also by activating DCs and remodeling of feeding arterioles of lymph nodes (LN), enlarge LN and recruit more CTLs to the inflamed area (Kumamoto et al., 2011).

1.3.1.3 Induction of memory CTLs

$CD8^+$ T cells primed in the absence of $CD4^+$ T cells are defective at mounting recall responses. $CD4^+$ T-cell help is required for the differentiation of $CD8^+$ effector cells into memory cells (Janssen et al., 2003; Janssen et al., 2005; Khanolkar et al., 2004; Shedlock et al., 2003; Sun et al., 2003; Sun et al., 2004; Williams et al., 2006). IL-2 is an essential input signal for programing and generation of $CD8^+$ memory T-cells and the major source of IL-2 during primary immune response is $CD4^+$ T cells (Williams et al., 2006). In the absence of $CD4^+$ T-cell help during priming, $CD8^+$ memory T cells can be developed but with lower frequency and with phenotypic and functional defects (Bevan 2004). Licensed DCs secrete IL-12 that its signaling reported to be crucial for generation of $CD8^+$ memory T cells (Agarwal et al., 2009; Pearce et al., 2007; Schmidt et al., 2002). Recent studies demonstrate that most unhelped $CD8^+$ T cells exhibit relatively high T-bet expression (Intlekofer et al., 2007); a pattern which is also exhibited by so-called short-lived effector $CD8^+$ T cells (Hamilton et al., 2007; Intlekofer et al., 2007; Joshi et al., 2007).

$CD8^+$ T cells primed in the absence of $CD4^+$ T cells are defective at mounting recall responses (Janssen et al., 2003; Shedlock et al., 2003; Sun et al., 2003). $CD8^+$ T cells that have been helped by $CD4^+$ T cells during priming downregulate TNF-related apoptosis-inducing ligand (TRAIL) expression on $CD8^+$ T cells. This makes them less susceptible to apoptosis (Janssen et al., 2005), or delays their susceptibility (Badovinac et al., 2006), to TRAIL-mediated apoptosis. By contrast, $CD8^+$ T cells that have not been helped undergo enhanced TRAIL-mediated apoptosis following antigen re-exposure. Paracrine IL-2 produced by $CD4^+$ T cells during the initial priming of $CD8^+$ T cells dramatically improved the $CD8^+$ T cell recall response (Williams et al., 2006). Furthermore, $CD4^+$ T cells have been shown to upregulate the expression of CD25 (IL-2Rα) on $CD8^+$ T cells (Obar et al., 2010). At later stages of the response, $CD4^+$ T cells produce additional cytokines, such as IL-21, which appears to be a crucial signal for downregulating TRAIL

expression on responding $CD8^{+}$T cells (Barker et al., 2010).

It has been shown that direct ligation of CD40 on naive $CD8^{+}$ T cells by CD40L on $CD4^{+}$ T cells can enhance the generation of memory $CD8^{+}$T cells (Bourgeois et al., 2002). $CD4^{+}$ T cells may promote the generation of effector and memory $CD8^{+}$ T cell populations through many possible pathways. One of these pathways is enhancing APC-mediated production of cytokines that augment initial $CD8^{+}$ T cell responses; these cytokines include IL-1, IL-6, TNF and IL-15 (Oh et al., 2008). Moreover, cognate $CD4^{+}$ memory T cells enhance the expansion of cognate $CD8^{+}$ memory T cells as well as the infiltration and accumulation of these cells within tumor (Hwang et al., 2007).

1.3.1.4 Maintenance of memory CTLs

The presence of $CD4^{+}$T cells during priming may influence the homing pattern and the tissue distribution of generated memory $CD8^{+}$ T cells which are important for their maintenance and life span (Azadniv et al., 2011). $CD4^{+}$ T cells promote stable maintenance of memory $CD8^{+}$ populations by regulating the expression of and hence signaling through the IL-7 and IL-15 receptors on $CD8^{+}$ T cells. IL-7 is required for the long-term survival of CD8 memory cells, while IL-15 supports basal homeostatic proliferation (Jameson, 2005; Surh et al., 2005). In the absence of $CD4^{+}$ help, memory $CD8^{+}$ T cells fail to express IL-7Ra (CD127) and also downregulate IL-15Rb (CD122), thus adversely impacting both survival and turnover of memory cells (Williams et al., 2006).

1.3.1.5 Maintenance of competent CD8+ T cells

It has been shown that $CD4^{+}$ T cells maintain strong and long-term effector function of $CD8^{+}$ T cells (Zajac et al., 1998). Recent studies suggest that IL-21 production by $CD4^{+}$ T cells during viral chronic infection is crucial for maintaining functional $CD8^{+}$ T cells which

are able to contain the infection (Elsaesser et al., 2009; Fröhlich et al., 2009; J. S. Yi, Du, & Zajac, 2009). Clinical evidence also correlates the presence of higher numbers of IL-21-producing $CD4^+$ T cells with improved $CD8^+$ T cell function and improved control of HIV infection (Chevalier et al., 2011; Williams et al., 2011).

$CD8^+$ T cells are able to produce IL-2 upon TCR ligation (D'Souza & Lefrançois, 2004); however after the initial activation the responding T cells enter a period termed "activation-induced non-responsiveness" (AINR). During this time T cells are still able to lyse target cells and produce IFNγ but are dependent on paracrine IL-2 supplied by $CD4^+$ T cells for further proliferation. After receiving these $CD4^+$ derived IL-2 signals, the responding $CD8^+$ T cells are rewired to allow IL-2 mRNA upregulation and proliferation in response to TCR signals in the absence of costimulation (Tham et al., 2002).

1.3.1.6 Upregulation of MHC molecules

One of the main immunoediting mechanism of malignant cells is down regulation or loss of MHC molecules that let them escape from immunosurveillance (Campoli et al., 2008; Zitvogel et al., 2006). Shankaran et al. have shown that the ability of IFNγ to promote tumor rejection is mediated, at least in part, through its capacity to upregulate the MHC class I pathway of antigen processing and presentation in tumor cells (Shankaran et al., 2001). Many tumors do not express MHC II molecules but most cell types, including those of non-bone marrow origin, upregulate the MHC II antigen presentation machinery in the presence of IFNγ (Boehm et al., 1997). In patients with cancer, DCs express significantly lower amount of MHC class II molecules due to upregulation of immunosuppressive cytokines like IL-10 (Choi et al., 2009). IFNγ neutralize this immune escape mechanism and upregualte MHC II molecules (Schroder et al., 2004).

IFNγ is predominantly produced by activated $CD4^+$ T, NK and NKT cells (Schoenborn et al., 2007). IL-12, secreted by licensed DCs, not only activates NK cells but also is the main and essential mediator of T_h1 differentiation in naive T cells. IL-12 directly induces IFNγ gene transcription and secretion in activated $CD4^+$ T cells. It stimulates IFNγ

secretion by NK cells as part of the activation process (Lederer et al., 1996; Trinchieri, 1995). IL-12 is in turn actively induced in macrophages and monocytes by IFNγ (Trinchieri, 1995; Yoshida et al., 1994). Thus it is a positive feedback loop between licensed DCs, activated CD4 T cells and cells of innate immunity that increases the secretion of IFNγ and IL-12 and upregulate the expression of MHC molecules.

1.3.1.7 Cytotoxic effects of $CD4^+$ T cells

$CD4^+$ T cells in addition to providing help and promote function of B lymphocytes and $CD8^+$ T cells, can work as an effector cell and attack their target directly and independently of their helper activities. Cytotoxic CD4 T cells have been first described in late 1970's (Billings et al., 1977) , but many early reports labeled CD4 CTL as an in vitro artifact and there was speculation whether these cells had any *in vivo* relevance.

Two major cytotoxic mechanism for effector $CD4^+$ T cells have been described: first, through the production of cytokines, most notably IFNγ and TNF (Brien et al., 2008; Iwashiro et al., 2001; Oxenius et al., 1996; Savarin et al., 2008; Stuller et al., 2010; Teijaro et al., 2010; Yauch et al., 2010); and second, through direct cytolytic activity mediated by both perforin and FAS (CD95) (Brien et al., 2008; Brown et al., 2006; Hou et al., 1992; Ishikawa et al., 2009; Iwashiro et al., 2001; Stuller et al., 2010; Yauch et al., 2010).

Brown et al. have shown that cytolytic CD4 cells develop during Th1 differentiation, but do not require IFN-γ to acquire this activity. Moreover, Th2 differentiation does not promote the cytolytic phenotype and IL-4 actively inhibits the ability of naïve CD4 cells to become cytolytic. APC were necessary to drive induction of cytolytic activity, however, blocking APC-derived cytokines such as IL-6, TGF-β, IL-10, IL-12 and TNF-α did not inhibit the generation of cytolytic CD4 cells. They identified IL-2 as the only exogenous cytokine that in low concentration of peptide was necessary for inducing cytolytic CD4 effector cells (Brown et al., 2009). Thus development of cytotoxic $CD4^+$ T cell does depend neither on Th1 cell polarization (T-bet transcription) nor on Th2 (GATA-3 transcription factor) (Eshima et al., 2012; Qui et al., 2011). Expression of the transcription factor eomesodermin (EOMES) seems be crucial in driving the development of cytotoxic $CD4^+$ T

cells *in vivo* (Eshima et al., 2012; Qui et al., 2011). Therefore, CD4^{+} T cells with cytotoxic activity could be considered a separate functional T cell subset.

The fact that MHC class II expression is largely restricted to professional APCs under steady-state conditions may limit the protective potential of virus-specific cytotoxic CD4^{+} T cells. However, cells other than professional APCs are capable of upregulating MHC class II expression following pathogen challenge and could therefore become targets of cytotoxic CD4^{+} T cells during viral infection. For example, epithelial cells that are activated by infection (Debbabi et al., 2005) or IFNγ-mediated signals (Cunningham et al., 1997) strongly upregulate their expression of MHC class II molecules.

1.3.1.7.1 **Cytotoxic CD4^{+} T cells - restricted to expression of MHC II on tumor**

MHC class II-restricted activity of cytotoxic CD4^{+} T cells have been shown (Brown et al., 2009; Quezada et al., 2010). While in infectious and viral models cytolytic activity of these cells was preferentially perforin mediated (over FasL) (D. M. Brown et al., 2009), in tumor model, tumors were also rejected in perforin$^{-/-}$ (PFN$^{-/-}$) recipient mice, suggesting that rejection is independent of perforin (Quezada et al., 2010). Interestingly, tumor rejection was independent of CD8^{+} and NK cells activity and their depletion failed to prevent tumor rejection.

1.3.1.7.2 **Cytotoxic CD4^{+} T cells - non-restricted to MHC II expression on tumor**

Most cancer cells lack MHC class II molecules and cannot present tumour-specific antigens directly to CD4^{+} T cells. It has been reported that macrophages and dendritic cells collaborate with tumour-specific CD4^{+} T cells to recognize tumor specific antigens (TSA) (Corthay et al., 2009). These professional antigen-presenting cells endocytose and process TSA to display antigenic peptides on their MHC class II molecules for indirect cancer cell recognition by CD4^{+} T cells.

Perez-Diez *et al*. compared the tumor clearance ability of CD4^{+} and CD8^{+} T cells using TCR transgenic mice containing pure population of CD4 or CD8 T cells against the same tumor antigen, in order to test each type of effector alone, without the effects of potential

contaminants (Perez-Diez et al., 2007). Surprisingly, they found that $CD4^+$ cells were better than $CD8^+$ cells at rejecting tumors, even when the $CD4^+$ effectors exhibited minimal in vitro or *in vivo* lytic activity against the tumor cells, and even when the tumor expressed major histocompatibility complex (MHC) class I but not class II molecules. Although the $CD4^+$ T cells did not require $CD8^+$ T cells, their partnership with other host cells and natural killer (NK) cells were essential for maximum effectiveness.

$CD4^+$ T cells attack MHC class II negative tumors whether alone (via T-cell–derived cytokines), or by activating NK cells and/or macrophages. They can also target stromal cells cross-presenting tumor antigens, on MHC class II due to upregulation of IFNγ.

1.3.1.8 Induction of tumor dormancy and Inhibition of angiogenesis

Tumor cells interact with their neighboring cells and microenvironment to program them for establishment of tumor’s microenvironment and neo-angiogenesis for feeding tumor (Bissell et al., 2000). This program is influenced by innate immune cells that promote aberrant growth of the $\alpha v\beta 3$ integrin-expressing vessels, required for the transition of premalignant dysplasias into carcinomas and further cancer progression (Bhowmick et al., 2004; Müller-Hermelink et al., 2008; de Visser et al., 2006). It has been shown that $CD4^+$ T cells induce tumor dormancy, preventing tumor escape (Müller-Hermelink et al., 2008). This tumor-growth-inhibiting effect strictly requires both IFN-γ and TNF-α signaling. In this scenario, the absence of IFN-γ or TNF-α could lead to tumor progression and transformation.

Furthermore, $CD4^+$ T cells inhibit tumor angiogenesis through a combined action of IFN-γ and TNF-α, which induces DCs to produce potent anti-angiogenic chemokines, CXCL10 and CXCL9 (Coughlin et al., 1998; Müller-Hermelink et al., 2008; Qin & Blankenstein, 2000).

1.3.1.9 Differentiation to regulatory T cells

Although the role of natural regulatory T cells (T_{reg}) has mostly been ascribed to maintain ‘‘self-tolerance’’ in the periphery, the induced regulatory T cells (iT_{reg}) from $CD4^+/CD25^-$ precursors has been proposed to be one of the important mechanisms

involved in the tumor immune evasion (Chattopadhyay et al., 2005; Sakaguchi et al., 2001).

It is known that T_{reg} hamper the functions of $CD8^{+}$ T cells and natural killer cells, the key effector cells of antitumor immunity (Ghiringhelli et al., 2005; Shimizu et al., 1999). The frequency of T_{reg} present in peripheral blood of patients with various cancers is higher than that of normal population (Curiel et al., 2004; Javia et al., 2003; Liyanage et al., 2002; A. M. Wolf et al., 2003; Woo et al., 2001). Notably, T_{reg} isolated from peripheral blood, ascites, or solid tumors remain suppressive of tumor-specific T cells in vitro (Curiel & Coukos et al. 2004). Likewise, T_{reg} from tumor-bearing mice inhibited tumor rejection (Berendt et al., 1980; Bursuker et al., 1984; North et al., 1984), indicating that T_{reg} suppress and limit tumor-specific immunity. In contrast, depletion of T_{reg} with anti-CD25 monoclonal antibody in animal models enhanced antitumor immunity and tumor regression (E. Jones et al., 2002; Prasad et al., 2005; Steitz et al., 2001; Sutmuller et al., 2001). Therefore, preventing induction and accumulation of iT_{reg} is necessary for a successful immunotherapy and active vaccination (Curiel et al. 2004; Liyanage et al. 2002; E. Y. Woo et al. 2001).

1.4 Dendritic cells and their application in tumor immunotherapy

1.4.1 DCs

Dendritic cells act as orchestrator of immune system and coordinate innate and adaptive immune responses. Due to this special property DCs have been used as vaccines against cancer. The aim of DC vaccination is to induce tumor-specific effector T cells that can reduce the tumor mass specifically and also induce immunological memory to control tumor relapse. DCs are bone marrow-derived cells that are distributed in all tissues. DCs sample the environment and transmit information to cells of the adaptive immune system (Banchereau et al., 1998). DCs initiate an immune response by presenting the captured antigen, as peptide-MHC complexes, to naive T cells in lymphoid tissues (Itano et al., 2003).

DCs in peripheral tissues are normally immature and have the ability to efficiently capture antigens. They can accumulate MHC II in the late endosome-lysosomal compartment and express low levels of co-stimulatory molecules. Such immature DCs (iDC) induce immune tolerance either through T cell deletion or through the expansion of regulatory T cells (Steinman et al. 2003). iDCs located in peripheral tissues express receptors for inflammatory chemokines (CCR1, CCR2, CCR5, CCR6, CXCR1) that guide them to inflammatory sites where they internalize antigens and receive maturation stimuli. Upon maturation, DCs down regulate receptors for inflammatory chemokines and up regulate receptors for homing to lymph nodes (CCR4, CXCR4, and CCR7) (Sallusto et al., 1999; Toebak et al., 2009).

The consequences of the maturation events include down-regulation of antigen-capture activity, increased expression of surface MHC II and co-stimulatory molecules and the ability to secrete cytokines (Trombetta et al. 2005). The engagement of the co-stimulatory receptor CD40 with its ligand is an essential signal for the differentiation of iDCs into fully mature DCs (mDCs) that are able to launch adaptive T cell-mediated immunity (Caux et al., 1994; Fujii et al., 2004). The different signals that are provided by different stimuli either directly or through the surrounding immune cells induce DCs to acquire distinct phenotypes that eventually contribute to different immune responses. Indeed, DC maturation varies according to different microbes expressing different pathogen-associated

molecular patterns (PAMPs) that trigger distinct pattern recognition receptors (PPRs) (Steinman et al., 2007; B Pulendran et al. 2001). DCs can also be polarized into distinct phenotypes by the products released from surrounding immune cells in response to injury. For example, γδ-T cells and NK cells release IFNγ, mast cells release pre-formed IL-4 and TNF, pDCs secrete IFNα, stromal cells secrete IL-15 and thymic stromal lymphopoietin (TSLP) that can differentiate DCs to distinct phenotypes (Cheng, Zhou et al., 2010; Ueno et al., 2010). These cytokines induce the differentiation of progenitor cells or of precursor cells such as monocytes into distinct inflammatory DCs that yield unique types of T cell.

Interaction of DCs with naive $CD4^+$ and $CD8^+$ T cells can trigger their differentiation into different effector cells with different functions. $CD4^+$ T cells can become T helper 1 (T_h1) cells, T_h2 cells, T_h17 cells or T follicular helper (T_{fh} cells that help B cells to differentiate into antibody-secreting cells), as well as regulatory T (Treg) cells that down-regulate the functions of other lymphocytes. Naive $CD8^+$ T cells can give rise to effector cytotoxic T lymphocytes (CTLs). DCs can also interact with cells of the innate immune system, including natural killer (NK) cells, phagocytes and mast cells (Banchereau et al., 1998; Steinman et al., 2007; Steinman 2011).

DCs regulate humoral immunity directly by interacting with B cells or indirectly by inducing the expansion and differentiation of $CD4^+$ helper T cells (Jego et al., 2005; H. Qi et al., 2006). These key properties of DCs, which activate both cellular and humoral arms of the adaptive immune system, make DCs the central candidates for antigen delivery and therapeutic vaccination against cancer.

1.4.2 DCs subtypes

DCs arise from hematopoietic stem cell precursors in the bone marrow; they share certain defining features, such as high levels of MHC II and a lack of other leukocyte lineage markers (CD3, CD14, CD19, CD20, CD16, and CD56). However, DCs constitute a heterogeneous cell population, with variability in immunophenotype, function and, potentially, hematopoietic lineage (G.-X. Yang et al., 2005). Two major subsets of peripheral blood DCs have been described: myeloid DCs (mDCs; also known as conventional DCs and classical DCs) and which are $CD14^-/CD11c^+/CD123^-$ and plasmacytoid DCs (pDCs; also known as lymphoid DCs) and are $CD14^-$/ $CD11c^-/CD123^+$.

Human DCs subsets in the blood can be distinguished by the differential expression of three cell-surface molecules: BDCA1 (CD1C), BDCA2 (CD303) and BDCA3 (CD141) (Dzionek et al., 2000). $BDCA2^+$ pDCs represent a front line of anti-viral immunity owing to their ability to secrete large amounts of IFNα in response to virus encounters (Siegal et al., 1999). Their pre-synthesized stores of MHC I molecules (Di, Chatterjee et al., 2008) may allow a rapid initial $CD8^+$ T cell response to viral infections. pDC-derived IFNα may also promote the immunogenic maturation of other subsets of DCs, thus helping to activate novel T cell clones. Furthermore, activated pDCs can induce the maturation of activated B cells into plasma cells through both cytokines and direct physical contact (Jego et al., 2003; Shaw et al., 2010). In their resting state, pDCs are considered to have an important role in immune tolerance, including in oral tolerance (Liu, 2005).

mDCs are potent activators of T cells and inducers of cytotoxic T lymphocyte (CTL) responses and are divided in two subsets in the blood that can be distinguished by expression of BDCA1 and BDCA3. Human $BDCA3^+$ DCs, like mouse $CD8^+$ DCs, have high capacity to capture exogenous antigens and present them on MHC I (cross-presentation). $BDCA3^+$ DCs express XCR1, which is the receptor for the chemokine XCL1 (lymphotactin) that is produced by NK cells and activated $CD8^+$ T cells (Crozat et al., 2010; Liu, 2005). Thus, mouse $CD8^+$ DCs and human $BDCA3^+$ DCs are equipped for the generation of $CD8^+$ T cell-mediated immune responses. In mice, gene knockdown studies have shown that the $CD8^+$ subset of DCs have an important role in cross-presentation (Shortman et al., 2010). However, in human, other DCs such as epidermal Langerhans cells also cross-present antigens (Klechevsky et al., 2008). The unique functions of $BDCA1^+$ DCs also continue to be analyzed.

There are two main subsets of mDCs in the human skin: epidermal Langerhans cells (LC) and dermal interstitial DCs (DDCs) (Valladeau & Saeland, 2005). The dermal DCs can be further subdivided into $CD1a^+$ DCs and $CD14^+$ DCs (Valladeau et al., 2005; Nestle et al., 1993). Human $CD14^+$ DCs can directly help activated B cells, as well as induce naive T cells to differentiate into cells with the properties of T_{fh} cells (Caux et al., 1997; Klechevsky et al., 2008). $CD14^+$ DCs may thus be specialized for the development of humoral responses (Klechevsky et al., 2008; Ueno et al., 2007). Langerhans cells are more

efficient in cross-presenting peptides from protein antigens to $CD8^+$ T cells and can prime the differentiation of $CD8^+$ T cells into effector CTLs.

In human studies, *in vitro* exposure of human monocytes to different cytokine combinations result in DCs with different phenotypes and functions. Granulocyte macrophage colony-stimulating factor (GM-CSF) together with IL-4 (Romani et al., 1994), IFNα (Paquette et al., 1998), TNF (Chomarat et al., 2003) or IL-15 (Mohamadzadeh et al., 2001) have been used and several of these combinations have been administered as vaccines to cancer patients.

1.4.3 DCs and tumor immunity

It has been shown that DCs can capture tumour antigens that are released from tumor cells, either from dying cells or 'nibbling' of live cells, and cross-present these antigens to T cells in tumor-draining lymph nodes (Dhodapkar et al., 2008; Diamond et al., 2011; Fuertes et al., 2011). This results in the generation of tumor-specific CTLs that contribute to tumor rejection (Diamond et al., 2011; Fuertes et al., 2011). Thus, DCs represent important targets for therapeutic interventions in cancer.

In the other hand, tumors can prevent antigen presentation and the establishment of tumour-specific immune responses by DCs through a variety of mechanisms. For example, tumors switch the differentiation of monocytes to macrophages rather than DCs, through the interplay of IL-6 and macrophage colony-stimulating factor (M-CSF) (P Chomarat et al., 2000). Alternatively, the tumor glycoproteins carcinoembryonic antigen (CEA) and mucin 1 (MUC1) that are endocytosed by DCs can be confined to early endosomes, which thus prevents efficient processing and presentation to T cells (Hiltbold et al., 2000). Tumors also interfere with DC maturation. They can inhibit DC maturation through the secretion of IL-10 (Fiorentino et al., 1991; Steinbrink et al., 1997), which leads to antigen-specific anergy. Tumor-derived factors can also alter the maturation of DCs and turn them to 'pro-tumour' DCs that indirectly promote tumor growth. For example, TSLP, which is produced by tumour cells, induces DCs to express OX40 ligand, which directs the generation of Th2 cells. These skewed $CD4^+$ T cells accelerate breast tumor development through the secretion of IL-4 and IL-13 (Aspord et al., 2007). These cytokines prevent tumor cell apoptosis and indirectly promote the proliferation of tumor cells by stimulating tumor-associated macrophages to secrete epidermal growth factor (EGF). A similar pathway

operates in pancreatic cancer (De Monte et al., 2011) and studies in mice corroborate the pro-tumorigenic effect of Th2 cells (DeNardo et al., 2009). Th2 cells can inhibit the generation of CTLs by secreting IL-4, which instead leads to the generation of a subpopulation of CTLs, termed TC2 cells, which have limited killing capacity owing to their low expression of granzymes and perforin (Sasaki et al., 2008).

Early studies showed that in comparison to mDC, pDC have weaker capacities for phagocytosis and T-cell stimulation (O'Doherty et al., 1994) and that upon stimulation, pDCs favor a T_h2 rather than T_h1 cytokine secretion profile (Rissoan et al., 1999). pDCs can contribute to tumour angiogenesis by secreting pro-angiogenic cytokines (Coukos et al., 2005; Curiel, Cheng, et al., 2004). Due to the well-characterized role of CTL in the destruction of tumor cells and the more potent CTL responses induced by mDCs than by pDCs, the former subset has been used in current DC vaccination approaches. However, IFN-secreting pDCs can also be induced to drive T_h1 polarization (Cella et al., 2000), and the synergy between pDCs and mDCs in stimulating T-cell activation and antitumor immune responses has been demonstrated (Lou et al., 2007). Moreover, appropriately stimulated pDCs play a more significant role in anticancer immune responses than once believed (Basner-Tschakarjan et al., 2006). Taken together, emerging evidence supports the idea that the interplay between DC subsets is important in eliciting appropriate immune responses and that any subset, given the appropriate conditions, may produce potent antitumor immune responses.

1.4.4 DC vaccine challenges

The goal of cancer vaccines and more specific DCs vaccines is to elicit robust and long-lasting tumor-specific $CD8^+$ T cell-mediated immune responses that generate durable tumor regression and/or eradication. To generate this ideal response, at least four components of the immune response are necessary: the presence of appropriate DCs; the quality of induced $CD4^+$ T helper cells; the elimination and/or non-activation of Treg cells; and the breakdown of the immunosuppressive tumour microenvironment (Zhang et al., 2011).

To date the most common DC vaccine strategy is pulsing of tumor-peptides on DCs and most trials of DC vaccines for cancer have utilized DCs pulsed with synthetic peptides

derived from a defined tumor antigen (TA) (E Gilboa, 1999; Nestle et al., 2005; Schuler et al., 2003; Wang et al., 1999). These synthetic peptides are well defined and are specific to certain type of tumors. They are short, immunogenic peptide sequences that can be synthesized with relative ease. Furthermore, since DCs are primed to present only one or a few specific epitopes, the risk of inducing autoimmune disease in patients is low (**Figure 1.12**). However, low availability of peptide-receptive MHC II on the surface of immature DCs attenuates the immune response and is a challenge in this common approach.

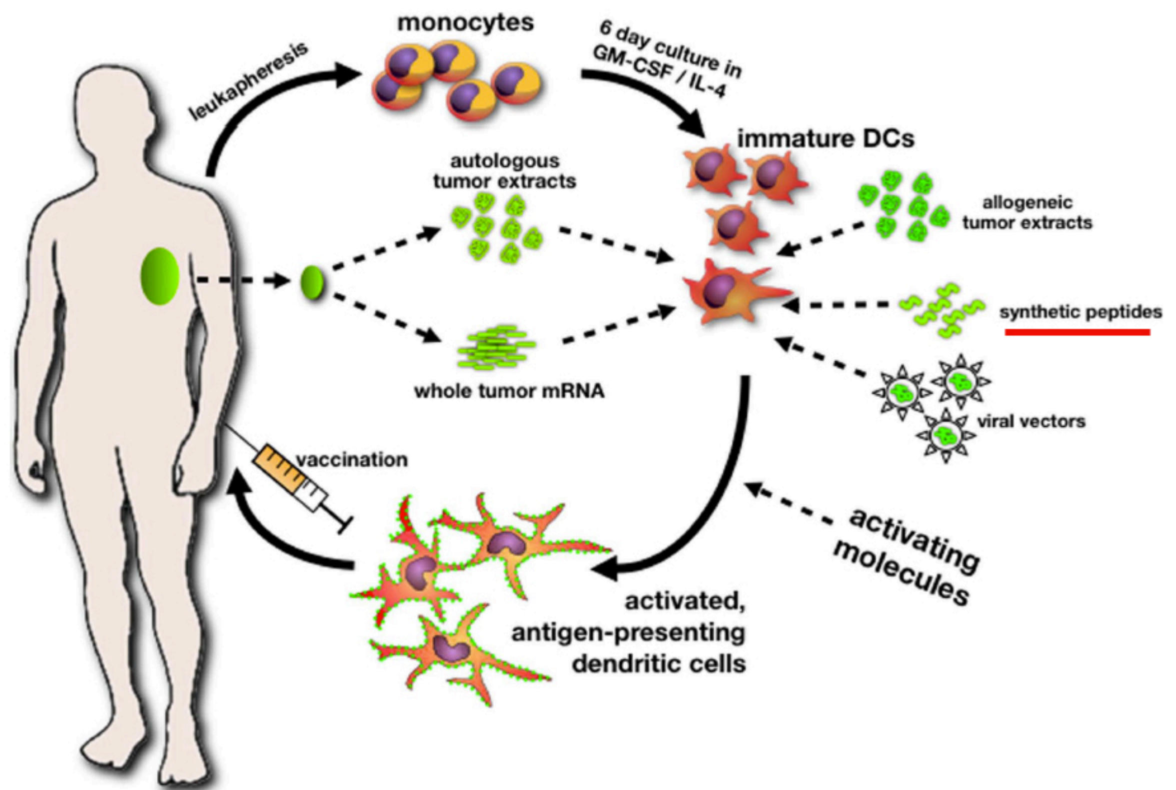


Figure 1.12 - Production of autologous DCs vaccines for cancer immunotherapy: Monocytes, harvested from a leukapheresis, are differentiated *in vitro* into immature dendritic cells by GM-CSF and IL-4 treatment. Immature DCs can subsequently be loaded with tumor-derived antigens using different approaches and most commonly pulsed by synthetic peptides (underlined with red line). A crucial final step in the whole process is the activation/maturation of DCs, typically using Toll-like receptor ligands and/or a cocktail of activating cytokines. The resulting activated, tumor antigen-presenting DCs constitute the vaccine. After injection, DCs migrate to draining lymph nodes and activate tumor-specific cytotoxic T-cells. (Adapted from Surmont VF 2011 Frontiers in Oncology).

Another challenge in peptide-based DC vaccines is the existence of type B conformers. Exogenous peptide pulsing on DCs in absence of functional DM on cell surface of APCs leads to the formation of type B conformers (Z Pu et al., 2004). In intracellular antigen presentation pathway by professional APCs, DM edits conformers in MIIC and remove type B conformers (Z Pu et al., 2004). Therefore, APCs in tumor microenvironment does

not present type B conformers to T cells. Administration of peptide-pulsed DCs vaccines with type B conformers on them would provoke type B T cells that are not tumor specific and divert the immune response against tumor.

1.4.5 Antigen loading on DCs

Loading MHC I and MHC II molecules on DCs with peptides derived from defined antigens is the most commonly used strategy for DC vaccination (Wang RF et al. 1999; Gilboa 1999; Nestle FO et al. 2005; Schuler G et al. 2003). In most clinical DC vaccination studies synthetic MHC I-binding peptides have been used (Fay et al., 2006; Thurner et al., 1999; de Vries et al., 2003), with class II-binding peptides being added in some case (Schuler-Thurner et al. 2002; Schadendorf et al., 2006). It has been shown that in monocyte derived DCs, only mDCs but not imDCs could be sufficiently loaded with exogenous class I-restricted peptides (Dieckmann et al., 2005). Primary stimulation with peptide-loaded-imDCs even down-regulated antigen-specific T cell responses (Dieckmann et al., 2005). In contrast to MHC I, much higher levels and longer half-lives of peptide-MHC II complexes were obtainable upon loading of imDCs with exogenous peptide (Dieckmann et al., 2005). However, mDCs significantly induced more T_h1 responses than imDCs (Dieckmann et al., 2005). Although simultaneous loading of several competitive peptides for the same class I or II molecule reduces the activation of their specific T cell clones, there is still significant stimulation of related T cells (Dieckmann et al., 2005). While simultaneous loading of several peptides on DCs and their presentation to T cells is possible, priming in this condition inhibits T cell activation (Dieckmann et al., 2005).

Instead of MHC-binding peptides, whole antigenic proteins can be used. The DC processes the protein into peptides, which has the advantage that multiple epitopes are presented in both MHC I and II and that there is no HLA-restriction (Lesterhuis et al., 2008). However, there are a few clinically grade available recombinant proteins (Gilboa, 2007). Autologous or allogeneic tumor cell lysates have also been used as a source of antigens (Nestle et al. 1998; Ridolfi et al., 2006; Berard et al., 2000; Salcedo et al., 2006). Application of tumor cell lysate has several advantages including, the antigen expression by the tumor does not need to be defined and a wide array of both MHC I and II epitopes are presented including tumor-unique antigens (Lesterhuis et al., 2008). Drawbacks of this

approach are the presentation of auto-antigens and the requirement of a sufficient volume of tumor tissue (Lesterhuis et al., 2008).

Transfection of DC with RNA is a more recent antigen loading approach (Sullenger et al., 2002). The antigen loading is either with tumor-derived RNA or synthetic RNA encoding specific tumor associated antigens (Kyte et al., 2005; Schaft et al., 2005). A benefit of this technique is presentation of several MHC I and MHC II epitopes, depending on the presence of lysosomal and endosomal targeting sequences (Bonehill et al., 2004). It also may lead to more prolonged antigen presentation as compared to peptide-loading on DCs (Laverman et al., 2006).

1.4.6 *In vivo* targeting of DCs

Antigens can be directly delivered to DCs *in vivo* using chimeric proteins that are comprised of an antibody that is specific for a DC receptor fused to a selected antigen. This would bypass the need for laborious ex vivo preparation of DCs (Tacken et al., 2007).

Ralph Steinman and colleagues demonstrated that the specific targeting of antigens to DCs *in vivo* elicits potent antigen-specific $CD4^+$ and $CD8^+$ T cell-mediated immunity (L. Bonifaz et al., 2002; L. C. Bonifaz et al., 2004; Hawiger et al., 2001). The induction of immunity also requires DC maturation, otherwise this strategy induces antigen-specific tolerance (Mahnke et al., 2003; L. C. Bonifaz et al., 2004; Hawiger et al., 2001). The use of vaccines that target surface molecules that are expressed on different subsets of DCs, demonstrated that distinct subsets of DCs elicit distinct immune responses (Dudziak et al., 2007). $CD8^+$ DCs that express the cell-surface protein CD205, presented the delivered antigens in the context of both MHC class I and MHC class II molecules; whereas, $CD8^-$ DCs, which express the DC marker DCIR2, are specialized for antigen presentation on MHC class II molecules (Dudziak et al., 2007). Furthermore, targeting antigen to these distinct receptors on DCs leads to the generation of T cell-mediated responses through independent pathways. $CD205^+$ DCs generate a Th1 immune response in an IL-12-independent, CD70-dependent mechanism; whereas, CD8- DCIR2+ DCs generate Th1 immune responses through the classical IL-12 pathway (Soares et al., 2007).

Another promising carrier for weak tumor antigens is superantigen. Superantigens (SAgs) are small microbial proteins that cross-link MHC II and TCR outside peptide-binding groove and cause strong T cell stimulation (H. Li et al., 1998; Malchiodi et al., 1995; Moza et al., 2007; Sundberg et al., 2002). In contrast to conventional antigen, they bypass the need for antigen processing and bind to a wide range of MHC II with no haplotype restriction. SAgs (SEA and SEB) have been used as adjuvant in DC-vaccine and pulsed on DCs with tumor peptides ($OVA_{257-264}$ [SIINFEKL]) (Kato et al., 2011). Administration of these DCs, strongly induced peptide-specific CD8+ T cell response against tumor (Kato et al., 2011). To avoid non-specific T cell stimulation, the mutant forms of SAgs have been used as vaccine carrier and conjugated to tumor-antigens (Radcliff et al., 2012). These mutant-SAgs have lost their T cell mutagenic function but kept their MHC II binding feature. Hybrid of these SAgs with tumor-peptides targets them to MHC II-bearing cells.

Better understanding of interaction between SAgs and antigen presentation pathway and to know which components of this pathway and how they affect the binding of SAgs to MHC II is an attractive field with potential applications in vaccine approaches.

1.5 Superantigens

Superantigens (SAgs) are conserved microbial proteins and are among the most powerful T cell mitogens (Proft & Fraser, 2003). SAgs are produced by many different pathogens, including bacteria, mycoplasmas, and viruses, and the responses they provoke are presumably helpful to the pathogen rather than the host. They cross-link TCR and MHC II outside peptide binding groove (on lateral surface of MHC II and Vβ of TCR) so, bypass the need of conventional antigens for processing and recognition by specific MHC and TCR (H. Li et al., 1998; Malchiodi et al., 1995; Moza et al., 2007; Sundberg et al., 2002) (**Figure 1.13)**.

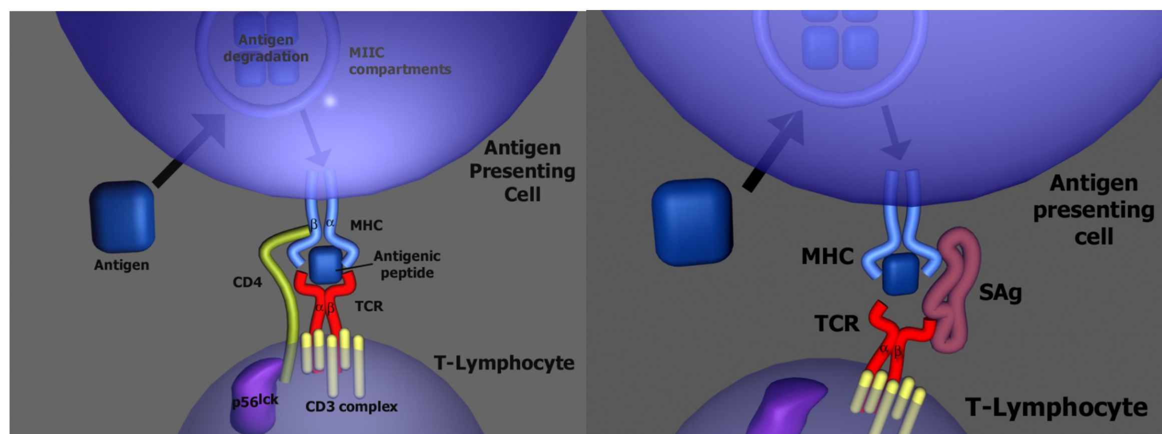


Figure 1.13 - SAg binding to MHC II and TCR: SAgs bind to lateral surface of MHC II (right) and TCR, outside peptide-binding groove and bypass the need for processing of conventional antigens (left)- (figure adapted from Jacquess Thibodeau)

The term 'superantigen' was first coined by the group of Philippa Marrack and John Kappler and described as a group of proteins with extremely high potency to stimulate human and other mammalian (mice) CD4 and CD8 T cells (White et al., 1989). Such a strong T cell response was first observed in mixed lymphocyte reactions using lymphocytes from strains of mice which were MHC identical but otherwise genetically distinct (Festenstein, 1973). The antigens stimulating this reaction were originally named minor lymphocyte stimulating (Mls) antigens, and thought to be functionally similar to the MHC molecules. However, now we know that they are of viral origin. The Mls antigens found in these mice strains are encoded by murine mammary tumor virus (MMTV) proviral DNA that had been integrated into the germline (Dyson et al., 1991; Frankel et al., 1991;

Woodland et al., 1991).

1.5.1 MMTV

Mouse mammary tumor virus (MMTV), was first characterized in the 1930s as a milk-transmitted agent associated with mammary tumors in mice (Bittner, 1936) and thus has long been used as an *in vivo* model for the study of human breast cancer (Callahan & Smith, 2008). The MMTV is a retrovirus with 9 kb in length and like all retroviruses, is flanked by 5' and 3' long terminal repeats (LTRs), which in the case of MMTV, are exceptionally long (approximately 1.3 kb). This is because the MMTV 3' LTR encodes the viral superantigen (vSAg) (Y. Choi, Kappler et al., 1991; Korman et al., 1992; Pullen et al., 1992). The protein encoded by this vSAg gene is a type II transmembrane molecule of about 45 kDa, with the C terminus outside the cell membrane and the N terminus in the cytoplasm (Golovkina et al., 1993; Launois et al., 1997; Pullen et al., 1992). In addition to the SAg coding region, there are a number of transcription factor binding sites in the LTR that determine tissue-specific, as well as glucocorticoid/progesterone-regulated virus expression (Mink et al., 1992; Q. Zhu et al., 2004). Because they direct high level, hormone regulated expression in mammary epithelial cells, the MMTV LTRs have been extensively used to drive oncogene expression in transgenic mice as a means of creating mouse models of breast cancer (Callahan et al., 2008).

MMTV binds to transferrin receptor 1 (TfR1) on the cell surface and is internalized and co-traffics with TfR1 to late endosomes with acidic pH (Ross et al., 2002; E. Wang et al., 2008). After uncoating, the viral genome is reverse-transcribed, transported to the nucleus, and the provirus integrates into the genome (Frankel et al., 1991; Woodland et al., 1991). The vSAg protein is translated from two different transcripts. The first transcript initiates in the 5' LTR and uses the same splice donor as the env mRNA. The second transcript uses a promoter and splice donor in env. Both transcripts use a splice acceptor just upstream of the open reading frame in the 3' LTR (Arroyo et al., 1997; Reuss et al., 1995; L. Xu et al, 1997).

MMTV is transmitted from infected mothers to pups through nursing, most likely as cell-free virus which is present at very high levels in milk (Golovkina et al., 1992). Susceptible strains that acquire exogenous MMTV through milk can be freed of the virus

by foster-nursing on uninfected mothers. In addition to the exogenous form of MMTV, all commonly used inbred mice as well as many wild mice inherit endogenous copies of MMTV which are thought to have entered the mouse genome between 10–20 million years ago (Baillie et al., 2004). The vast majority of endogenous MMTVs do not produce infectious virus due to deletions or mutations in the proviral genome.

vSAgs encoded by endogenous MMTVs cause the deletion of cognate T cells during shaping of the immune repertoire, while those encoded by exogenous virus produce a slower but none-the-less almost complete deletion of such lymphocytes (Ignatowicz et al., 1992). This results in mice that are immune to infection by exogenous MMTVs with the same SAgs because they lack responding T cells (Golovkina et al., 1993). The endogenous proviral DNAs are named Mtv loci and are numbered so far from 1 to 54 (with several gaps) (Acha-Orbea et al., 1995; Lee et al., 2011). The average number of Mtv loci for common laboratory mice is 2 to 8 (Kozak et al., 1987).

1.5.2 Bacterial SAgs

The first bacterial SAg was isolated in the late 1960s by Bergdoll and coworkers as a secreted toxin of S. aureus and was named staphylococcal enterotoxins A (SEA) for its potent enterotoxic properties (Chu et al., 1966). Over 30 bSAgs have now been described including staphylococcal toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SE) A to R, the streptococcal pyrogenic exotoxins (SPE) A, C, G–M and streptococcal mitogenic exotoxin Z (Fraser & Proft 2008).

The staphylococcal enterotoxins (SE) are the causative agent in staphylococcal food poisoning and induce vomiting and diarrhea within 1–2 h following ingestion. More than 18 different SEs have been described in the literature and all are potent T cell mitogens with half maximum stimulation values as low as 0.1 pg/ml (Proft & Fraser 2003). The S. aureus toxic shock syndrome toxin (TSST) is the causative agent of toxic shock syndrome (Bonventre et al., 1993). Twelve SAgs have been identified in Group A Streptococci (GAS), predominantly but not exclusively produced by S. pyogenes (Proft & Fraser 2003). These are the streptococcal pyrogenic exotoxins (SPEs) A, C, G-M, the streptococcal superantigen (SSA) and the streptococcal mitogenic exotoxin (SMEZ) 1 and 2.

These toxins are ribosomally synthesized proteins of relatively similar molecular weight (approx. 22–28 kDa). Although staphylococcal and streptococcal SAgs generally share only

a low percentage of sequence homology, they all seem to share a similar three-dimensional fold based on crystallographic studies (H. Li et al., 1999). Each characterized structure contains an N-terminal β-barrel oligosaccharide/oligonucleotide-binding fold domain, and a C-terminal β-sheet structure known as the β-grasp domain, with the two domains being joined by a central α-helix (H. Li et al., 1999).

The staphylococcal enterotoxins constitute a family of eight single-chain polypeptides (26-28 kDa, 228-239 amino acid residues) with a typical disulfide loop (Müller-Alouf et al., 2001). TSST-1 (22 kDA, 194 amino acid residues) lacks disulfide loop and shows no significant homology to any of the other staphylococcal SAgs, despite their similar biological activities (Acharya et al., 1994).

1.5.2.1 Other bSAgs

Clostridium perfringens enterotoxin (CPET) involved in food poisoning has been reported to behave as a SAg inducing the proliferation of human T cells expressing Vβ6-9 and Vβ22 motifs on their TCRs (Bowness et al., 1992).

The only Gram negative bacteria known so far to produce SAg is *Yersinia pseudotuberculosis*, a pathogenic bacterium responsible for enteritis and mesenteric lymphadenitis. The SAg produced by *Y. pseudotuberculosis,* designated YPM for *Y. pseudotuberculosis-derived mitogen,* is a 14.5-kDa protein (131 amino acid residues) which selectively stimulates human T cells bearing Vβ3, Vβ9, Vβ13.1 and Vβ13.2 TCR variable regions (Ito et al., 1995; Miyoshi-Akiyama et al., 1995). In mice, YPM expands T-cells with Vβ7 and Vβ8 TCR variable regions (Miyoshi-Akiyama et al., 1997).

Mycoplasma arthritidis, a wall-less bacterium, which indices chronic form of arthritis in rodent resembling human rheumatoid arthritis, produces a 25-kDa (213 amino acid residues) SAg designated MAM (*M. arthritidis mitogen*). MAM, which was first described early eighties (Cole et al., 1981), strongly activates murine T cells bearing Vβ6 and Vβ8 TCR variable regions. MAM also stimulates human T cell through Vβ17 region but to a less extent compared to its activation of murine T cell or the human T cell activation by staphylococcal enterotoxins (Cole et al., 1981).

The binding of MAM to MHC II is Zn dependent and cytokine production by monocytes requires a dimerization of MHC II as for SEA (Bernatchez et al., 1997; Zhao et al., 2004). Although MAM binds preferentially to murine H2-E and its human MHC homologue, HLA-DR (Cole et al., 1990), it also interacts with H2-A and HLA-DQ (Cole et al., 1997).

1.5.2.2 Binding of bSAgs to MHC II

MHC class II binding occurs at two conserved sites away from Ag-presenting regions of the molecule—a high-affinity interaction with the class II β-chain and a low-affinity interaction with the class II α-chain (J. Kim et al., 1994; Petersson et al., 2001; Wen at al., 1996).

bSAgs based on their interaction with MHC II have been classified into three main groups (Fraser & Proft 2008). One group binds the α-chain with (e.g. TSST-1) (J. Kim et al., 1994; Wen et al., 1996) or without contact with antigenic peptide (e.g. SEB) (Jardetzky et al., 1994) **(Figure 1.14)**. This group binds MHC II α-chain through an N-terminal, low affinity binding.

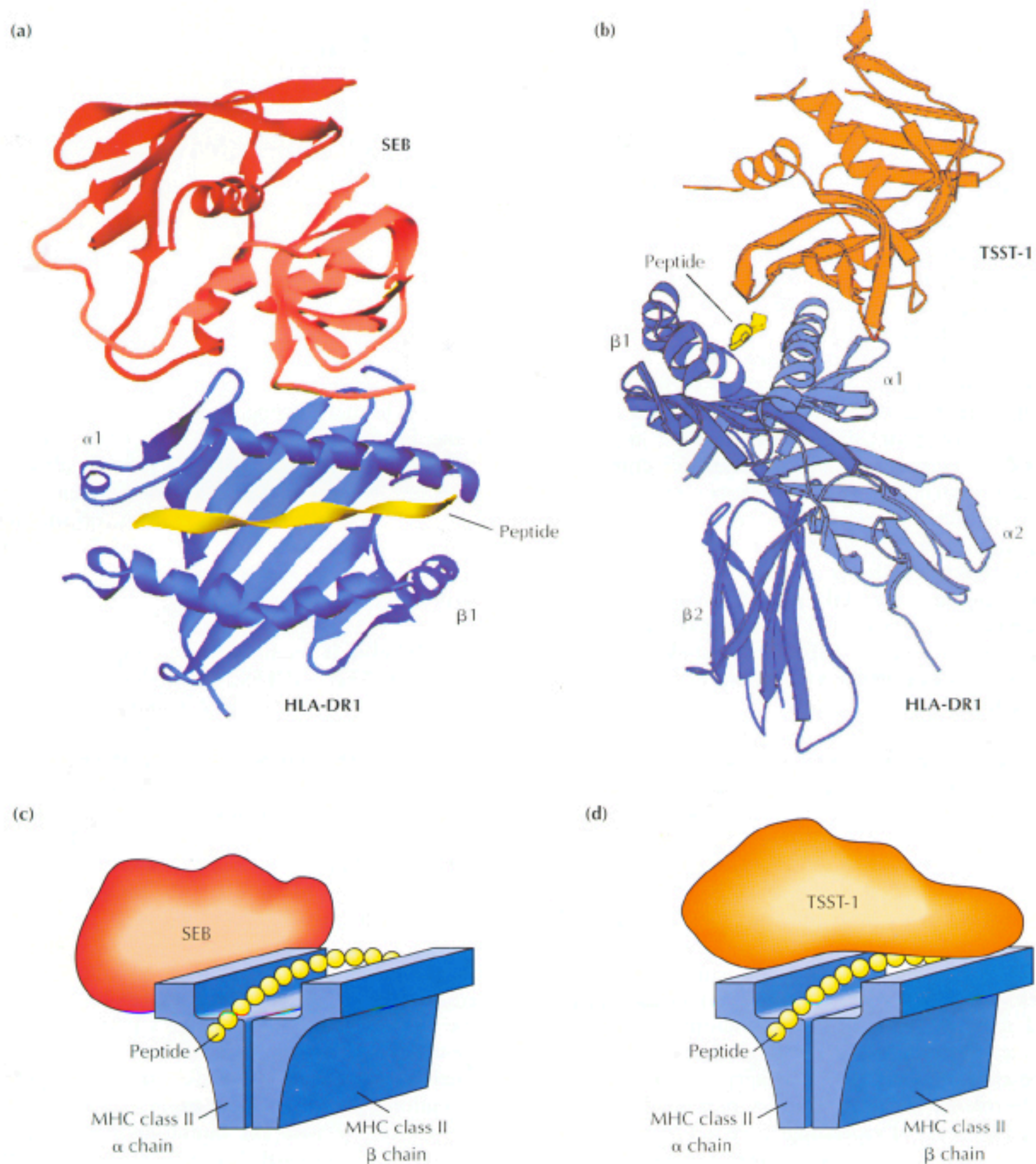


Figure 1.14 - Binding of SEB and TSST-1 to MHC II: SEB and TSST-1 both bind to α-chain of MHC II but TSST-1 bends over the binding-groove and contacts peptide - **(a)** Crystal structure of the SEB-DR1 complex. **(b)** Crystal structure of the TSST-1-DR1 complex. **(c)** Schematic structure of the SEB-DR1 complex, showing the exclusive binding of SEB to the α chain of DR1, primarily off one edge of the peptide-binding groove. **(d)** Schematic structure of the TSST-1-DR1 complex, showing how TSST-1 bends over the binding-groove and contacts part of the bound peptide. Adapted from (Hsu & Huber, 1995).

Members of the second group bind the MHC II β-chain (e.g., SPE-C and SEH) (Y. Li et al., 2001; Petersson et al., 2001). The interaction with the β-chain is of high-affinity and zinc-dependent (Y. Li et al., 2001; Petersson et al., 2001). The third group binds to both sites and cross-links MHC class II (e.g., SEA, SED and SEE) (Abrahmsen et al., 1995; Kozono et al., 1995; Y. Li et al., 2001; Petersson et al., 2001). These SAgs appear to bind to both the α-chain and the β-chain of MHC class II using their N- and C-terminal domains, respectively (Y. Li et al., 2001; Petersson et al., 2001) (**Figure 1.15)**.

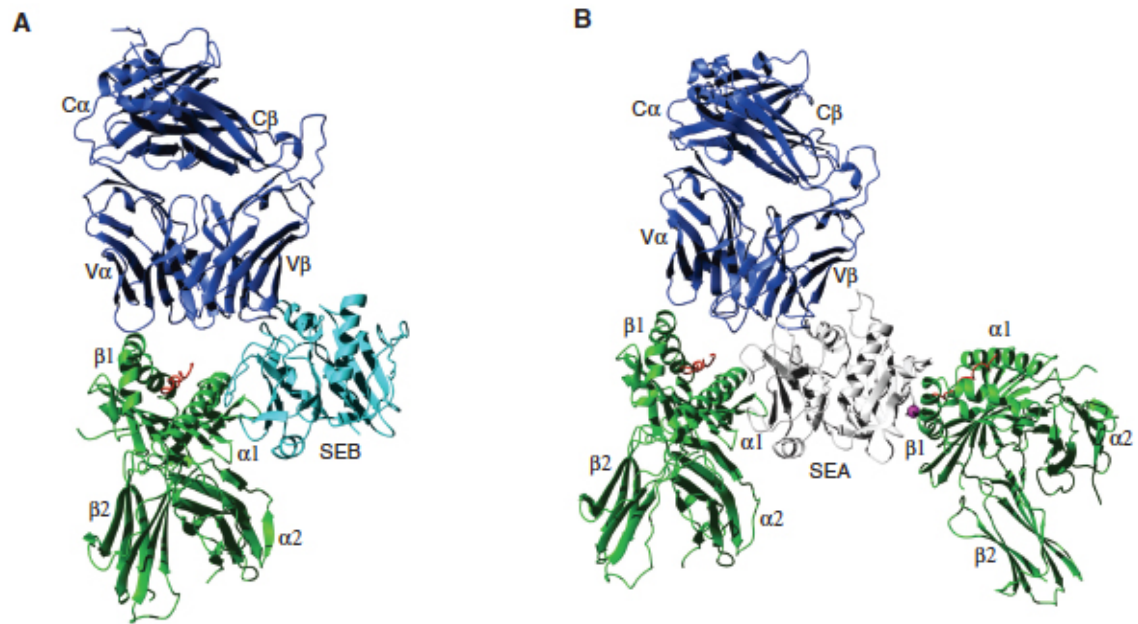


Figure 1.15 - SEA cross-link two MHC II: (**A**) SEB binds to α-chain of MHC II and β-chain of TCR. (**B**) SEA binds to β-chain of TCR but is able to bind to both α and β-chains of MHC II and cross-links two MHC II molecules - Adapted from (Petersson et al., 2004).

It has been shown that the affinity of SEA and TSST-1, but not SEB, is modulated by MHC class II-bound peptide (Kozono et al., 1995; Hogan et al., 2001; McCormick et al., 2001). Using soluble DR1 molecules that were covalently linked to a peptide, Kozono et al. have shown that N-terminal end of the peptide directly affected SEA binding to MHC II (Kozono et al., 1995). A peptide derived from hemagglutinin, enhanced binding of SEA to MHC II molecule (Wen et al., 1996). Wen *et al.* have shown that loading of a special peptide on DM deficient cell line, T2-I-A^b, increases presentation of TSST-1 (Wen et al., 1997). Addressing the question that whether the increase in TSST-1 presentation is just

limited to that special peptide or extend to other non-identified peptides, Hogan et al. found two other peptides that improved presentation of TSST-1 (Hogan et al., 2001). They also demonstrated that presentation of SEA, like TSST-1, is peptide dependent whereas SEB presentation is peptide independent.

1.5.2.3 bSAgs binding to TCR

While a conventional peptide antigen requires a very specific interaction with the third hypervariable region of the TCR, the Vβ chain of the TCR is sufficient for recognition of a superantigen (White et al., 1989). TCR binding is, in most cases, through the Vβ region and independently of the CDR3 sequence (Kappler et al., 1989), although some SAgs such as SEH, interact with the TCR Vα region (Petersson et al., 2003). SAgs vary in their TCR Vβ specificity and this is determined primarily by interactions with the TCR Vβ CDR2 loop. Some SAgs are more specific for TCR Vβ than others as a result of interactions with other hyper-variable regions of the TCR Vβ region; CDR1, CDR3 and HV4. For example, TSST-1 is highly specific for TCR Vβ2 whereas SEB and SEA activate several structurally related TCR Vβ types (TCR Vβ1, 3.2, 12, 13.2, 14, 6.4, 15, 17, 20 and TCR Vβ1, 5.3, 6.3-4, 7.3-4, 9.1, 16, 18, 21, 22, 23 respectively) particularly at higher concentrations (Alouf et al., 2003; Petersson et al. 2004; Llewelyn et al. 2006).

The immediate outcome of the T cell recognition of bSAgs is the large expansion of T cells with the resulting massive release of cytokines by the host, which are believed to be responsible for the most severe consequences of SAg-associated diseases (McCormick et al., 2001). Furthermore, following the initial expansion, the host may become unresponsive to the SAg by several mechanisms including T cell anergy and activation-induced clonal T cell deletion (White et al., 1989).

1.5.2.4 Benefits of bSAgs for Bacteria

After more than two decades of intensive research, it is still largely unknown, why bacteria produce toxins that stimulate the immune system. Currently, there are 40 staphylococcal and streptococcal SAgs that, despite their often minor sequence homology, share a common protein fold and target the same host cell receptors, MHC class II and

TCR. The best-established biologic relevance of SAgs is the non-antigen restricted activation of up to 50% of the whole T cell repertoire, which causes toxic shock syndrome (TSS) (Y. Choi et al., 1990; Todd et al., 1978).

A possible advantage conferred by SAg production might involve the corruption of the host immune system. SAgs interfere with the adaptive immune response driving profound Th1-type responses characterized by high levels of type 1 cytokines, such as IL-2, IFN-γ and TNF-α, and nonspecific T cell proliferation. It is possible that the prime function of SAgs is to suppress a Th2-type response thereby preventing the production of high-affinity cytotoxic antibodies by promoting a Th1-mediated response.

Another possible mechanism of how SAgs corrupt the immune response might be through their ability to cause T cell anergy. Systemic stimulation of T cells by SAgs results in a nonresponsive (anergic) state, which can be reversed in vitro by application of IL-2. Since anergic T cells also fail to produce IL-2 (O'Hehir & Lamb, 1990), SAg stimulation might also lead to local IL-2 deficiency, which in turn could limit the expansion of antigen-specific T cells (Lavoie et al., 1999).

In addition to MHC class II and TCR, Arad et al. found that SEB possesses a third binding site for CD28 (Arad G et al. 2011). The authors showed that SEB binds to a soluble form of CD28 through a relatively conserved region that is distinct from both the MHC class II binding site and the TCR binding site (Arad G et al. 2011). They have mutated the predicted CD28 binding site on SEB and showed that this mutant fails to stimulate secretion of IL-2, TNF-α, and INF-γ in T cells. SEB is also shown to bind to cells transfected with CD28 that lack either MHC class II or TCR. Importantly the authors show that the same CD28 binding site can be found on other SAgs such as SEA and TSST and the synthetic peptides that inhibit SEB also inhibit SEA- and TSST-induced cytokine production (Arad G et al. 2011; G Arad et al., 2000). This suggests that CD28 is a general target for all bacterial SAgs. On the opposing side of the complex, the SEB binding site on CD28 is mapped to a region needed for homo-dimerization that is distinct from the B7 binding site. CD28 may therefore be bound by both SEB and B7 in the resulting membrane complex.

Early studies of T cell responses to bacterial superantigens described proliferation

followed by anergy (Gaus et al., 1994; Rellahan et al., 1990). Recently, it has been shown that superantigens expand human FOXP3^{+} T cells in a dose-dependent, Vβ-specific manner and these cells arise from CD25^{-} FOXP3^{-} cells rather than through expansion of natural regulatory T cells (nTregs) (Taylor et al., 2010). More recently, the Fraser group have shown that bacterial superantigens are also potent inducers of human FOXP3^{+} CTLA-4^{+} regulatory CD8^{+} T cells (Taylor et al., 2012). They used SEA, TSST-1 and SPE-A and K/L at concentrations below 1 ng/ml. All bSAgs trigger concentration- dependent T-cell receptor Vβ-specific expression of CD25 and FOXP3 on CD8^{+} T cells. This effect was independent of CD4^{+} T-cell help but required APCs for maximum effect. These findings indicate that expansion of regulatory T cells may be a feature of acute bacterial infections contributing to immune evasion by the microbe.

1.6 Adenoviral transduction

1.6.1 Viral gene therapy

Gene therapy is the process of introducing genetic material into a patient's cells in order to achieve a therapeutic effect. Since the inception of this approach in 1989 by Rosenberg et al. (Rosenberg et al., 1990) the basic goal is to alter the transcription products of a cell by inserting new DNA, but can also involve insertion of RNA sequences or deactivation of existing genes. Many novel methods have been proposed or even used for getting DNA into the target cell, but viruses are the most common (Edelstein et al., 2007). One of the advantages of viral vectors over other vectors is their higher transfection rate (Gray et al., 2008). This means that fewer vector particles are needed to achieve the same results and reducing the production time. All viral vectors are recombinant which means target genes add to or replace the genes of viruses (Edelstein et al., 2007).

Retroviruses and adenoviruses are among the most commonly used viral vectors in trials (Tani et al., 2011). The retrovirus introduces the gene it carries into the target cell genome while the adenovirus introduces the gene into the target cell nucleus without incorporating it into the target cell genome. A retrovirus is a RNA virus that integrates itself into its host's genome during its course of infection. After the virus gains entry into the host cell, its reverse transcriptase enzyme will produce a DNA copy of viral positive mRNA, and then the viral integrase enzyme, integrate the DNA copy into the host genome (Goff, 2007). To introduce specific gene material into the target cell, the intended gene is inserted into the retroviral gene sequence. A defective virus is produces and used to infect the target cell with the modified retrovirus. The retrovirus would then introduce the gene it carries into the target cell genome. Once the intended gene is integrated into the host cell's genome, all offspring of those cells are expected to contain the inserted gene. This property of retroviruses made it a preferred vector for gene therapy treatments that require long-term, sustained gene expression. However, as retroviruses can insert the gene in any position in the host's genome, it could disrupt a gene if inserted in the middle of a certain gene. Retroviral integration in proximity of some pro-oncogenes could also induce unleashed proliferation and ultimately cancer (Bushman, 2007). Another drawback of retroviral vectors is that they target dividing cells and make them non-applicable for DCs vaccines (Miller et al., 1990). Lentiviral vectors are developed from lentiviruses, a subset of

retrovirus family, and are able to transduce non-dividing cells with preferential targeting of myeloid cells (Durand et al., 2011; Naldini et al., 1996).

While retroviral and lentiviral vectors would result in the integration of the gene they carry into the host genome with the risk of insertional mutagenesis, an adenovirus would introduce the gene into the host cell's nucleus without incorporating the gene into the host genome. As a result, the introduced gene would be transcribed and expressed on the host cell for a certain period of time, but will not be present in the genome of the host cell's offspring after the host cell undergoes replication. This property of adenovirus makes it useful in certain applications of gene therapy (Douglas, 2007).

1.6.1.1 Adenovirus

Adenovirus (Ad) was first discovered in 1953, isolated from cultures of human adipose tissue (adenoid tissue) (ROWE et al., 1953). This virus has been classified into six species (A–F) that infect humans, and these species are subdivided into over 51 infective serotypes (Russell, 2009). Ad2 and Ad5 viruses of species C are the most effective for creating viral vectors for use in gene therapy (Kovesdi et al., 1997). The Ad capsid is a nonenveloped, icosahedral protein shell that surrounds the DNA-containing core. The capsid primarily consists of three proteins; hexon, penton base and fibre-knob (Campos et al., 2007) (**Figure 1.16**). The bulk of Ad particles are ~900 Å in diameter (van Oostrum et al., 1985). The genome of the Ad is a linear, double-stranded DNA (dsDNA) ranging from 26 to 40 kb in length (Campos & Barry, 2007). This linear form is organized into a compact, nucleosome-like structure within the viral capsid and is known to have inverted terminal repeat (ITR) sequences on each end of the strand (Douglas, 2007; Shenk, 1996). The viral genome comprises two major transcription regions, termed the early region and the late region. The early region of the genome contains four important transcription units (E1, E2, E3, and E4) (Douglas, 2007; Shenk, 1996).

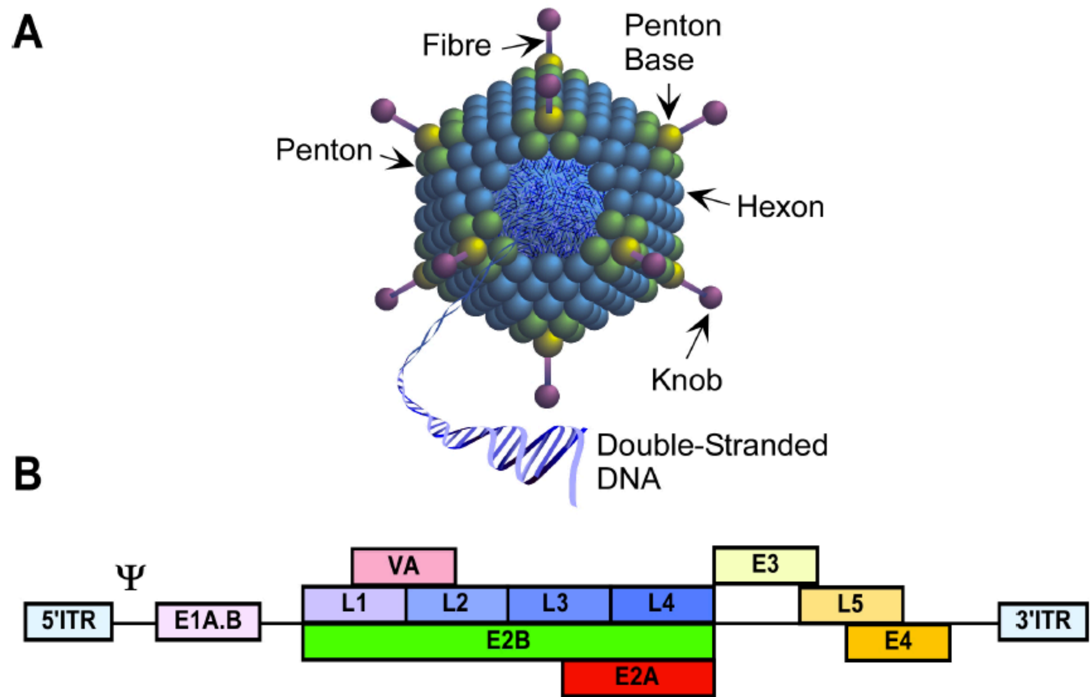


Figure 1.16 – Adenovirus particle: A) structural fiture of adenovirus B) genomic organization of wild type adenovirus – Adopted from (Al-Allaf et al., 2010).

The knob domain of the viral capsid has a high affinity for the coxsackievirus and adenovirus receptors (CAR), which are expressed on a variety of cells in the human body (Bergelson et al., 1997; Tomko et al., 1997). When the virus finds a host cell, the process of binding and internalization begins. The affinity between the fibrous knob and the CAR is heightened by the interaction of the penton base protein with secondary cellular receptors (α_v integrins). Binding of penton to α_v integrins stimulates endocytosis of viral particles (Wickham et al., 1993). The virus then passes through the cell membrane via receptor-mediated endocytosis, the virion is released, and the genome escapes the protein capsid and makes its way into the nucleus.

Transcription of viral DNA begins when the genome enters the host cell nucleus. E1A transcription unit is transcribed, followed quickly by the E1B unit. Together, these two units help to prepare the genome for further transcription, shift the host cell into the S phase of replication, and inhibit apoptosis of the host cell. The E2 unit, the next to transcribe, encodes for DNA polymerase, a preterminal protein, and a DNA-binding protein, all of which are necessary for DNA replication. This process is followed by the transcription of

the E3 unit, which inhibits the host cell from responding to the viral invasion. Finally, the E4 unit is transcribed to produce a variety of proteins required for DNA replication. Viral DNA replication begins at the origins of replication in the ITRs on either end of the viral genome, and the terminal protein at each end of the chromosome acts as the DNA primer (Shenk, 1996; Shenk & Flint, 1991).

1.6.1.2 Adenoviral vectors

The *first-generation* of ad vectors were based on Ads 2 and 5 of species C and had the E1 region of the genome deleted to allow more genomic space for foreign DNA (Berkner 1988; Kovesdi et al., 1997). The El genes are necessary for expression of E2 and late genes that are required for Ad DNA synthesis, capsid protein expression, and viral replication and also encodes the oncogenic transforming functions of the virus. The E1 deleted vectors become replication-defective and packaging cell lines (e.g. 293), which complement the deleted E1 gene, are used for in vitro propagation and production of recombinant ad vectors. The 293 cell line is a human embryonic kidney derived cell that has been transformed by the adenovirus E1 gene (Graham et al., 1977). Removal of the E1 region provides approximately 5.1 kb for insertion of therapeutic gene (Bett et al., 1993). Ad vectors can be grown to extremely high titers in the HEK293 cell line, typically between 10^3 - 10^4 viral particles (VP) per cell and the final concentrations could reach 10^{13} VP/ml, after purification by Cesium chloride (CsCl) density gradient ultracentrifugation.

Many of the *first-generation* vectors also contain a deletion in the E3 region (Massie et al., 1998). The E3 gene encodes proteins involved in evading host immunity and is dispensable. These deletions provide space for up to 8.2 kb for transgene insertion (McConnell et al., 2004). Because expression of E3 genes dampens host immune response, it has been suggested that E3 expression may be beneficial *in vivo* and increases persistence and expression of the transgene (Ilan et al., 1997). Although the first-generation of Ad vectors has been proven to be very promising but the main drawback is the emergence of replication efficient virus in the stocks of replication deficient viruses. During production of viral stocks, recombination between the E1 region sequences of the complementing cell line and the recombinant virus can produce replication competent viruses with functional E1 genes (Lochmüller et al., 1994). The other and more troublesome problem associated

with the *first-generation* of ad vectors is stimulation of cellular immune response, which attacks and destroys transduced cells that are expressing therapeutic transgenes (Dai et al., 1995; Yang et al., 1994a; Yang et al., 1995). It is theorized that the stimulation of immune response is due to low levels of replication that occur even in the absence of E1 gene and this idea is supported by findings that genome replication and late gene expression can occur from E1-deleted vectors *in vivo* (Yang et al., 1994b; Yang et al., 1994a).

In the *second generation* of Ad vectors, to prevent the immune response generated by low-level replication in E1-deleted viruses, multiple genes were deleted to inhibit viral gene expression more effectively. Primarily this second generation of Ad vectors has been constructed by the removal of E2 and E4 regions that also provide larger space for transgene insertion. The main drawback for construction of these multiple genes deleted vectors is the need for establishment of cell lines expressing the missing genes functions in trans. For example for E2 deleted vectors, cell lines that stably express the viral DNA polymerase, pre-terminal protein and the single stranded DNA-binding protein alone or all together have been established (Amalfitano et al., 1998; Amalfitano et al., 1997; Gorziglia et al., 1996; Schaack et al., 1995). Despite the time consuming process, the vectors produced in these cell lines are less likely to undergo recombination and create replication efficient viruses.

The *third generation* of Ad vectors perhaps is the most promising Ad vectors for long-term expression of therapeutic genes in the absence of complicating effects of viral genes. In these vectors, that are named helper-dependent Ad (HD-Ad) or "gutless" vectors, to avoid stimulation of unwanted immune response, all of the viral structural genes are deleted and only the cis-acting elements are left, i.e., the packaging signal (ψ) and the two ITRs (H. H. Chen et al., 1997; Clemens et al., 1996) (Figure 1.15.). This approach completely eliminates the production of viral proteins in transduced cells and elicits a negligible CTL response therefore is capable of producing long-term gene expression (Morral et al., 1998; Morsy et al., 1998). These vectors have up to 37 kb of cloning capacity (Mitani et al., 1995). (**Figure 1.17**)

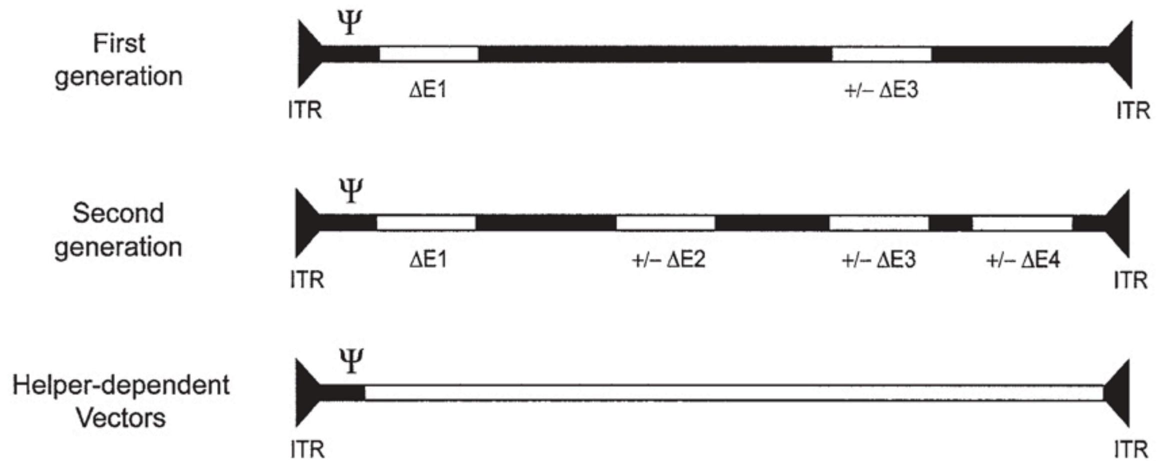


Figure 1.17 Genome structure of *first-generation, second-generation and third-generation* (helper-dependent) Ad vectors. Open boxes indicate regions that have been deleted. Adapted from (McConnell et al., 2004).

Because production of a completing cell line that provide all necessary functions for replication and assembly has not been possible, propagation of HD-Ad vectors requires the presence of a helper virus that provides trans-acting elements for replication and assembly. The main drawbacks of HD-Ad vectors are cumbersome processes for production of virus at a scale necessary for clinical use and more importantly to completely purify HD-Ad viruses from contaminating helper viruses stocks (Dormond et al., 2011; McConnell et al., 2004; Sandig et al., 2000; Steinwaerder et al., 1999). Helper viruses, even lacking their packaging signal, still can be packed and contaminate the HD-Ad stocks (Sakhuja et al., 2003).

1.7 Aims

The general objective of this thesis is improvement of dendritic cell vaccines by manipulation of MHC class II antigen presentation pathway. This thesis includes two projects: "Enforcing of HLA-DM expression on cell membrane of DCs" and "HLA-DO effect on bSAgs binding to MHC II".

1.7.1 Enforcing of HLA-DM expression on cell membrane of DCs

Although, *in vivo* targeting of DCs obviates the laborious ex vivo preparation of DCs, immature targeted DCs need maturation signal otherwise they induce T_{reg} and tolerance to inoculated antigen (Mahnke et al., 2003; L. C. Bonifaz et al., 2004; Hawiger et al., 2001). $CD8^{+}$ ($BDCA3^{+}$) subset of DCs in steady state induces $FOXP3^{+}$ T cells and targeting of antigen to this subset of DCs induces expansion of antigen specific T_{reg} (Yamazaki et al., 2008). The whole antigenic protein can also be used for loading on DCs but there are a few available clinically grade recombinant proteins (Gilboa, 2007).

Ex vivo loading of defined tumor peptides is the most common immunotherapy approach in DC vaccines (Wang RF et al. 1999; Gilboa 1999; Nestle et al. 2005; Schuler G et al. 2003) however, this approach is hampered by low number of peptide-receptive MHC II on the surface of DCs. HLA-DM (DM), in acidic endosomal compartments, grants a peptide receptive conformation to MHC II. We hypothesized that mutant DM (DM_{Y248A} or DMY) will be functional on cell surface and will generate peptide receptive MHC II and improve exogenous peptide loading.

Evidence point out that increase in peptide dose polarizes T helper response toward T_h1 (Hosken et al., 1995) while loading of synthetic CLIP peptide reverses the polarization toward T_h2 (Rohn et al., 2004). We also hypothesized that DMY by removing CLIP and improving peptide loading will drive T_h cell response toward T_h1.

It is also been shown that DM in late endosomes, edit peptide-MHC II conformational isomers (conformers) in favor of more rigid and stable conformers (type A) (Z Pu et al., 2004). Formation of type B conformers is a pitfall in exogenous peptide loading and a challenge in peptide-based vaccines (Z Pu et al., 2004). We hypothesized that DMY can perform a fine tuning in favor of type A conformers at the cell surface level.

To prepare DCs, we used the most common approach in current DC vaccines that is to prepare DCs from autologous monocytes by culturing them in the presence of a cocktail of cytokines. These monocyte derived DCs (moDCs) are a uniform population of mature DCs with enhanced expression of MHC II and costimulatory molecules. To express DMY in these moDCs, we transduced them with recombinant adenoviral vector. Natural adenoviral infection occurs in early childhood, result in immunity to subsequent adenoviral infection so, there is very low risk of self-contamination during manipulation (Tebruegge et al., 2012). This vector can infect most cell types and is well tolerated by cells (Khare et al., 2011; Sharma et al., 2009; Z.-L. Xu et al., 2005).

Better understanding of molecules that bind to MHC II has the potential for application in DCs vaccine. Superantigens (SAgs) with their strong MHC II binding ability could be one of these candidates.

1.7.2 HLA-DO effect on bSAgs binding to MHC II

Bacterial Superantigens (bSAgs) are small proteins that bind to MHC II molecules outside the peptide-binding groove. It has been shown that the MHC II-bound peptide modulates the affinity of SEA and TSST-1, but not SEB, for MHC class II molecules (Kozono et al., 1995; Hogan et al., 2001; McCormick et al., 2001). HLA-DO (H2-O in mice) modulates the peptide repertoire of MHC II molecules (van Ham et al., 2000). Qi et al. have shown that transfection of H2-O in class II+ mouse SaI sarcoma cell line inhibited the binding and presentation of SEA and SEB (L. Qi et al., 2001). We addressed the impact of HLA-DO on binding of SAgs to human MHC II molecules by using various cell lines and knocking-down gene expression using siRNAs. We also evaluated the effect of H2-O on binding of bSAgs to mice MHC II using splenocytes from H2-O-deficient and proficient mice.

CHAPTER .2: Article 1

FORCED EXPRESSION OF HLA-DM AT THE SURFACE OF DENDRITIC CELLS INCREASES LOADING OF SYNTHETIC PEPTIDES ON MHC CLASS II MOLECULES AND MODULATES T CELL RESPONSES

Abdul Mohammad Pezeshki, Marie-Hélène Côté, George Azar, Jean-Pierre Routy, Rachid Boulassel and Jacques Thibodeau

Published in Journal of Immunology

Contributions

Abdul Mohammad Pezeshki did most of the experiments and wrote the paper.

Marie-Hélène Côté and Georges A. Azar performed preliminary experiments to show that DMY is functional at the cell surface of cell lines. Marie-Hélène Côté is also contributed to Figures 1 and 2.

Jean-Pierre Routy and Mohamed-Rachid Boulassel provided PBMCs from healthy donors.

Jacques Thibodeau planed the experiments, analysed the data and wrote the paper.

Forced Expression of HLA-DM at the Surface of Dendritic Cells Increases Loading of Synthetic Peptides on MHC Class II Molecules and Modulates T Cell Responses

Abdul Mohammad Pezeshki,* Marie-Hélène Côté,* Georges A. Azar,* Jean-Pierre Routy,† Mohamed-Rachid Boulassel,† and Jacques Thibodeau*

Adoptive transfer of autologous dendritic cells (DCs) loaded with tumor-associated CD4 and CD8 T cell epitopes represents a promising avenue for the immunotherapy of cancer. In an effort to increase the loading of therapeutic synthetic peptides on MHC II molecules, we used a mutant of HLA-DM (DMY) devoid of its lysosomal sorting motif and that accumulates at the cell surface. Transfection of DMY into HLA-DR$^+$ cells resulted in increased loading of the exogenously supplied HA$_{307–318}$ peptide, as well as increased stimulation of HA-specific T cells. Also, on transduction in mouse and human DCs, DMY increased loading of HEL$_{48–61}$ and of the tumor Ag-derived gp100$_{174–190}$ peptides, respectively. Interestingly, expression of DMY at the surface of APCs favored Th1 differentiation over Th2. Finally, we found that DMY$^-$ and DMY$^+$ mouse APCs differentially stimulated T cell hybridomas sensitive to the fine conformation of peptide–MHC II complexes. Taken together, our results suggest that the overexpression of HLA-DMY at the plasma membrane of DCs may improve quantitatively, but also qualitatively, the presentation of CD4 T cell epitopes in cellular vaccine therapies for cancer. *The Journal of Immunology*, **2011, 187: 74–81.**

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Although immunization with tumor Ags can activate CTLs in patients, it has become clear that cancer immunotherapy efforts solely based on class I-specific epitopes will not deliver reliable vaccines. Indeed, the critical role of CD4$^+$ T cells in the immune response against tumors has now been well established in a number of experimental animal models (1). Th cells, more specifically Th1, specific for tumor-associated Ags (TAAs) provide help to CTLs and dendritic cells (DCs). They also mediate important effector functions, even in the case of tumors initially negative for MHC II expression (2). Efforts were made in recent years to identify new tumor Ags and to incorporate MHC II-restricted peptides in vaccines to stimulate CD4 T cells in cancer patients (3). However, the simultaneous injection of class I- and class II-restricted peptides did not clearly demonstrate the benefit of including class II epitopes, highlighting the importance of carefully controlling and monitoring the quality of the CD4$^+$ T cell response (4).

Autologous DCs are powerful adjuvants for therapeutic cancer vaccines, and they have been pulsed with various sources of tumor Ags ranging from apoptotic bodies to recombinant TAAs (5). Genetic approaches aiming at the endogenous expression of tumor Ags are also being explored. In all cases, tumor Ags must be processed into the endocytic pathway to generate peptides or polypeptides exposing MHC II-binding sequences. At that point, under the control of a chaperone called HLA-DM (DM), the class II-associated invariant chain (Ii) peptide (CLIP) is removed from the MHC II groove and replaced by an antigenic peptide (6). DM is a highly conserved, nonclassical class II molecule containing a tyrosine-based lysosomal sorting motif (YxxL) in its β-chain cytoplasmic tail (7). DM activity is optimal at the acidic pH of late endosomes, and it edits the repertoire of antigenic peptides following molecular rules that are still under investigation (6).

Of the different protocols being developed to increase the display of tumor T cell epitopes on MHC II, pulsing APCs with synthetic oncopeptides is the most convenient one (8). Peptides bypass the need for intracellular processing and can be loaded directly onto cell surface MHC II in a DM-independent manner. However, it soon became apparent that such Ags could have serious limitations because of their rather inefficient loading (9). Also, binding of synthetic peptides can create MHC II isomers that are distinguished by T cells. Indeed, Unanue and collaborators have shown that synthetic HEL$_{48–61}$ peptides generate two distinct isomers when bound to I-A^k. The more stable conformers are referred to as type A and the less stable ones as type B. Although some T cells (also called type A) can recognize both conformers, other clones (type B) are specific for the type B peptide–MHC II complexes. Interestingly, the type B-specific effector T cells do not recognize the epitope coming from intracellular processing of the native Ag and that are loaded under the control of H2-DM (10). Thus, it is predicted that a substantial fraction of the T cell repertoire activated by peptide-loaded DC vaccines will not recognize tumor-resident APCs displaying the target native epitopes, as these are loaded on MHC II in the presence of DM.

Empty MHC II and functional DM accumulate, albeit at low levels, at the plasma membrane of B lymphocytes and immature DCs (11, 12). This minor peptide loading pathway depends on the presence of extracellular proteases capable of processing Ags. In immature DCs, one can speculate that the caspase-mediated inactivation of AP-2, the adaptor protein complex that mediates

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; CLIP, class II-associated invariant chain peptide; DC, dendritic cell; DM, HLA-DM; Dyn, dynamin; Ii, invariant chain; MoDCs, monocyte-derived dendritic cells; SEB, staphylococcal enterotoxin B; TAA, tumor-associated Ag.

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internalization of proteins with tyrosine-based sorting motifs, causes the accumulation of DM at the plasma membrane (13). On maturation, internalization of DM to endocytic compartments resumes and most MHC II become filled with peptides (11).

In this study, in an effort to increase the efficiency of peptide-based vaccine strategies, we have tested the hypothesis that a DM molecule devoid of its lysosomal sorting signal (DMY) would accumulate by default at the surface of APCs and would improve Ag loading (9). Our results demonstrate that cell surface loading of synthetic peptides in the presence of DMY modulates both quantitatively and qualitatively the T cell response, creating a shift toward a Th1 response and favoring the accumulation of type A peptide-MHC isomers.

Materials and Methods

Plasmids and Abs

Wild-type (pSVL-Dyn2.myc) and dominant-negative (pSVL-DynK44E.myc) dynamin 2 cDNAs were provided by Dr. R. B. Vallee (14). The AdDMY was constructed in pAdenoVator-CMV5 (CuO) from pBud-DMY (15). The pBud-DMY, pcDNA3-CIITA, pREP4-CIITA, pBud-DRα+DRβ, pREP4-Ii p33, and pREP4-Ii p35 plasmids have been described previously (16, 17). L243, CerCLIP.1, MaP.DM1, and Mags.DO5 mAbs have been described previously (16, 17). Abs for mouse CD80, CD86, CD11c, and for human CD80, CD83, and CD86 were purchased from BD Pharmingen (Mississauga, ON). Anti-mouse I-A^K was from BioLegend (San Diego, CA). Anti-human IFN-γ, anti-human IL-4, and CD4 were purchased from Miltenyi Biotec (Auburn, CA). Anti-mouse IFN-γ and IL-4 were from eBioscience (San Diego, CA). Cell surface and intracellular staining were performed as described previously (16).

Peptides, peptide loading, and superantigens

Biotinylated and nonbiotinylated $HA_{306-318}$ (PK***Y***VKQNTLKLAT) (PKY), $HA_{306-318}$ (PK***L***VKQNTLKLAT) (PKL), $gp100_{174-190}$ (TGRAMLGTHT-MEVTVYH), $CLIP_{81-105}$ (LPKPPKPVSKMRMATPLLMQALPMG), and HEL_{48-61} (DGSTDYGILQINSR) were purchased from Centre de recherche du Centre Hospitalier de l'Université Laval (Québec, QC, Canada). Biotin was added to the N terminus using an ε-aminocaproic acid linker. The peptides were purified by HPLC, and purity was >95%. Staphylococcal enterotoxin B (SEB) was from Toxin Technology (Sarasota, FL). For binding assays, cells were incubated for 5 h at 37°C with peptides in culture medium containing 2% of FBS. Where indicated, *n*-propanol was added at a final concentration of 2%. Cells were washed four times with PBS and either used directly for functional assays or incubated with streptavidin-PE (Jackson ImmunoResearch, West Grove, PA) for flow cytometry.

Cells, mice, and transfections

HEK 293T, HEK 293T DR1, HEK 293T DR Ii, HeLa CIITA, HeLa CIITA DO, and HeLa DR1 Ii cells have been described previously (16, 17). The murine fibroblast cell line DAP (ATCC CRL-1949) was transfected using Fugene6 (Roche Diagnostics, Canada). Cells stably expressing CIITA or both DMY and CIITA were sorted using magnetic beads (Dynal ASA, Oslo, Norway) based on I-A^K and DMY surface expression. Human naive $CD4^+$ T cells were isolated by negative selection (Miltenyi Biotec, Auburn, CA) from PBMCs of healthy donors. C3H and 3A9 TCR transgenic BALB.k mice were obtained from Dr. Nathalie Labrecque and Dr. Sylvie Lesage, respectively (University of Montreal). Mouse $CD4^+$ T cells were isolated from splenocytes of 3A9 TCR transgenic BALB-k mice by negative selection (Miltenyi Biotec). 3A9 (type A), CP 1.7 (type B), and MLA 11.2 (type B) T cell hybridomas were provided by Dr. E. Unanue (10). Jurkat HA1.7 T cells were obtained from Dr. R. P. Sékaly (University of Montreal) (18). Human monocyte isolation and preparation of DCs (MoDCs) was described previously (15). Mouse bone marrow-derived DCs (BMDCs) were prepared as described using GM-CSF and IL-4 (19).

Adenovirus production and cell transduction

Recombinant AdDMY was prepared in HEK293 cells as described previously and purified on CsCl gradients (15). For transduction, cells were distributed in 24-well plates at 2.5×10^5 cells/well in 250 μl DMEM. Viruses were added for 3 h at a multiplicity of infection of 50 for HeLa cells and 500 for DCs. Complete media was added and cells were cultivated for 2 d. For DC transduction, viruses were added with LPS.

Priming of $CD4^+$ T cells and functional assays

Human naive $CD4^+$ T cells were cocultured at a ratio of 10:1 with peptide- or SEB-pulsed APCs in 24-well plates. At day 5, SEB-stimulated cells were expanded in 1000 U/ml IL-2 (eBioscience), and at day 8, IFN-γ and IL-4 production was determined by intracellular cytokine staining. Cells were stimulated with 60 ng/ml PMA and 1 μg/ml ionomycin (Sigma, Oakville, ON) for 4 h. Cells were treated with 10 μg/ml brefeldin A (Sigma) 3 h before harvesting, staining, and analysis on a FACSCalibur (Becton Dickinson, Mississauga, ON). For functional assays using HEL_{48-61}, 5×10^4 APCs were cocultured for 12 h with 5×10^4 T cells in 96-well plates. IL-2 production was determined by the ability of the supernatants to sustain the proliferation of the IL-2–dependent T cell line CTLL-2 as measured by [^{3}H]thymidine incorporation (18).

Results

DMY increases peptide loading

Using cells in which a small subset of DM resides at the plasma membrane, it has been shown that MHC II Ag processing can occur extracellularly (11, 12). We have readdressed the issue of DM-mediated peptide exchange at the cell surface by using a dominant-negative form of dynamin (Dyn K44E). As opposed to DR, DM reaches the endosomes after its rapid clathrin-mediated internalization from the cell surface (20, 21). Thus, transient expression of Dyn K44E causes the selective accumulation of DM at the plasma membrane (Fig. 1*A*). HEK 293T cells stably expressing DR1 and Ii (HEK293T DR1 Ii) were transiently transfected with DM and either the wild-type dynamin (Dyn) or the Dyn K44E mutant. In line with a functional role for cell surface DM, there was a 2-fold increase in the binding of the biotinylated $HA_{307-318}$ synthetic peptide (HA-PKY) on cells expressing the Dyn K44E mutant (Fig. 1*A*). These results suggest that DM-assisted peptide loading occurred directly at the plasma membrane and not in the endocytic pathway.

To further increase the levels of DM at the plasma membrane without the pleiotropic effects associated with the expression of the DynK44E, we used a DM variant (DMY) that bears a point mutation destroying its YxxL lysosomal sorting motif. On transient transfection into HEK293T DR1 Ii cells, DMY was found mostly at the plasma membrane (Fig. 1*B*, surface counts) but is still detectable in the endocytic pathway by confocal microscopy (data not shown). In contrast, the control wild-type DM could only be detected after membrane permeabilization (Fig. 1*B*, total counts). Interestingly, DM and DMY appeared equally effective in removing CLIP from DR (Fig. 1*B*). Then, we compared the capacity of these DM isoforms to assist the loading of HA-PKY on live cells. Peptide loading was greatly increased (>6-fold at 50 μM HA-PKY) in the presence of DMY, in line with the results obtained earlier with the DYN K44E mutant (Fig. 1*C*, 1*D*). This translated also into a more efficient stimulation of the HA-specific Jurkat T cells, confirming that the peptide–MHC II complexes are properly folded (Fig. 1*E*). Conversely, peptide binding was only marginally enhanced (<2-fold) after expression of wild-type DM (Fig. 1*C*, 1*D*). Although this small increase could be caused by the very transient cell surface expression of few recycling wild-type DM molecules, it is most likely indirect and the result of the intracellular editing of the peptide repertoire. Increased peptide loading was also observed on HeLa DR1 cells transduced with an adenoviral vector encoding DMY (Fig. 2*A*; compare mock and adDMY conditions). Peptides loaded with or without DMY remained stably associated over a period of 24 h after shifting the cells to 4°C (Supplemental Fig. 1). To confirm the direct role of DMY in mediating peptide exchange, we conducted inhibition experiments using a DM-specific mAb or by cotransduction with another adenovirus encoding HLA-DO (DO). Presence of the DM-specific mAb during peptide loading almost completely

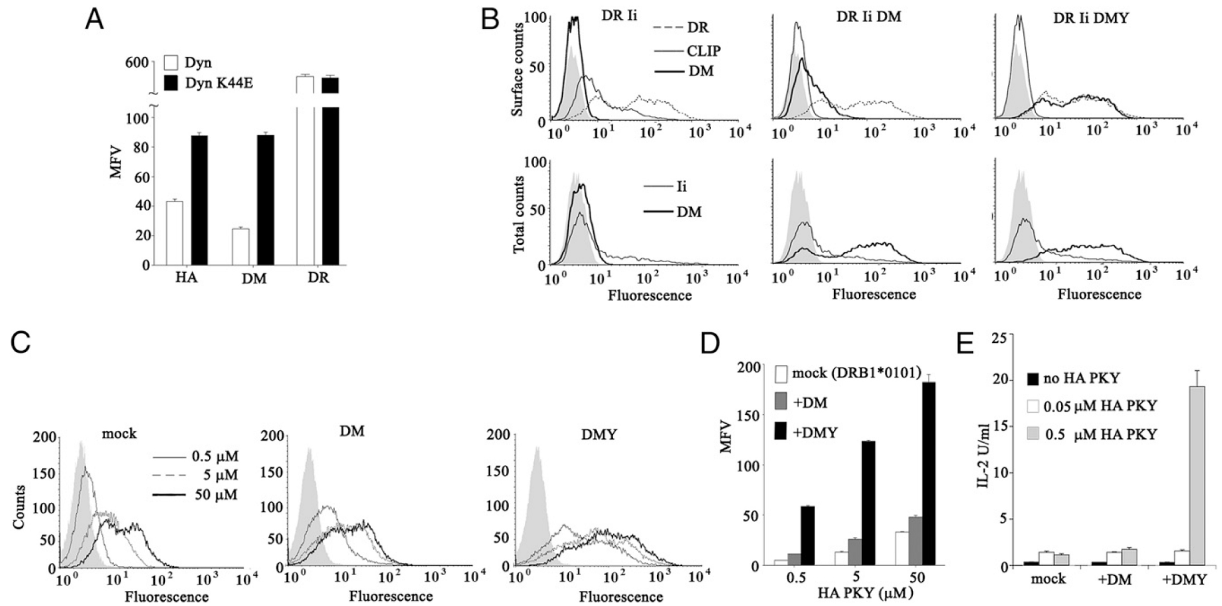

FIGURE 1. DMY increases peptide binding to MHC II. *A*, HEK293T DR Ii cells were transfected with DM together with wild-type dynamin (Dyn) or the dominant-negative mutant (DYN K44E). After 48 h, cells were stained for DM or DR, and biotinylated HA-PKY peptide (50 μM) was loaded for 4 h before flow cytometry. Mean fluorescence values (MFV) are plotted. *B*, HEK293T DR Ii cells (DRB1*0101) were transfected with empty vector (mock), DM, or DMY and stained for cell surface expression of DR, CLIP, and DM (*upper panels*). Alternatively, cells were permeabilized and stained for total expression of Ii and DM (*lower panels*). Filled curves represent the fluorescence background of cells incubated with the secondary Ab alone. *C*, Cells from *B* were loaded with bio-HA-PKY peptides and analyzed by flow cytometry. Filled curves represent the fluorescence background of cells incubated with the PE-coupled avidin alone. *D*, MFV from *C* were plotted as bar charts. *E*, Functional assay using cells described in *B* and Jurkat HA 1.7 T cells.

inhibited DM-mediated peptide loading, presumably by preventing the interactions with DR. Although its exact mechanism of action remains to be identified, DO associates in the endoplasmic reticulum with DM and is a very potent inhibitor of peptide loading at near neutral pH values (20). Indeed, cotransduced DO totally inhibited the DMY-assisted loading of exogenous synthetic peptides on HeLa DR1 cells, further demonstrating the functionality of DMY (Fig. 2*A*, *right panel*).

The effect of DMY was tested using a second peptide-MHC II combination. HeLa cells transfected with the MHC CIITA (HeLa CIITA) express the product of the homozygous DRB*0102 allele and bind more efficiently a $HA_{307-318}$ peptide variant with a tyrosine-to-leucine substitution at position 3 (HA-PKL) (22, 23). As expected, in the absence of DMY, binding of HA-PKL was slightly more efficient than HA-PKY on HeLa CIITA (Fig. 2*B*). This contrasts with the fact that both peptides bind equally well to the DRB*0101 gene product expressed on transfected HeLa DR1 cells (Supplemental Fig. 2). Transduction of DMY into HeLa CIITA increased 2- and 5-fold the binding of HA-PKL and HA-PKY, respectively (Fig. 2*B*). This suggests that DMY not only favors peptide exchange but also stabilizes weak interactions. Altogether, our results demonstrate that expression of DM at the plasma membrane increases the loading of exogenously supplied synthetic peptides.

Modulation of DMY function

Because the main physiological role of DM is the removal of CLIP from the groove of classical MHC II, we have addressed the impact of Ii on peptide exchange. The binding of HA-PKY was measured on HEK293T DR1 cells transfected with Ii, DMY, or both (Fig. 3*A*). Because Ii slightly increased DR expression at the plasma membrane, peptide binding results were normalized to account for this fact (Fig. 3*A*, *right panel*). Loading of HA-PKY was slightly decreased by Ii, most likely because CLIP engages into more stable complexes at neutral pH or because it prevents the loading of low-affinity ligands (peptides or polypeptides) in the

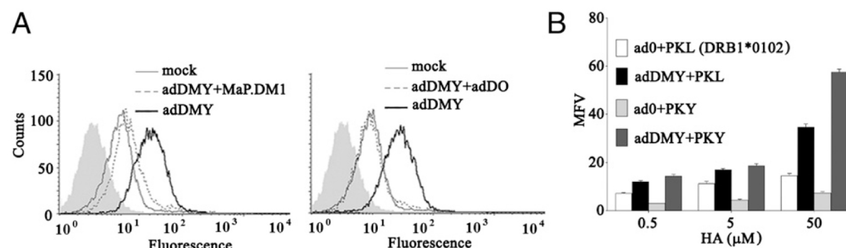

FIGURE 2. DMY increases peptide binding of low-affinity peptides. *A*, HeLa DR1 cells (mock) were transduced with a DMY-expressing adenoviral vector (adDMY) and loaded with HA-PKY peptide (50 μM) in the presence or absence of the DM-specific Map.DM1 mAb (*left panel*). Alternatively, HeLa DR1 cells were cotransduced with a DMY-expressing adenoviral vector and a second adenovirus encoding DO (adDO, *right panel*). Filled curves represent the fluorescence background of cells incubated with the PE-coupled avidin alone. *B*, HeLa CIITA cells (DRB1*0102) were transduced or not with adDMY and loaded with biotinylated HA-PKY or HA-PKL peptides. Cells were analyzed by flow cytometry. All conditions were done in triplicate, and error bars indicate the SDs to the means. Each experiment was done at least twice and a representative experiment is shown.

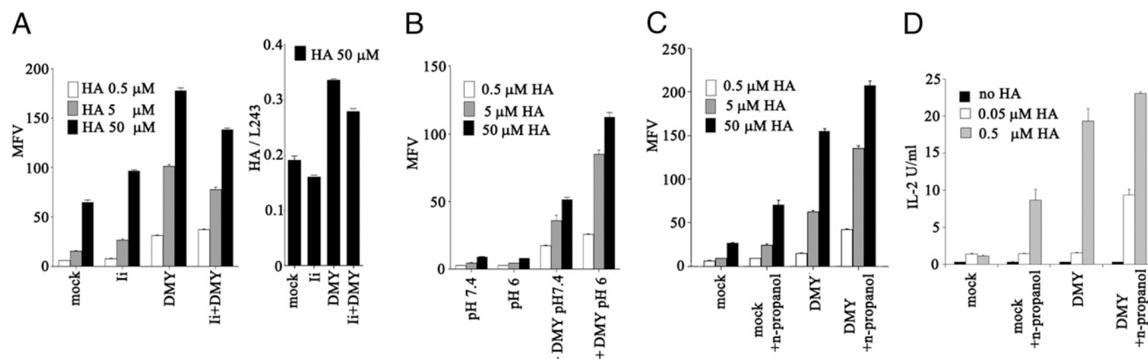

FIGURE 3. DMY activity is modulated by Ii, pH, and *n*-propanol. *A*, HEK293T DR cells (mock) were transfected with either Ii, DMY, or a combination of both molecules. Binding of biotinylated HA-PKY was measured by flow cytometry (*left panel*), and the mean fluorescence values (MFV) were plotted as a ratio (HA/L243) to account for variations in the levels of DR expression (*right panel*). *B*, HEK293T DR1 Ii cells were transfected with DMY, and peptide loading was performed at the indicated pH values. *C*, HEK293T DR1 Ii cells (mock) were transfected with DMY, and peptide loading was performed in the presence or absence of 2% *n*-propanol. *D*, Functional assay using peptide-loaded cells cocultured with Jurkat HA 1.7 T cells. All conditions were done in triplicate, and error bars indicate the SDs to the means. Each experiment was done at least twice and a representative experiment is shown.

endoplasmic reticulum or endosomes (6, 24). In contrast, DMY was more efficient on cells devoid of Ii, suggesting again that low-affinity peptides are associated with DR1 in these conditions. Altogether, our results demonstrate that DMY acts as a general editor of the peptide repertoire, independent of Ii expression (25).

Because DM is most functional at pH values ranging from 5 to 6, we reasoned that lowering the pH during the assay may potentiate peptide loading (6). We tested the resistance of APCs to acidic pH using propidium iodide. Although >50% of the cells died after a 4-h incubation period at pH 5, viability was not affected at pH 6 (9) (Supplemental Fig. 3). Thus, we repeated peptide loading and found that HA-PKY binding was increased ~2-fold when performed at pH 6, but only in the presence of DMY (Fig. 3*B*).

We tested the possibility of combining DMY with small molecules capable of forming H-bonds and that act as chemical analogs of DM (26). Such MHC-loading enhancers, like *n*-propanol, modulate both off and on rates of peptides on soluble and cell-bound DR (27). Fig. 3*C* shows that addition of 2% *n*-propanol to HEK293 DR Ii cells increased >3-fold HA-PKY loading, in line with the ability of such compounds to induce a peptide-receptive state of MHC II. Interestingly, the effects of *n*-propanol and DMY were additive. The peptide–MHC II complexes generated in all these conditions were properly folded and functional, as judged by their ability to stimulate the HA-specific Jurkat T cells (Fig. 3*D*).

Transduction of DMY in DCs

The capacity of DMY to increase peptide loading was tested on DR1$^+$ MoDCs after adenoviral transduction and maturation with LPS. Expression of DM at the plasma membrane was increased dramatically in these conditions and CLIP disappeared from the cell surface (Fig. 4*A*). Peptide loading of HA-PKY was increased (Fig. 4*B*) and so was the T cell stimulation of HA-specific Jurkat T cells (Supplemental Fig. 3). We also tested the binding of a synthetic melanoma oncopeptide, gp100$_{174–190}$, to DCs prepared from another donor expressing the DR7 allotype (15). Again, the presence of DMY at the plasma membrane was sufficient to eliminate all traces of CLIP and resulted in increased peptide loading (Fig. 4*C*, 4*D*).

DMY polarizes T cell responses

Synthetic CLIP peptides have been used in the past to modulate T cell responses. For example, loading of CLIP at the surface of human DCs induced a Th2 bias in vitro in response to the superantigen SEB (28). In addition, injection of mice with the CLIP peptide and a conventional Ag caused a shift from a Th1 to a Th2 type of response (29). We tested the hypothesis that DMY, by removing CLIP from the cell surface and by increasing the loading of exogenously added peptides, would polarize T cells toward Th1. First, we used HeLa CIITA cells transduced with DMY to stimulate primary naive human T cells with SEB. DMY transduction removed most of the CLIP at the plasma membrane (Fig. 5*A*). SEB-pulsed APCs were then incubated with T cells, and cytokine production was measured by flow cytometry after intracellular staining. The results demonstrated that DMY expression caused a Th1 shift in the T cell population responding to SEB (Fig. 5*B*). Although 27.3% of the cells produced IL-4 in the mock-transduced population, the proportion of these cells declined to

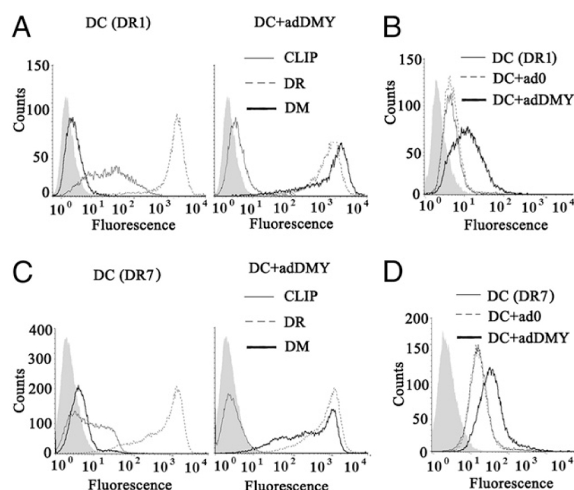

FIGURE 4. DMY increases peptide binding on primary DCs. *A*, Human DR1$^+$ MoDCs (DRB1*01) were transduced with adDMY or mock virus (Ad0) in the presence of LPS. After 48 h, cells were stained and analyzed by flow cytometry for surface expression of CLIP, DR, and DM. *B*, Binding of HA-PKY (50 μM) was measured on nontransduced DCs (DR1), DCs transduced with an empty adenovirus (ad0), or with DMY (adDMY). *C*, Human DR7$^+$ MoDCs (DRB1*07) were treated as in *A*. *D*, Binding of the gp100$_{174–190}$ peptide (50 μM) was measured on nontransduced DCs (DR7), DCs transduced with empty adenovirus (ad0), or transduced with DMY (adDMY). An independent experiment gave similar results.

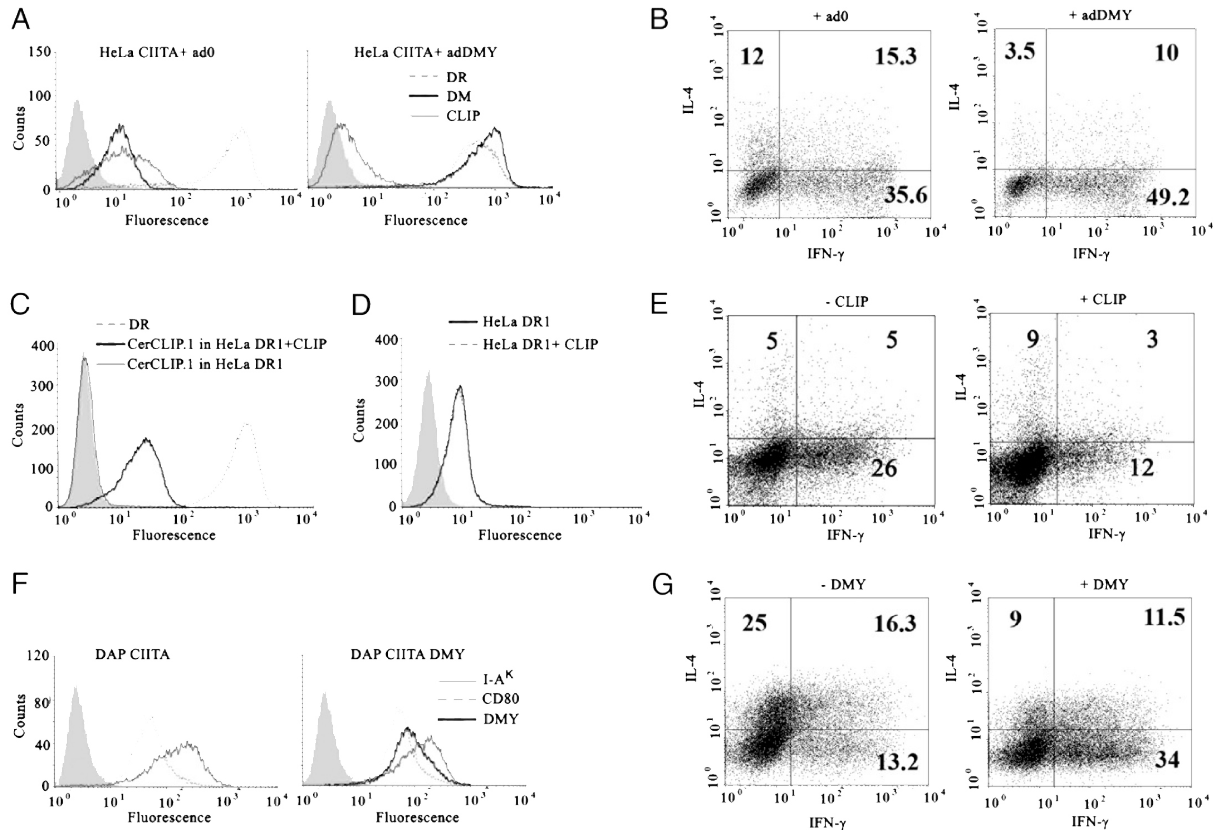

FIGURE 5. DMY favors Th1 responses. *A*, HeLa CIITA cells were transduced with ad0 or AdDMY and stained after 48 h for DR, DM, and CLIP expression. *B*, Transduced cells (HeLa CIITA, *left panel*; HeLa CIITA DMY, *right panel*) were cocultured with naive human $CD4^+$ T cells in the presence of SEB (1 μg/ml). After 5 d, activated cells were expanded with 1000 U/ml IL-2 and analyzed at day 8 for IFN-γ and IL-4 production by intracellular cytokine staining. *C*, HeLa DR1 cells were pulsed with $CLIP_{81-105}$ peptide for 5 h at 37°C and washed and stained for cell surface expression of DR and CLIP. *D*, HeLa DR1 cells loaded or not with the CLIP peptide were pulsed with SEB (10 μg/ml) and stained with PE-coupled avidin. *E*, HeLa DR1 cells loaded or not with the CLIP peptide were co-cultured with naive human $CD4^+$ T cells in the presence of SEB (1 μg/ml) and stained as described in *B*. *F*, DAP CIITA and DAP CIITA DMY cells were stained for MHC II ($I\text{-}A^k$), CD80, and DMY. *G*, Cells in *F* were pulsed with HEL_{48-61} peptide (1 μM) and incubated with $CD4^+$ T cells isolated from 3A9 TCR transgenic mice. Because of a strong inherent Th1 response in these mice, 150 U/ml IL-4 and 20 U/ml IL-2 were added to the cultures on day 1 to skew the response toward Th2. Intracellular cytokine staining for IL-4 and IFN-γ was performed at day 5. Data are representative of three independent experiments.

13.5% on DMY expression. However, the percentage of cells producing IFN-γ was increased from 50.9 to 59.2% in these conditions.

To confirm the role of CLIP in T cell polarization (28), we loaded DR-transfected HeLa cells with exogenous CLIP before pulsing with SEB (Fig. 5*C*, 5*D*). SEB binding was not affected by the presence of cell surface DR/CLIP complexes, but we observed that the Th1/Th2 ratio in responding cells was biased by CLIP (Fig. 5*E*). Although five times more T cells produced solely IFN-γ as compared with IL-4 when stimulated with HeLa DR1 cells, the presence of CLIP resulted in almost equal numbers of cells producing IFN-γ (12%) or IL-4 (9%).

To further investigate the effect of DMY on Th1/Th2 cell polarization, we used a second approach based on the recognition of the HEL_{48-61} peptide by mouse primary T cells. We generated APCs using a stable mouse cell line, DAP CIITA, transfected or not with DMY. The DAP CIITA cells are derived from C_3H/An mice and express MHC II of the $H\text{-}2^k$ haplotype (30). Although we did not monitor for mouse CLIP expression, it has been shown previously that human DM interacts with mouse MHC II (31). The transfected DAP cells express endogenous costimulatory molecule CD80 and were used to activate purified naive $CD4^+$ T cells from the 3A9 TCR transgenic BALB.k mouse (Fig. 5*F*, 5*G*). The 3A9 TCR is specific for the HEL_{48-61} peptide presented in the context of $I\text{-}A^k$. As observed earlier for SEB, the presence of DMY at the plasma membrane during peptide loading polarized the HEL_{48-61} T cell response toward a Th_1 phenotype (Fig. 5*G*). The proportion of cells secreting IFN-γ augmented from 29.5 to 45.5% when APCs were transfected with DMY. Although it is difficult to dissociate the relative contribution of CLIP removal and increased peptide loading, these results suggest that DMY will preferentially polarize toward a Th1 response.

DMY edits peptide–MHC II conformation

Recently, Unanue and collaborators (10) have demonstrated that peptide loading in the presence of DM in late endosomes prevents formation of type B peptide–MHC II conformers. To determine whether DMY can affect peptide–MHC isomerization, we tested the response of type A and B mouse T cells specific for HEL_{48-61}. It has been previously shown that type A 3A9 T cell hybridoma recognizes both type A and B $HEL\text{-}I\text{-}A^k$ complexes, whereas CP1.7 and MLA11.2 can respond only to type B (10). Transduction of DMY in DAP CIITA cells did not affect the expression of $I\text{-}A^k$ (Fig. 6*A*). However, loading of HEL peptide was strongly

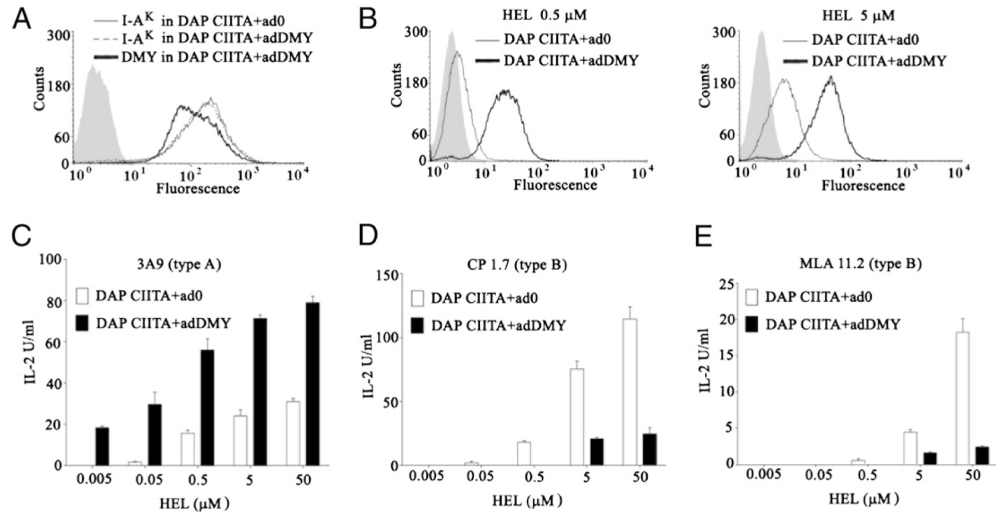

FIGURE 6. DMY edits peptide–MHC II conformation on DAP cells. DAP CIITA cells were transduced with Ad0 or AdDMY and stained for MHC II or DMY surface expression (*A*). Binding of bio-$HEL_{48–61}$ peptide to DAP CIITA cells was monitored by flow cytometry (*B*). DAP cells were used as APCs to present the $HEL_{48–61}$ peptide to type A (*C*) or type B T cell hybridomas (*D*, *E*). All conditions were done in triplicate, and error bars indicate the SDs to the means. Each experiment was done at least twice and a representative set of data is shown.

increased in the presence of DMY (Fig. 6*B*). We cocultured DMY^+ or DMY^- DAP CIITA cells with T cell hybridomas in the presence of the $HEL_{48–61}$ peptide. Fig. 6*C* shows that DMY-expressing DAP cells stimulated the type A 3A9 T cells more efficiently, in line with the observed increase in peptide loading. Interestingly, the presence of DMY had the opposite effect on both type B-specific T cell lines. (Fig. 6*D*, 6*E*).

The impact of DMY on the presentation of $HEL_{48–61}$ was also tested using mouse BMDCs. Although the expression of DMY did not affect the level of I-A^k at the plasma membrane, it enhanced the binding of the HEL peptide (Fig. 7*A*, 7*B*). As observed earlier for DAP CIITA cells, the presence of DMY on BMDCs increased the stimulation of 3A9 whereas reducing the response of both type B T cell lines (Fig. 7*C*–*E*). These results demonstrate that DMY edits the conformation of peptide–MHC II complexes, preventing accumulation of type B isomers in favor of type A. Also, because DMY operates at the plasma membrane, the data suggest that the acidic pH of late endosomal vesicles is not a prerequisite for DM to impact on the fine structure of MHC II–peptide complexes and to eliminate type B conformers.

Discussion

The possibility of loading MHC II exogenously with synthetic peptides or Ags is attractive in the context of cellular vaccine

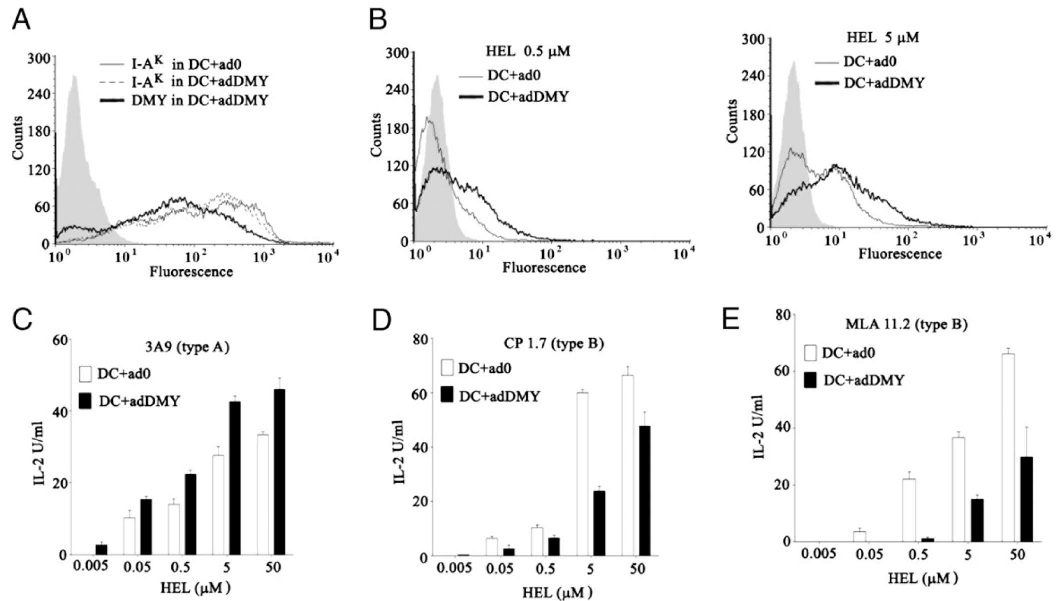

FIGURE 7. DMY edits peptide–MHC II conformation on mouse DCs. BMDCs were transduced with Ad0 or AdDMY and stained for MHC II or DMY surface expression (*A*). Binding of bio-$HEL_{48–61}$ peptide to BMDCs was monitored by flow cytometry (*B*). Transduced BMDCs were used as APCs to present the $HEL_{48–61}$ peptide to type A (*C*) or type B T cell hybridomas (*D*, *E*). All conditions were done in triplicate, and error bars indicate the SDs to the means. Each experiment was done at least twice and a representative set of data is shown.

development. Although experimental conditions may greatly affect peptide loading, little optimization has been done to standardize the procedures (32). The capture of short synthetic epitopes is inefficient, and it has been estimated that only 1% of the MHC II on the surface of a B cell line can be successfully loaded (9, 33). Exogenously pulsed peptides bind mostly to cell surface MHC II, but there are examples of internalization and loading inside various compartments of the endocytic pathway (12, 33–36). On a mechanistic basis, DM most likely operates similarly at the plasma membrane and in the endocytic pathway. Indeed, peptide loading was more efficient when cells were maintained at pH 6, a value optimal for DM activity (6). However, we have not thoroughly investigated the impact of endogenous DM expression or the cellular background on the activity of DMY. For example, although DMY clearly increased peptide binding on DCs, the loading appears more efficient on DR1-transfected HEK 293T cells. Also, some MHC II isotypic or allelic polymorphisms may affect the efficiency of DMY. Future experiments should shed light on the impact of peptide affinity for MHC II molecules. Our initial analysis using PKY and the substituted PKL peptides suggest that empirical studies will be needed to assess the efficiency of binding to different alleles in the presence of DMY. The impact of DMY on low-affinity peptides will need to be addressed to rule out the possibility that these are edited out of the groove or that they simply do not persist in the body because of peptide exchange. We have shown that the high-affinity $HA_{307–318}$ peptide remained stably associated over a period of 24 h at 4°C (Supplemental Fig. 1), but the fate of low-affinity peptides in the presence of competitor peptides may well be different. In this context, the effect of DMY on the loading of low-affinity tumor peptides could be detrimental. Such peptides would necessitate modifications of their anchoring amino acids to increase the affinity for MHC II molecules.

APCs expressing DMY polarize specific T cells toward a Th1 phenotype. Our results using superantigens suggest that the mechanism of action of DMY is independent of a trivial role in increasing avidity of superantigen–TCR interactions. Indeed, SEB binding to MHC II was not affected by DMY (Supplemental Fig. 4). The fact that DMY increased the proportion of cells producing IFN-γ confirms that CLIP has an inhibitory effect on Th1 polarization. This was first proposed by Röhn et al. (28), who found that CLIP-pulsed DCs skewed the SEB response of naive human T cells toward Th2. In this context, CLIP probably finds itself in the immunological synapse and acts as an antagonist peptide, diminishing the impact of pseudodimers (37). A direct role for CLIP in T cell polarization was also inferred from in vivo experiments where coinjections of CLIP and antigenic peptides favored a Th2 response in mice (29). Interestingly, low levels of CLIP and increased DM expression on various tumors predicted better survival of afflicted individuals (38, 39). This may relate to Th1 polarization of tumor-specific T cells as shown in DM^{+} breast carcinoma patients (40).

In the case of nominal Ags such as OVA, the role of DMY could be 2-fold. First, the more efficient peptide loading may favor Th1 responses as Ag load has been shown to affect T cell priming (41). Second, as described above for SEB, the dramatic increase in overall DM expression and the ensuing reduction in CLIP levels may affect molecular events at the immunological synapse.

In addition to its role in editing the epitope repertoire, we have shown that DMY, just like H2-DM (10), can modulate the fine structure of a given peptide–MHC II complex (Figs. 6, 7). DMY reduced the display of type B HEL-I-A^{k} conformers at the cell surface of APCs, causing the poor stimulation of CP1.7 and MLA11.2 type B T cell hybridomas. Unanue and collaborators (10) showed that $HEL_{48–61}$ binds MHC II in at least two different conformations depending on the folding of the N-terminal extension, especially at position -2. One of these conformers (type B) is nonoptimal, and thus readily dissociates in the presence of H2-DM. This activity of H2-DM (and DMY) is likely to affect T cell responses to a large array of natural peptides with N-terminal extensions. In contrast, as shown for HEL, some short synthetic peptides devoid of N-terminal extensions will exist in both type A and B conformations independent of the presence of DM (10). We can speculate that in physiological conditions, professional APCs in the tumor environment will process exogenous TAAs and load nominal peptides in the context of DM, thereby eliminating type B complexes. Because vaccination protocols using short synthetic peptides will generate some type B complexes, the activated effector T cells specific for this conformer would thus not be recruited by APCs in the vicinity of the tumor.

Various chemicals have been investigated to increase Ag presentation and availability of peptide-receptive MHC II species. Early in vitro studies have shown that certain detergent formulations could dissociate CLIP–MHC II complexes and increase peptide loading (42). More recently, DM mimics capable of catalyzing peptide exchange have been described. These simple organic chemicals or peptidic molecules operate at neutral pH and enhance T cell activation (26, 27, 43–45). Interestingly, a better loading was obtained by combining *n*-propanol and DMY, suggesting that the former acts in a DM-independent and -dependent fashion. However, we cannot rule out that *n*-propanol and DMY target separate MHC II–peptide complexes. Future experiments should address the capacity of MHC-loading enhancers to polarize T cells toward Th1 and to eliminate type B complexes, as shown in this article for DMY. Collectively, our results argue that DMY is functional at the plasma membrane of APCs, and that it can be used to improve, both quantitatively and qualitatively, the loading of exogenous peptides. More experiments will be needed to determine in vivo whether DMY boosts Ag-specific Th1 responses and whether our findings can be generalized to other tumor peptides or MHC II alleles and isotypes. In this context, it is interesting to note that the monomorphic nature of this nonclassical DMY class II molecule would allow its universal use in immunotherapy protocols based on adoptive transfer of peptide-loaded DCs.

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Disclosures

The authors have no financial conflicts of interest.

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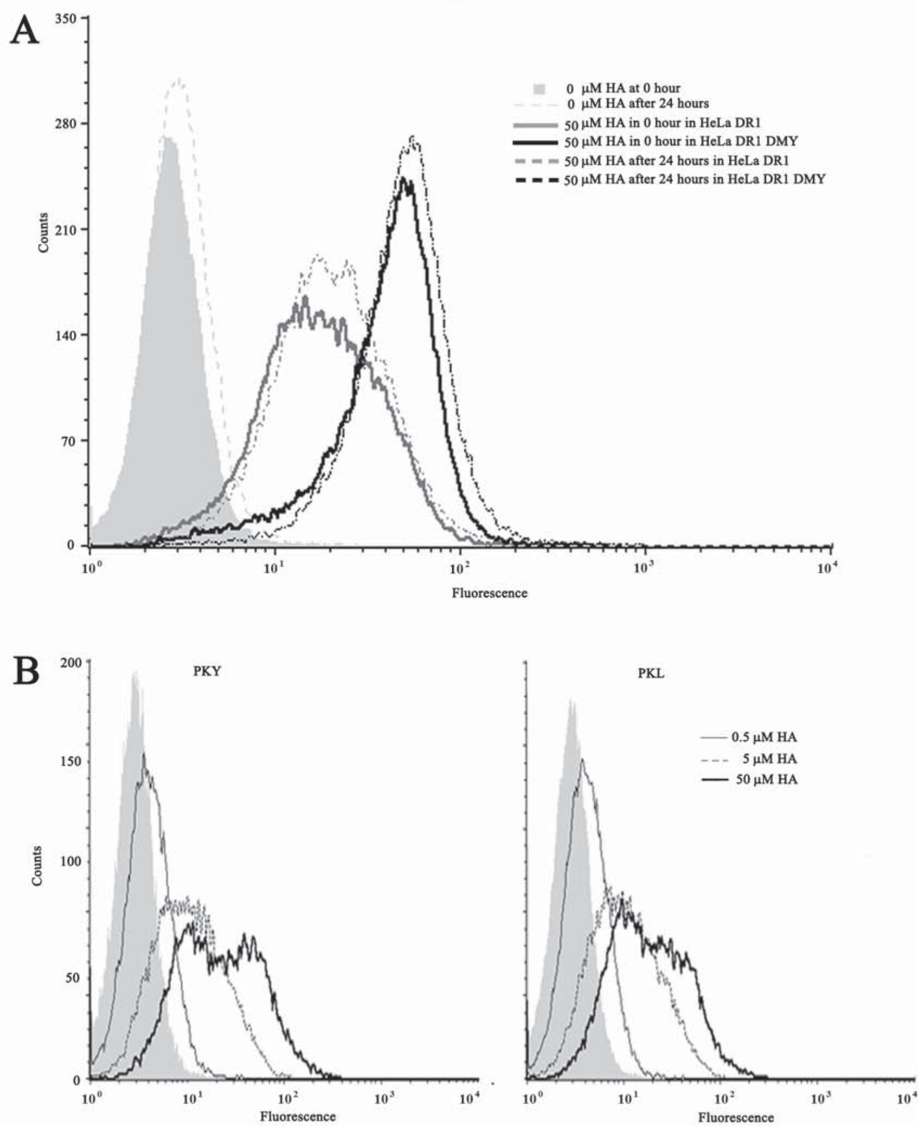

(A) HeLa DR1 and HeLa DR1 DMY stable cell lines were pulsed with $HA_{306-318}$ (50 μM) for 5 hours at 37°C and washed 3 times with PBS. A part of cells were stained with PE-coupled avidin and recorded as time 0 and the rest were kept in 4°C for 24 hours and then washed with PBS and stained with PE-coupled avidin.

(B) Peptide loading with 0.5, 5 and 50 μg/mL of $HA_{306-318}$ (PKY) and $HA_{306-318}$ (PKL) on 293T DR1 Ii stable cell line that has DR B1*0101 allotype.

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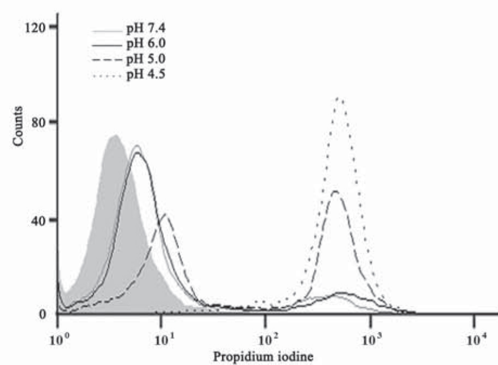
Figure S2



Cell viability of 293TDR1Ii stable cell line in different pH. Cells were incubated for 5 hours in media at pH 4.5, 5.0, 6.0 and 7.4 and the cell viablity were determinated by propidium iodine staining.

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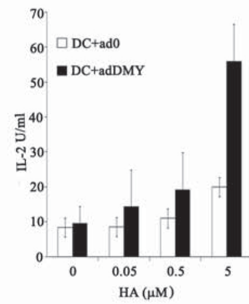
Figure S3



Jurkat HA1.7 T cell hybridoma incubated overnight with peptide pulsed DCs transduced with DMY or mock virus. IL-2 production by hybridoma was determined by the ability of the co-culture supernatant to sustain the proliferation of IL-2-dependent T cell line CTLL-2 as measured by ^{3}H-thymidine incorporation. Data is representative of three experiments.

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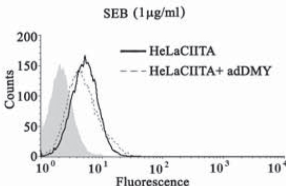
Figure S4



HeLa CIITA and DMY transduced HeLa CIITA cells were pulsed with biotinilated-SEB (1μg/ml) for 4 hours. Flow cytometric analysis performed after staining with PE-labled strep-Avidin.

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CHAPTER .3: Article 2

HLA-DO increases bacterial superantigen binding to human MHC molecules by inhibiting dissociation of class II-associated invariant chain peptides

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Submitted to Journal of Immunology

Contributions

Abdul Mohammad Pezeshki did most of the experiments and wrote the paper.

Georges A. Azar obtained preliminary data and planed experiments.

Walid Mourad provided superantigens

Jean-Pierre Routy and Mohamed-Rachid Boulassel provided PBMCs from healthy donors.

Lisa K. Denzin provided splenocytes of H2-O KO and wild type mice.

Jacques Thibodeau Jacques Thibodeau planed the experiments, analysed the data and wrote the paper.

HLA-DO INCREASES BACTERIAL SUPERANTIGEN BINDING TO HUMAN MHC MOLECULES BY INHIBITING DISSOCIATION OF CLASS II-ASSOCIATED INVARIANT CHAIN PEPTIDES [1]

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Running title: HLA-DO regulates bacterial superantigens

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ABSTRACT

HLA-DO (H2-O in mice) is an intracellular non-classical MHC class II molecule (MHCII). It forms a stable complex with HLA-DM (H2-M in mice) and shapes the MHC class II-associated peptide repertoire. Here, we tested the impact of HLA-DO and H2-O on the binding of superantigens, which has been shown previously to be sensitive to the structural nature of the class II-bound peptides. We found that the binding of staphylococcal enterotoxin (SE) A and B, as well as toxic shock syndrome toxin 1 (TSST-1), was similar on the HLA-DO^{+} human B cell lines 721.45 and its HLA-DO^{-} counterpart. However, overexpressing HLA-DO in MHC class II^{+} HeLa cells (HeLa-CIITA-DO) improved binding of SEA and TSST-1. Accordingly, knocking down HLA-DO expression using specific siRNAs decreased SEA and TSST-1 binding. We tested directly the impact of the class II-associated invariant chain peptide (CLIP), which dissociation from MHC class II molecules is inhibited by overexpressed HLA-DO. Loading of synthetic CLIP on HLA-DR^{+} cells increased SEA and TSST-1 binding. Accordingly, knocking down HLA-DM had a similar effect. In mice, H2-O deficiency had no impact on bSAgs binding to isolated splenocytes. Finally, the presentation of a mouse mammary tumor virus SAg was not modulated by the overexpression of HLA-DO in transfected cells. Altogether, our results demonstrate that the sensitivity of bSAgs to the MHCII–associated peptide has physiological basis and that the effect of HLA-DO on SEA and TSST-1 is mediated through the inhibition of CLIP release.

INTRODUCTION

Superantigens (SAgs) are small bacterial (bSAgs) or viral (vSAgs) proteins that bind MHC class II molecules outside the peptide-binding groove and activate up to 20% of the T cell repertoire in a Vβ-specific manner (1,2). This results in secretion of high levels of cytokines and leads to either anergy or deletion of those activated T cells (3,4). The best-characterized SAgs are those secreted by *Staphyloccocus aureus* as well as the product of the 3' open reading frame (ORF) of the mouse mammary tumor viruses (MMTV) (5).

bSAgs are soluble toxins and more than a dozen have been crystallized so far. While they all show very similar tertiary structures, various modes of interaction with MHC class II molecules (abbreviated MHC II) and TCRs have been described (6-8). Some bSAgs, such as SEB and TSST-1, rely on their N-terminal domain to bind the α chain of MHC II (9,10). Others (SEH, SPE-C, SPE-G - J and SMEZ) engage in a Zn-dependent manner the histidine 81 on the MHC II β chain. Finally, some (SEA, SED, SEE and MAM) engage both class II chains and possibly crosslink the receptors (11-14).

SAgs have long been implicated in various diseases such as toxic shock syndrome and autoimmune conditions (15). They also proved to be useful tools for the appreciation of the peptide-driven structural heterogeneity of HLA-DR (abbreviated: DR) molecules (16,17). It has been demonstrated by many groups and through a variety of read-outs that the class II-bound peptides modulate the affinity of SAGs, especially that of SEA and TSST-1, for MHC class II molecules (6,18-20).

Using soluble DR1 molecules covalently linked to a peptide, Kozono et al. have shown that the N-terminal end of the peptide directly affected SEA binding to MHC II (18). Interestingly, SEA binding to MHC II was enhanced by a peptide ($HN_{421-436}$) derived from the Sendai virus hemagglutinin-neuraminidase (HN) (21). Wen et al. have shown that loading of a special peptide ($SEB_{121-136}$) on a DM deficient cell line, T2-I-A^b, increases presentation of TSST-1 (22). Hogan et al. addressed the question that whether the increase in TSST-1 presentation was just limited to that special peptide or other non-identified peptides could enhance presentation of TSST-1 (20). They found two other peptides that improved presentation of TSST-1. They also demonstrated that presentation of SEA, like TSST-1, is peptide-dependent whereas SEB presentation is not. As for bSAgs, it has been suggested that vSAgs presentation is sensitive to the MHC class II peptide repertoire (23,24). Accordingly, molecules implicated in the MHC class II antigen-processing pathway have been shown to modulate the binding of some SAgs. Interestingly, it was suggested that HLA-DM (abbreviated: DM) affects SAgs binding not only through its modulation of the peptide repertoire but also due to a possible direct effect on the fine conformation of class II molecules (24,25).

The peptide repertoire of MHC II molecules is finely tuned by HLA-DO (H2-O in mice) (26-28). This non-classical MHC II is expressed in B cells, dendritic cells and subsets of thymic epithelial cells (29-31). HLA-DO (abbreviated: DO) does not directly bind peptides (32) and requires association with DM to egress the ER and gain access to MHC class II compartments (MIICs) (33,34). DO remains engaged in

DO:DM complexes during intracellular transport and recycles between MIICs and the cell surface (35). In human peripheral blood B cells, around 50% of the total pool of DM is bound to DO (36). Although its physiological role remains to be firmly established, DO clearly regulates the activity of DM (37-40). Recently, it has been shown that DO prevents autoimmunity by dampening presentation of self-antigens (41). Interestingly, the group of Ostrand-Rosenberg has shown that transfection of H2-O in the class II-expressing mouse SaI sarcoma cell line variant inhibited the binding and presentation of SEA and SEB (42).

Here, using various cell lines and by knocking down gene expression using siRNAs, we addressed in human cells the impact of specific components of the class II antigen presentation pathway on the binding of SAgs. We also evaluated the binding of bSAGs on splenocytes from H2-O-deficient mice. Our results suggest that although physiological levels of H2-O did not appear to have a major influence, overexpression of human DO led to the up-regulation of CLIP and enhanced SEA and TSST-1 binding. The potential implications of these findings for the pathophysiology of SAgs are discussed.

MATERIAL AND METHODS

Antibodies and reagents

L243 (ATCC HB-55) mAb (IgG_{2a}) binds a DRα conformational determinant (43). BU45 (IgG_1) is a mAb specific to the C-terminal portion of the human Ii (44). Pin.1 (IgG_1) recognizes the cytoplasmic tail of Ii (45). CerCLIP.1 (IgG_1) is directed against the N-terminal segment of CLIP bound to MHC class II molecules (46). MaP.DM1 (IgG_1) is directed against the luminal portion of HLA-DM (47). Mags.DO5 (IgG1) recognizes a conformational determinant on HLA-DO (48).

Biotinylated and non-biotinylated SEA, SEB and TSST-1 were from Toxin Technology (Sarasota, FL, USA).

Cells and mice

Kmls 13.11 (mVβ6) murine T cell hybridomas were grown in DMEM and RPMI 1640, respectively, containing 10% FBS and 4μM 2-ME (49). HeLa-CIITA and HeLa-CIITA-DO cells have been described previously (50). The human B-cell line 721.61 (.61) is derived from the Epstein–Barr virus-transformed HLA-hemizygous B-cell line 721.45 (.45), and were cultured in RPMI 1640 medium with 10% FBS and 4μM 2-ME (51). Human naive CD4+ T cells were isolated by negative selection (Miltenyi Biotec, Auburn, CA) from peripheral blood mononuclear cells (PBMCs) of healthy donors.

Mice with both H2-OA genes inactivated (H2-Oα$^{-/-}$) were a kind gift from Dr. L. Karlsson (39). Splenocytes from these mice and from age-matched C57Bl/6 mice were cultured in complete RPMI 1640 10% FBS.

Peptide, siRNA and transfections

$CLIP_{81-105}$ peptide (LPKPPKPVSKMRMATPLLMQALPMG) was purchased from Centre de recherche du Centre Hospitalier de l'Universite´ Laval (Québec, QC, Canada). DO and DM siRNAs were purchased from QIAGEN (Ontario). The AllStars negative control siRNA has no homology to any known mammalian gene. We used HiPerfect transfection reagent according to manufacturer's recommendations (Qiagen). vSAg7 was expressed following transfection of SRαMTV7 encoding the resistance gene to puromycin (52).

Flow cytometry analysis

Cells were washed in PBS and incubated with biotinylated bSAg (bSAg-bio) in PBS for 4 hours at 37°C. Stainings using antibodies were carried out for 30 minutes. Then, cells were washed in PBS and incubated in PBS for another 30 min. at 4°C with Streptavidin-PE for the SAG-bio or Alexa 488-conjugated goat anti-mouse IgG (Cedarlane, Canada) for the mAbs. Cells were washed twice and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, CA). Intracellular staining for DO, DM and Ii were carried out on cells fixed in 4% paraformaldehyde and permeabilized with saponin (53,54).

Priming of $CD4^+$ T cells, cytokine intracellular staining and functional assays

Human naive CD4+ T cells were co-cultured at a ratio of 10:1 with SEB-pulsed APCs in 24-well plates. At day 5, SEB-stimulated cells were expanded in 1000 U/ml IL-2 (eBioscience), and at day 8, IFN-γ and IL-4 production was determined by intracellular cytokine staining (Miltenyi Biotec, Auburn, CA) (55). Cells were stimulated with 60

ng/ml PMA and 1 mg/ml ionomycin (Sigma, Oakville, ON) for 4 h. Cells were treated with 10 mg/ml brefeldin A (Sigma) 3 h before harvesting, staining, and analysis on a FACSCalibur (Becton Dickinson, Mississauga, ON). For functional assays, splenocytes were cultured for 3 days at 10^5 cells per well in presence of bSAgs before adding the ^{3}H-thymidine to measure the bSAg induced T cell proliferation. The vSAg7-expressing APCs were co-cultured at different ratios with $6x10^4$ T cell hybridomas. IL-2 production was determined by the ability of the co-culture supernatant to sustain the proliferation of the IL-2 dependent T-cell line CTLL-2 (ATCC TIB-214) as measured by ^{3}H-thymidine incorporation (56).

5,6-Carboxylfluorescein diacetate succinimidyl ester (CFSE) was obtained from Invitrogen (Burlington, ON) and labeling and analysis was performed according to the manufacturer's protocol. Briefly, Naïve CD4 T cells were resuspended in PBS at a concentration of $2x10^7$/ml and were labeled with CFSE at a final concentration of 10 μM. After stimulation, T cells were incubated for 5-6 days before analysis by flow cytometry.

RESULTS

bSAgs binding on B cell lines

To evaluate the impact of DO on bSAgs binding to human classical MHC II, we first relied on two members of the *721* series of mutant cell lines. The 721.61 B cell line expresses all three classical MHC class II isotypes, DR, DQ and DP. It is identical to its 721.45 counterpart except for a small deletion, which eliminates the DOB gene in 721.61 cells (57). Figure 1A shows that the loss of HLA-DO did not affect the expression of HLA-DR and the proportion of MHC II molecules associated to the CLIP peptide. The expression of DM was lower in DO-deficient cells but it is not clear if this relates to the loss of DO. Cells were incubated with biotinylated SEA, SEB and TSST-1 and analyzed by flow cytometry. Although SEA stainings were stronger, the binding of all SAgs was dose-dependent and uniform at the cell surface (Fig. 1B). Importantly, the expression of DO did not influence the overall binding of the different SAgs as the relative flow cytometry profiles between 721.45 and 721.61 matched the one observed for MHC II molecules.

Overexpression of DO increases SEA and TSST-1 binding

Although the exact physiological role of DO is still nebulous, its overexpression leads to the inhibition of HLA-DM and to the accumulation of CLIP/MHCII complexes at the plasma membrane (27,37,58). To fully appreciate the impact of a DO-induced change in the peptide repertoire on the binding of bSAgs, we turned to MHCII^{+} HeLa-CIITA cells super-transfected or not with DO. The class II transactivator CIITA is the

master regulator of MHC II genes and under its control, cells up-regulate not only the expression of MHCII but also Ii and the non classical class II DM and DO (50,59,60). DO levels are low in HeLa-CIITA cells and overexpression of DO (HeLa-CIITA-DO) led to a 10 fold increase in the mean fluorescence value (MFV) of cell surface CLIP-MHCII complexes, as monitored by CerCLIP.1 staining (Fig. 2A). While the relative SEB binding appeared to closely match the HLA-DR expression on the two cell lines, more binding of SEA and TSST-1 was observed in DO-overexpressing cells (Fig. 2B and C). These results demonstrate that DO overexpression affects binding of bSAgs to MHCII molecules, in line with an important role of the associated peptides in this receptor-ligand interaction (16,18,20,22).

Knocking down DO expression decreased bSAgs binding

To rule out the possibility that the differences between HeLa-CIITA and HeLa-CIITA-DO cell populations were simply due to clonal variations, the binding of bSAgs was measured following the transient knockdown of DO in HeLa-CIITA-DO cells using specific small interfering RNAs (siRNAs). The expression of DO was markedly reduced two days after transfection of siRNAs (Fig. 3 A). While the overall cell surface level of DR was not affected, the proportion of CLIP-bound MHC II molecules decreased about five fold. Interestingly, bSAgs binding, especially for SEA and TSST-1, was decreased on cells expressing lower levels of DO (Fig. 3B). Again, the effect on SEB appeared very limited. DO silencing by siRNAs confirmed our previous results

in HeLa-CIITA and HeLa-CIITA-DO cell lines, which showed that DO increases SEA and TSST-1 binding.

To help determine if SEB presentation was affected quantitatively and/or qualitatively by the presence of DO, we turned to a sensitive functional readout using PBMCs from healthy donors. Others and we have shown that APCs exogenously pulsed with CLIP could induce T_h2 polarization in naïve CD4 T cells responding to nominal Ags or SEB (55,61,62). As CLIP levels vary dramatically on HeLa cells overexpressing or not DO, we tested the T cell polarization in response to SEB. Naïve $CD4^+$ T cells from healthy donors were stimulated and analyzed for IL-4 and IFN-γ expression. Intracellular cytokine staining showed that the ratio of cells producing exclusively IFN-γ over IL-4 is very similar between HeLa-CIITA (5.7) and HeLa-CIITA-DO (4.8) (Fig. 4A). This experiment was repeated with HeLa-CIITA-DO cells treated or not with DO-specific siRNAs (Fig. 4B). Again, there was no strong shift in polarization in these conditions.

Interestingly, we noted that the overexpression of DO resulted in increased proportions of double-negative cells for the production of IL-4 and IFN-γ (Fig. 4A and B). As it has been reported that bSAgs could induce $FoxP3^+$, IL-10-producing regulatory T cell (63), we performed FoxP3 and IL-10 staining. However, we did not observe any difference in prevalence of $FoxP3^+$ or IL-10 producing $CD4^+$ T cells in conditions where APCs were overexpressing or not DO (data not shown). To determine if the increase in the double negative population of T cells is the

consequence of a higher threshold of T cell activation induced by the expression of DO, we compared the proliferation of T cells using carboxyfluorescein diacetate succinimidyl ester (CFSE). Figure 4C shows that a high dose of SEB triggered a very potent T cell stimulation and up to six cell divisions can be observed in these conditions. The MFVs obtained for CFSE under different SEB concentrations were plotted and the results demonstrate that the binding of SEB and the T cell response are independent of the presence of DO (Fig. 4D). Altogether, these results suggest that DO does not affect the T_h1/ T_h2 balance.

Exogenous CLIP increases SEA and TSST-1 binding

Given the importance of MHCII-bound peptides on bSAgs binding, we surmised that DO mediates its effect, at least in part, by modulating CLIP levels. To test this hypothesis, we loaded cell surface MHC II molecules exogenously with the synthetic $CLIP_{81-105}$ peptide. For these experiments, we used a reductionist approach based on HEK293T cells transfected with HLA-DR1 $\alpha\beta$ cDNAs. As these cells do not express Ii nor HLA-DM, MHC II molecules are loaded with easily displaceable polypeptides. Loading of the $CLIP_{81-105}$ was quiet efficient after 3h at 37°C as monitored by flow cytometry using the CerCLIP.1 mAb (Fig. 5A). Then, cells were washed and incubated with bSAgs as above. Figure 5B shows that the presence of $CLIP_{81-105}$ enhanced the binding of bSAgs, especially SEA and TSST-1. These results are in line with a role for CLIP in the DO-induced increase in bSAgs binding to MHC II molecules.

Knocking down DM affects bSAgs binding

A corollary to our findings on the role of DO and exogenous CLIP is that tempering with DM expression would also increase CLIP levels and bSAgs binding. To test this hypothesis, we knocked down DM in HeLa-CIITA using siRNAs. These cells express high levels of DM proteins and silencing of DM led to a seven fold increase in CLIP-MHC II complexes on cell surface (Fig. 6A). As expected, this increase in CLIP level translated in an augmentation of SEA and TSST-1 binding (Fig. 6B). SEB was not affected in these conditions.

Effect of H2-O on bSAgs binding

Qi et al. have reported that H2-O transfection in I-A^{k} -expressing SaI cells inhibits binding and presentation of SEA and SEB (42). The availability of DO-deficient animals offers the possibility of looking at primary cells to evaluate the impact of H2-O on bSAgs binding. For these ex vivo experiments, we used fresh splenocytes as H2-O is expressed by about 50% of the B cells and 1-3% of $CD11c^{+}$ DCs (31,64). Although none of the three bSAgs was affected by the loss of H2-O, the T cell response to TSST-1 was reduced in its intensity but not in its sensitivity as the T cells detect the SAg at the same concentration with or without DO (Fig. 7A and 7B). This is most likely due to variations in the T cell repertoire between the two mice.

Indeed, while minor, differences were observed in the proportions of some Vβs between DO-deficient and -proficient animals (26).

Perraudeau et al. noted that the small variations in the Vβ repertoire of T cells did not correlate with the nature of the endogenous MMTV SAgs (26). The H2-O-deficient mice studied here are on a C57BL/6 background and express I-A^b, which is inefficient in the presentation of MMTV vSAgs (65). Therefore, to determine if DO could alter MMTV vSAgs presentation, we knocked down DO expression in HeLa-CIITA-DO stably expressing vSAg7 (Fig. 8A). These APCs were co-cultured at different ratios with vSAg7-responsive Kmls 13.11 mouse T cell hybridoma and IL-2 production was measured. The results provided no evidence for a role of DO in vSAg presentation (Fig. 8B).

DISCUSSION

Unlike nominal Ags, binding of SAgs to MHC class II molecules and their presentation to T cells is not strictly MHC-restricted. Indeed, some MHCII isotypic and allotypic polymorphisms affect bSAgs binding as well as T cell recognition of the complex (1,16). For example, it was shown that one polymorphic amino acid in the MHCII β chain binding site for SEA is responsible for the almost complete lack of binding to DRw53 (DRB4*0101) as compared to the DR1 (DRB1 "0101) allele (66,67). In addition to variations altering the binding site of bSAGs, other polymorphisms rather affect the recognition of the complex by the TCR. This is best exemplified by the fact that the mouse Vβ repertoire of responsive T cells will vary depending if the SAg is presented by murine versus human MHC II molecules (68).

However, allelic variations could not explain the observation that only a fraction of all the cell surface molecules of a given MHC II allotype were apt at binding SEB or TSST-1 (16). The structural basis for this phenomenon was addressed in many subsequent studies. It was shown that the MHCII microheterogeneity sensed by bSAgs was due to the nature of the peptides found in the groove (17). The three-dimensional structure of various SAgs-MHC combinations has been resolved and clearly established that peptides can directly interact with SAgs (9,69,70). Also, the peptides anchor side-chains can indirectly affect SAgs presentation by modifying the fine conformation of the binding site (18,20,22,70).

Although the exact function of DO remains to be identified, it is clearly a regulator of the MHCII peptide repertoire (26,27). As such, we hypothesized that it could impact

on the presentation of peptide-sensitive bSAgs. SEB was used mainly as a negative control as although it binds only a subset of the total pool of MHCII molecules, no clear evidence has been provided to show that it is influenced by peptide sequence or length. Our results showed that DO expression affects SEA and TSST-1 binding only in experimental settings where CLIP is up-regulated. It appears that the minor modifications of the peptide repertoire resulting from weak DO expression are not sufficient to alter the SAgs-binding profiles. This was clearly the case in mouse B cells, where H2-O expression does not impact on CLIP removal. Accordingly, H2-O-deficient spleen cells were as efficient as wild-type splenocytes in binding bSAgs. Knock-out mice or mice made transgenic with H2-O showed no variations in the level of I-A^b/CLIP complexes (26,28,39,71). The reasons for the lack of CLIP up-regulation is unknown and may relate, amongst other factors, to the H2-O/H2-M ratio or the cell type studied. Interestingly, DCs from transgenic mice expressing the human HLA-DO displayed more CLIP at their surface (64).

Our results on mouse cells contrast with those of Qi and Ostrand-Rosenberg, who found that H2-O expression diminished presentation of SEA and SEB (42). These authors used I-A^k -expressing SaI cells and found that the presentation of bSAgs was Ii-independent but DM-dependent. Interestingly, Albert et al. have shown that the effect of DM on bSAgs/I-A^k interactions is peptide-independent and probably the result of a DM-induced conformation change in the MHCII (25). We can speculate that the effect of H2-O on bSAgs reflects the inhibition of a conformation change in I-A^k, rather than the modulation of CLIP levels. Thus, as SAgs binding to I-A^b was not

affected by H2-O, it appears that allelic and isotypic polymorphisms may explain variations in the susceptibility to H2-M/O.

Our results are difficult to reconcile with those obtained previously using T2-DR3 cells reconstituted or not with HLA-DM (19,72). In these Ii^+ cells, others and we have shown that DM expression increases the binding of TSST-1. This may represent another example of allelic variations which, under the influence of DM, allow DR3 to bind a set of peptides that greatly enhance TSST-1 binding as compared to CLIP. In the case of DRB* 0102 expressed by HeLa-CIITA cells or DRB* 0101-transfected cells, it appears that knocking down DM prevents the replacement of CLIP by non-permissive peptides. Thus, CLIP affinity for SAgs is probably intermediate and DM will replace it with more or less permissive peptides, depending on the allelic polymorphisms. At this point, for similar MFVs, we cannot discriminate between cells expressing many low affinity binding sites and those having less receptors, but of higher affinity. A more detailed analysis will be required to discriminate between these possibilities. Interestingly, expression of Ii in DM-negative cells allowed transfected DR1 to bind more efficiently SEA (72). A likely explanation for such result is that although CLIP has low affinity for SEA in these conditions, it displaced non-permissive peptides. Altogether these results are in line with the fact that panels of peptides most likely generate MHCII complexes spanning a large scale of affinities for bSAgs.

As for $I\text{-}A^k$, the binding of SAgs may also be affected by the activity of DM on the fine structure of human MHC II. Indeed, DM was shown to be responsible for the

uncovering of the peptide-independent 16.23 mAb epitope and for the TCR-sensitive isomerisation of some peptide/MHC II complexes (73,74). TSST-1, in addition to being sensitive to CLIP and other peptides, could also be affected by the DM-induced conformation changes, just like some mAbs and TCRs.

In 721.45 cells, as DO expression appears to be compensated by increased levels of DM, no net accumulation of CLIP was observed as compared to 721.45 cells (Fig. 1A). However, as demonstrated here using MHCII$^+$ HeLa transfectants, most human cell lines and primary B cells studied so far showed a direct correlation between DO expression levels and cell surface display of CLIP (75-79). This suggests that DO, in the context of certain MHC II alleles, is likely to influence bSAgs binding and possibly play a role in the pathophysiology of these molecules in humans. DO is mostly expressed in resting mature B cells, causing the accumulation of CLIP at the plasma membrane (36). Upon activation with CD40L and IL-4 or through the B cell receptor, DO expression is negatively regulated and so are CLIP levels (36,80,81). Presentation of SAgs by naïve resting B cells may contribute to the induction of anergy and activation-induced cell death observed in responding T cells (3,4). Recently, it was shown that B cells stimulated through their TLR will ectopically express CLIP (82). Many pathogens cause an early polyclonal activation of B cells and the ectopic expression of CLIP might favor the amplification of SAgs binding in some individuals (83). Finally, although upregulation of cell surface CLIP by DO did not influence T cell polarization in our assay, SAg presentation in vivo in the context

of CLIP may divert the immune response away from the more protective Th1 immunity (55).

In conclusion, DO and H2-O seem to affect differentially the capacity of MHCII to bind bSAgs. DO overexpression modulated the binding of some bSAgs through cell surface accumulation of CLIP. Future studies should investigate the impact of DO and H2-O overexpression on other murine and human classical MHC II isotypes and in vivo.

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FIGURE LEGENDS

Figure 1. Loss of DO in B cell lines does not affect bSAgs binding. (**A**) 721.61 (DO-negative) and 721.45 (DO-positive) B cell lines were stained for expression of DR (L243 Ab), CLIP (CerCLIP.1 Ab), DO (Mags.DO5 Ab) and DM (MaP.DM1). (**B**) Cells were pulsed with bSAgs for 4h at 37°C, washed and stained with StrepAvidin-PE for detection of biotinylated bSAgs.

Figure 2. Overexpression of DO increases SEA and TSST-1 binding to MHC II^{+} HeLa cells. (**A**) HeLa-CIITA and HeLa-CIITA-DO cells were stained for cell surface expression of DR (L243 Ab) and CLIP (CerCLIP.1 Ab). Permeabilized cells were used to measure DO (Mags.DO5 Ab) and DM (MaP.DM1) expression. (**B and C**) Cells were pulsed with bio-bSAgs for 4h at 37°C, washed and then stained with StrepAvidin-PE. (**C**) MFVs were plotted as bar charts. Error bars represent the standard deviations (SD) obtained from triplicate staining. Data are representative of three independent experiments.

Figure 3. DO knockdown decreases SEA and TSST-1 binding on HeLa-CIITA-DO cells. (**A**) HeLa-CIITA-DO cells were transfected with control or DO-specific siRNAs, cultured for 48h and then stained for surface expression of DR and CLIP. Cells were also permeabilized (total) and stained for DM and DO. (**B**) Cells were pulsed with biotinylated SEA, TSST-1 and SEB for 4h at 37 °C. Cells were washed and stained with StrepAvidin-PE for bio-bSAgs detection. Error bars represent the standard deviations (SD) obtained from triplicate staining. Data are representative of three independent experiments.

Figure 4. SEB presentation is not affected by DO expression. Naïve CD4 T cells were isolated from PBMCs of healthy donors. SEB (1µg/ml) was pulsed on (**A**) HeLa-CIITA and HeLa-CIITA-DO cells or (**B**) HeLa-CIITA-DO and DO-silenced cells. These APCs were co-cultured with naïve CD4^{+} T cells. After 5 days, activated T cells were expanded with IL-2 (1000 U/ml) and analysed for IL-4 and IFN-γ at day 8. **(C)** Naïve

CD4 T cells were labeled by CFSE and co-cultured with HeLa-CIITA-DO cells beforehand pulsed with different concentrations of SEB. HeLa-CIITA-DO cells were treated either with DO-specific or control siRNAs. After 5 days at 37°C, T cell proliferation was monitored by flow cytometry. **(D)** The MFVs obtained for CFSE-loaded cells were plotted. Error bars represent the standard deviations (SD) obtained from triplicate staining. Data are representative of three independent experiments.

Figure 5. CLIP favors bSAgs binding to MHCII. (**A**) HEK293-DR1 cells were pulsed with synthetic $CLIP_{81-105}$ peptide for 3h at 37 °C. Cells were washed and stained for DR and CLIP expression at cell surface and (**B**) incubated with bio-bSAgs for 4h at 37 °C. Cells were washed and stained with StrepAvidin-PE. Error bars represent the standard deviations (SD) obtained from triplicate staining. Data are representative of three independent experiments.

Figure 6. Knocking-down of DM expression increases binding of bSAgs. (**A**) HeLa-CIITA cells were transfected with control or DM-specific siRNAs, cultured for 48h and stained for cell surface DM and CLIP. Cells were also permeabilized (total) and stained for DM and DO. (**B**) Cells were pulsed with SEA, TSST-1 and SEB for 4h at 37 °C, washed and stained with StrepAvidin-PE for bio-bSAgs detection. Error bars represent the standard deviations (SD) obtained from triplicate staining. Data are representative of three independent experiments.

Figure 7. H2-O deficient splenocytes bind bSAgs. (**A**) Splenocytes were pulsed with biotinylated SEA, TSST-1 and SEB for 4h at 37 °C. Cells were washed and stained with StrepAvidin-PE. (**B**) For functional assays, splenocytes were cultured in 96 well plates for 3 days at 10^5 cells per well in presence of bSAgs before adding the ^{3}H-thymidine to measure T cell proliferation. Error bars represent the standard deviations (SD) obtained from triplicate staining and assays. Data are representative of three independent experiments.

Figure 8. Endogenous vSAgs presentation is DO-independent. (A) HeLa-CIITA-DO cells were transfected with vSAg7 as well as DO-specific or control siRNAs. DR and CLIP expression were monitored at cell surface after 48h. **(B)** Transfected cells were co-cultured with the Kmls 13.11 (mVβ6) T cell hybridoma at different ratios of T cells/APC. The error bars indicate the standard deviation (SD) obtained for all IL-2 assays performed in triplicate. Data are representative of three independent experiments.

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Figure 1

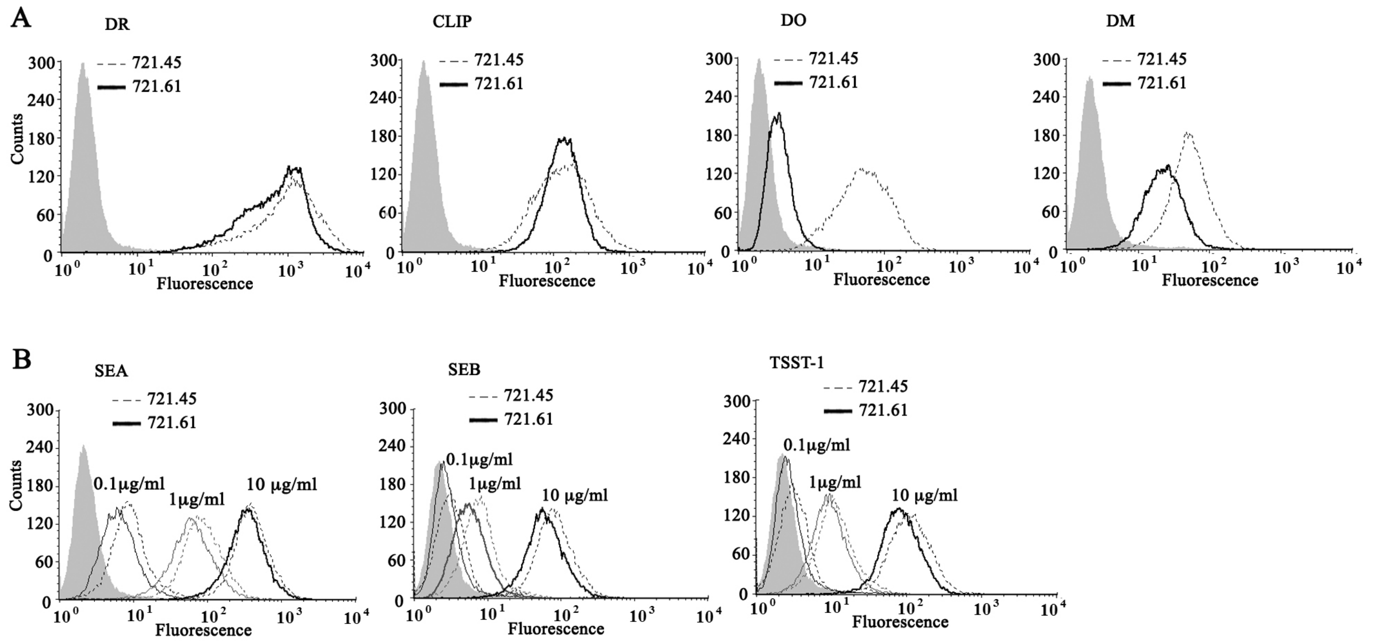


Figure 2

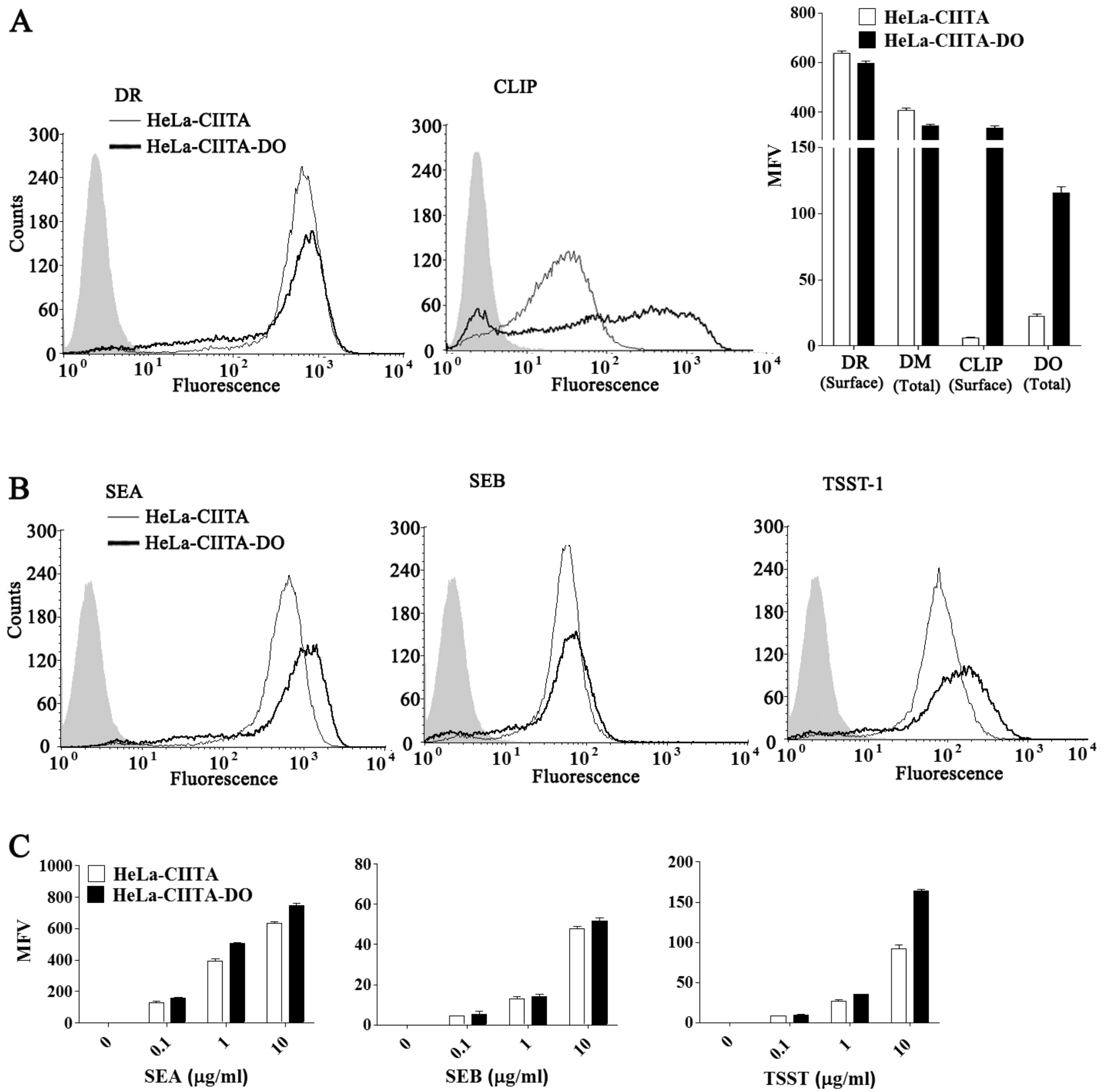


Figure 3

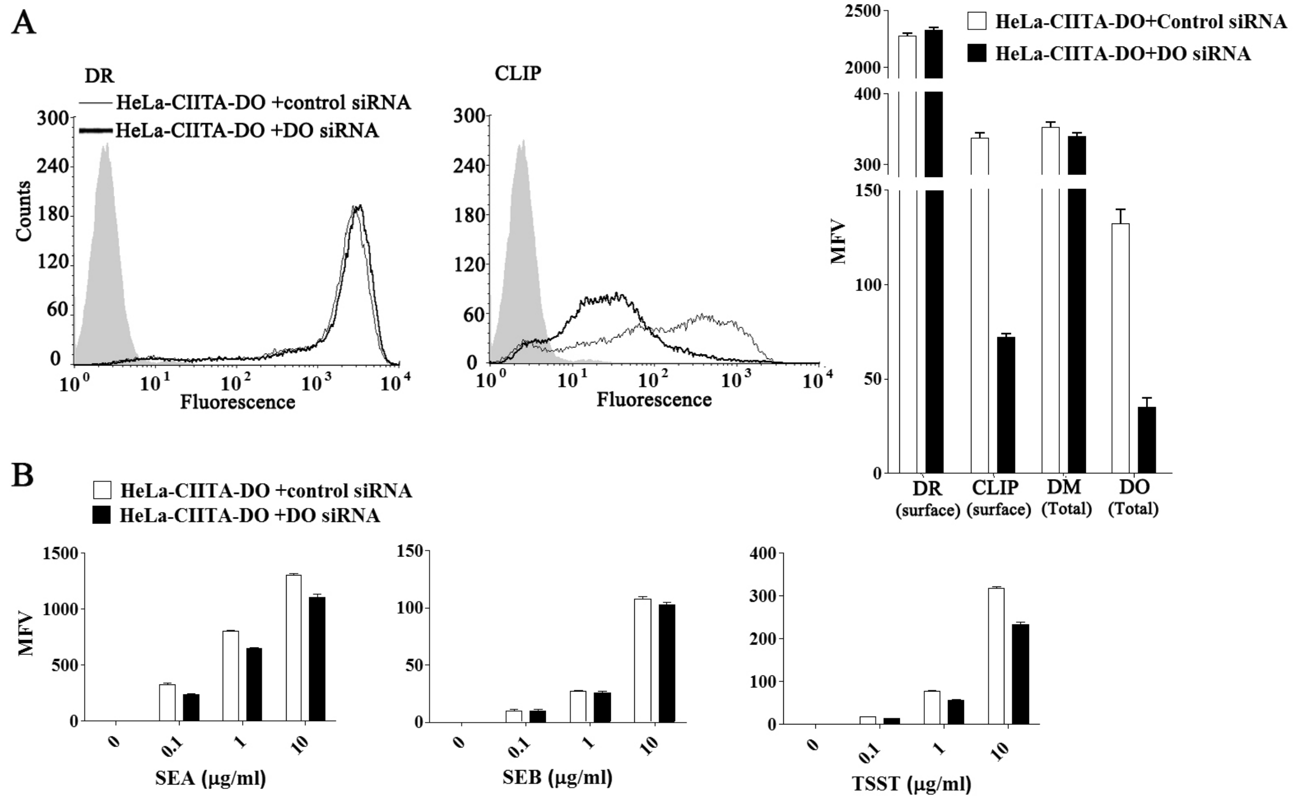
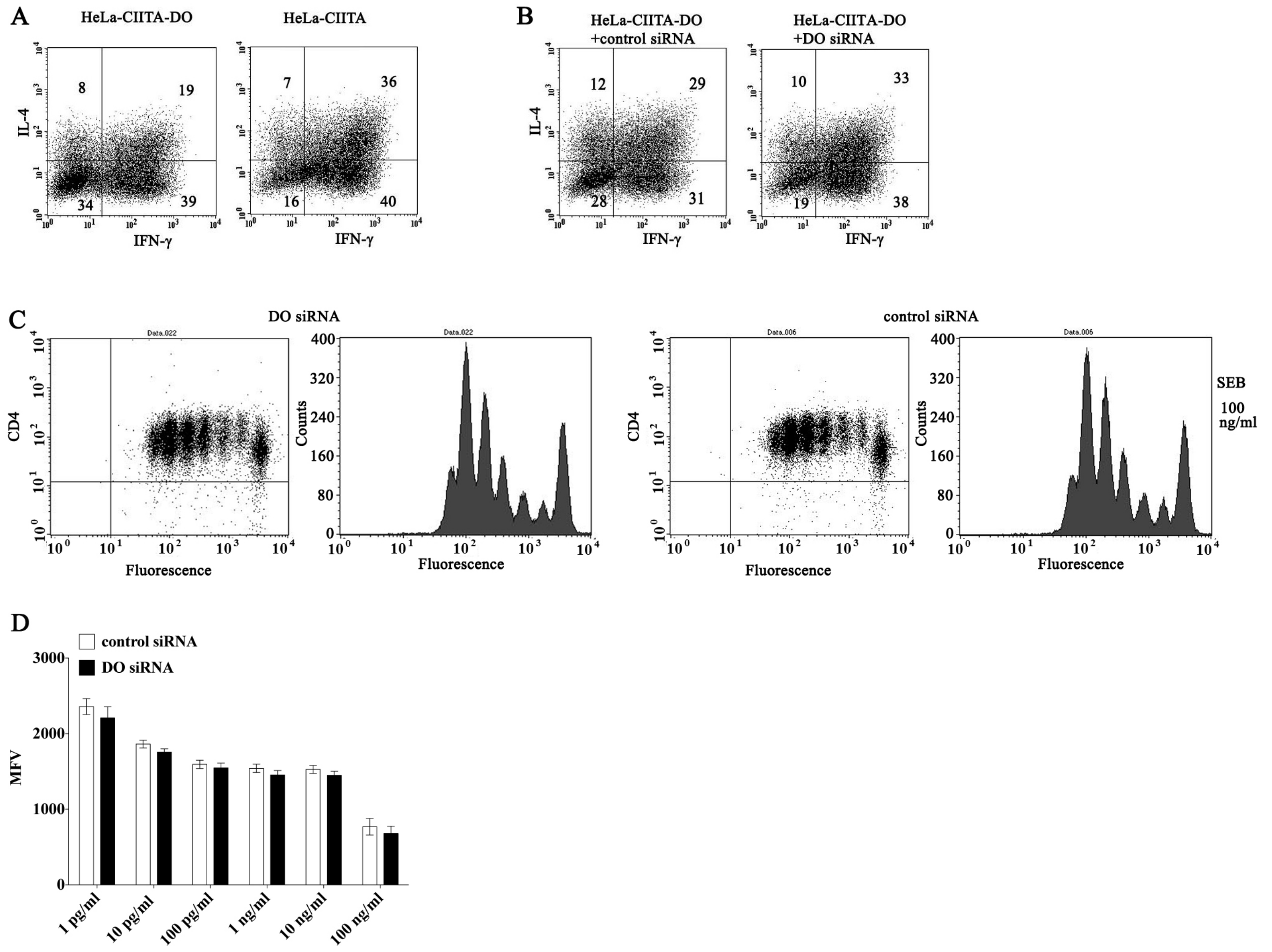


Figure 4



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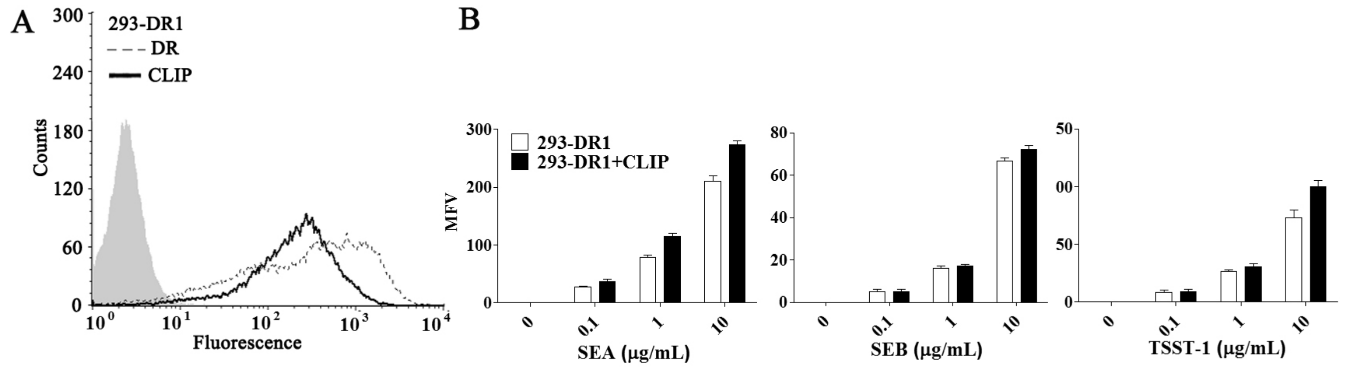


Figure 6

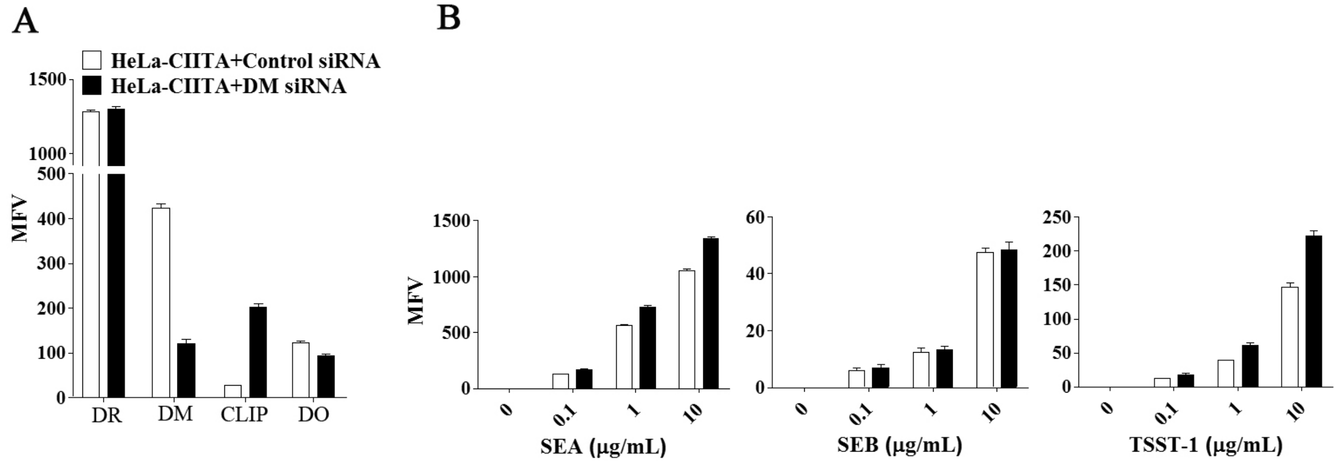


Figure 7

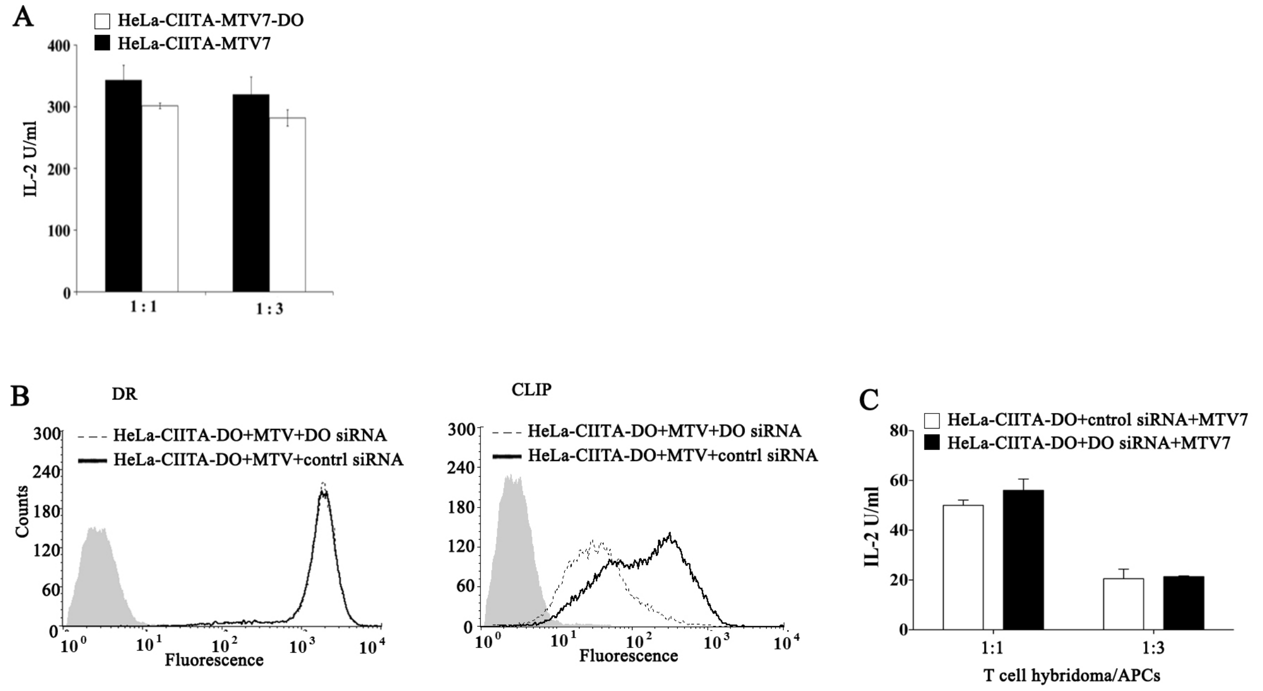
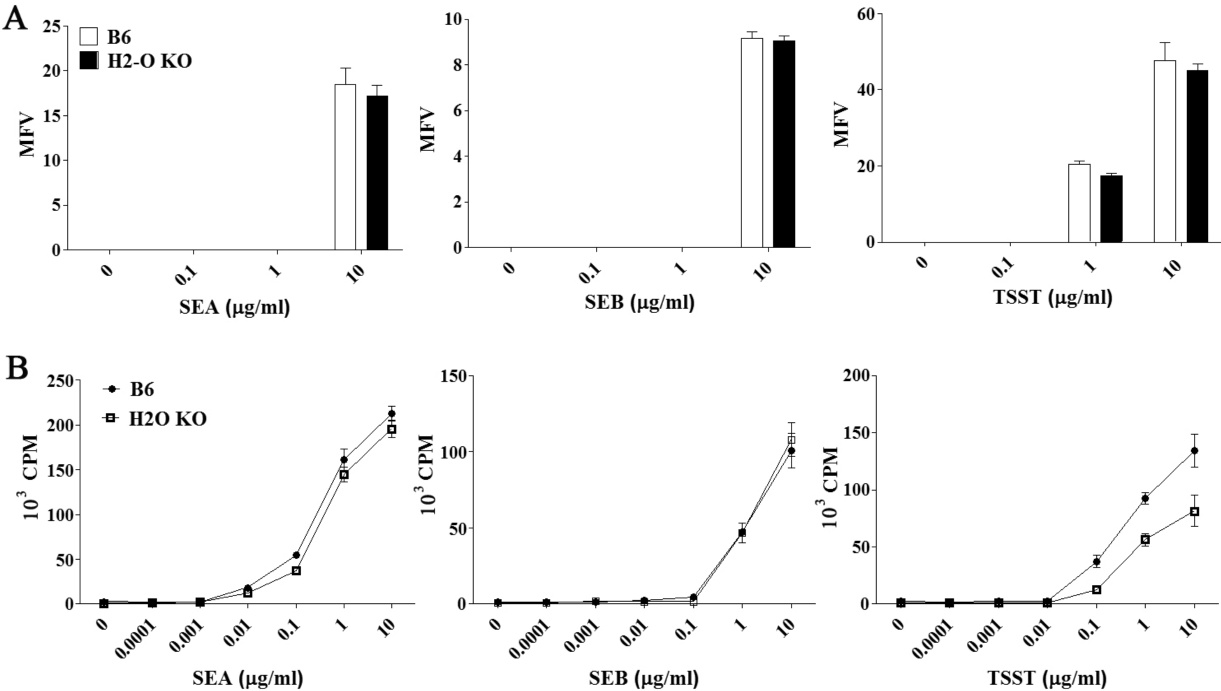


Figure 8



CHAPTER .4

Discussion and perspectives

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Vaccines improve the immunity against particular diseases. They stimulate the immune system to recognize the vaccine components as foreign material, to destroy them and to remember their structure in case of future encounter. The vaccines could be prophylactic (to prepare for a future encounter with disease) or therapeutic (to cure a current disease). For now most cancer vaccines are therapeutic and used to treat patients diagnosed with cancer. The aim of cancer vaccines is to stimulate an immune response to selectively eradicate tumor cells, typically by involving cytotoxic T cells (CTLs). To achieve this goal and have a strong, long lasting CTLs response, involvement of helper T cells (T_h) is necessary. A more efficient T_h response would result in more effective and persistent CTL responses.

To induce a strong and durable T_h response by DCs vaccines, we need efficient peptide loading on MHC II molecules at the surface of DC. Quantitative and qualitative improvement of peptide loading on DCs were the major goal of this thesis. We have shown that expression of a mutant DM molecule (DMY), which accumulates on the cell surface, highly improves loading of peptides on DCs. We also provided evidence that this quantitative improvement in peptide loading translates into more robust T_h cell response. The strengthen T_h response was T_h1 that is the most effective subset of T_h response against tumors. The quantitative increase in exogenous peptide loading is narrowed to stable and rigid conformers of MHC-peptide – type A. This stable type A conformer is most likely the type of conformer that gets presented by professional APCs to T cells in the tumor microenvironment. Moreover, we have shown that DMY has synergistic effect when coupled to its chemical analogues and enhance their catalytic potential.

We also presented evidence that DO alters binding of bSAgs. DO expression enhances binding of peptide-dependent bSAgs (SEA & TSST-1) to human MHC II molecules. DO probably plays a role through accumulation of CLIP on the cell surface of APCs. However, expression of H2-O did not increase binding of bSAgs to murine MHC II. Overexpression of DO did not affect binding of vSAgs to MHC II molecules. DO overexpression also did not polarize helper T cells response. DO overexpression also did not induce peripheral T_{reg}

development after stimulation with bSAgs. This finding has the potential to be used in vaccination approaches, as discussed below.

To express DMY in primary APCs, we used the first generation of adenoviral (Ad) vectors. Although, these vectors are safe and one of the most efficient systems for gene delivery *in vivo* for gene therapy and vaccine applications, there is still concern about pre-existing or later host immune responses against viral antigens (Ahi, Bangari et al., 2011; Dharmapuri et al., 2009; Z.-L. Xu et al., 2005). This anti-Ad immunity diminishes the duration of transgene expression, especially in the booster doses of administrated vaccine. To circumvent these drawbacks, several approaches have been applied, such as development of Ad vectors based on less prevalent human Ad serotypes and nonhuman Ad or deletion of all of the viral structural genes (gutless Ad) (Ahi et al., 2011; Dharmapuri et al., 2009; Z.-L. Xu et al., 2005). Increasing transduction efficiency of DCs by manipulation of viral capsid proteins such as fiber knob and penton base could be another approach that expose host to lower dose of viral antigens (Arnberg, 2009; Khare et al., 2011; Sharma et al., 2009; Z.-L. Xu et al., 2005). More efficient transduction decreases the multiplicity of infection (MOI) of administrated Ad vectors. Besides weakening the anti-vector immune response, the lower MOI has lower toxicity for DCs and extends their life span. This is especially important for transgenes that need high level of expression. DMY needs high level of expression to compensate lack of endosomal acidic pH on the cell surface level.

To bypass the use of viral vectors for expression of DMY in clinical trials, one could envisage the use of non-viral vectors (Gao et al., 2007; X. Guo et al., 2012). Viral vectors offer greater efficiency of gene delivery and long-term expression of transgene by genomic insertion. However, non-viral vectors are preferred due to safety concerns for clinical trials (Gao et al., 2007; X. Guo & Huang, 2012). For DC vaccines and expression of DMY, non-viral vectors might represent a good option. Preparation of DCs and peptide-loading are in vitro protocols moreover DCs are not proliferative cells (after differentiation to DC) to dilute the transduced gene and the viral vectors could be replaced with non-viral ones. Several non-viral methods for gene transfer had been applied, including physical methods and using of chemical vectors (Gao et al., 2007; X. Guo et al., 2012). Genes can be delivered by exerting physical forces that permeates the cell membrane. Methods such as needle and jet injection, hydrodynamic gene transfer, gene gun delivery, sonoporation,

electoporation and its advanced formats such as nucleofection are in this category. For chemical vectors, researchers use cationic lipids (form lipoplexes upon mixing with DNA), cationic polymers (form polyplexes upon mixing with DNA) and inorganic nanoparticles (Gao et al., 2007; X. Guo et al., 2012).

Another hurdle for DCs-immunotherapy is the preparation of sufficient quantity of DCs. Preparation of large quantity of autologous DCs to be transduced by DMY for clinical use is difficult, expensive and time consuming. An alternative source of autologous APCs in large quantity might be activated T cells, that could be easily generated from PBMC (Foster et al., 2007). Activated T cells up-regulate MHC II, the costimulatory molecules CD80 and CD86, and the adhesion molecules ICAM-3, ICAM-1 and LFA-3, making a proper surrogate professional APCs (Cooper et al., 2005; Foster et al., 2007; Naota et al., 2006; Salgado et al., 2002). These activated T cells are potentially used for *in vivo* boosting of adoptively transferred T cells and are called T-APC (C. Berger et al., 2008). However, T-APCs can act as antigen carrier and migrate to lymphoid organs and deliver antigen to DCs in tumor-bearing mice (Russo et al., 2007). T-APC were modified to express melanoma antigens and used as a vaccine in melanoma patients, demonstrating induction of T cell immunity to a self/tumor antigen (Fontana et al., 2009). These studies indicate that T-APC has the potential to be used as DC's surrogate or complement in peptide-based DCs vaccines. DMY would be a good candidate to improve exogenous peptide loading in this new approach, since MHC II levels remain low on activated T cells. DMY by removing endogenous peptides from MHC II peptide-binding groove can improve peptide loading even in absence of Ii.

Another challenge in immunotherapy with DC vaccines is the limited half-life of DCs (Kukutsch et al., 2000). Dendritic cells are not proliferative cells and have a limited life-span (Merad et al., 2009). Transduction and overexpression of a recombinant gene decreases this short half-life and therefore their *in vivo* efficiency. To improve exogenous peptide loading in DC vaccines, instead of DMY expression, one can take another approach and target DM internalization pathway in DCs. Newly synthesized DM rapidly internalize from plasma membrane to endosomes via clathrin-mediated endocytosis (Bonifacino et al., 1999; Marks et al. 1995; Lindstedt et al. 1995; Copier et al. 1996). We have shown that by transfecting dominant-negative Dynamin, DM stays at cell surface and acts as DMY.

Dominant negative Dynamin prevents clathrin-mediated internalization and leads to accumulation of DM at cell surface. Dynamin could be knocked down by siRNA transfection in DCs. Although the surface accumulated DM never reaches the levels of DMY expression at cell surface, this accumulation of DM could improve exogenous peptide loading. However, due to lower levels of DM expression, this approach may not benefit from other advantages of DMY such as conformer editing and T_h1 polarization. This requirement for high surface expression of DMY could be compensated by chemical analogues of DM. Combination of Dynamin specific siRNA and chemical compounds could be a surrogate for DMY with its all benefits and worthy to test (Call et al., 2009; Dickhaut et al., 2009).

DMY and Th polarization

T_h1 cells are clearly important for immunotherapy and T_h1 polarization is an important issue in this field. We have shown that DMY, by releasing CLIP from MHC II, shifts the T_h1/T_h2 balance toward T_h1. The mechanism of T_h2 polarization by CLIP is not clear yet. CLIP is a low-affinity self-ligand that could decrease threshold of TCR stimulation by the inhibition of ERK activation and elevate expression of the inhibitory tyrosine phosphatase SHP-1 (Stephen et al., 2009). Although this decline in threshold of TCR is used to explain thymic positive selection and survival of peripheral T cells, it may explain T_h2 polarization by CLIP. Strong TCR signalling results in T_h1 differentiation while weak TCR signalling favours T_h2 differentiation (Constant et al., 1997). CLIP engagement with TCR may decrease the TCR threshold and deliver a weak signal to T cells leading to T_h2. Weakening strength of the TCR signal by CLIP, follows the same principal as low concentration of cognate peptide that preferentially develops T_h2 response (Yamane, Zhu, & Paul, 2005). Stimulation of naive $CD4^+$ T cells with low concentrations of cognate peptide causes weak stimulation of TCR and induces IL-4-independent GATA-binding protein 3 (GATA3) upregulation and early IL-4 production (Yamane et al., 2005). This also brings weak and transient ERK activation. Such weak ERK activation is necessary for inducing sufficient amounts of IL-2 needed for further T_h2 cell polarization but is not sufficient to repress

TCR-mediated GATA3 induction. CLIP by weakening ERK activation and elevating the inhibitory tyrosine phosphatase SHP-1, could follow the same path and differentiates naïve T cells to T_h2. DMY removes CLIP and replaces it with cognate peptide. Stimulation of naive $CD4^+$ T cells with high concentrations of cognate peptide causes strong and prolonged ERK activation. Such strong ERK activation suppresses TCR-mediated GATA3 upregulation and IL-2-mediated signal transducer and activator of transcription 5 (STAT5) phosphorylation, two crucial events for inducing IL-4 production (Yamane et al., 2005; J. Zhu et al., 2010). Cross-regulation between transcription factors is important during T_h1/T_h2 differentiation. GATA3 and STAT5 down regulate expression of STAT4 and T-bet, respectively and promote T_h2 differentiation (Usui et al., 2003; J. Zhu et al., 2003). On the other hand, GATA3 expression is suppressed by T-bet during T_h1 differentiation (Usui et al., 2006).

According to our study, DMY favors T_h1 polarization. This skewing of helper T cell responses might occur through two different mechanisms. The first mechanism could be through exchange of CLIP with a high affinity peptide and increase in the quantity of that specific peptide. This primary mechanism is more evident in our mice model of polarization where we stimulated T cells with a specific peptide (HEL_{48-61}). The second mechanism could be through immunological synapse or other unknown mechanism. This mechanism seems to be more involved in our human model of T_h polarization; because for stimulation of T cells we used superantigen. The strong T cell stimulation by SAg, fades the peptide-dependent mechanism, either through removal of CLIP or high dose of specific peptide, and underlines involvement of another mechanism. The T cell polarization experiments show that, DMY shifts T_h response toward T_h1 and CLIP-accumulation shifts it to T_h2.

Future experiments could address the role of CLIP and DM in the regulation of transcription factors. One way to address that would be knocking down of DM in DCs to stimulate naïve CD4 T cells from TCR transgenic mice. Transcriptome of these T cells could be analyzed and compared with T cells stimulated with DM-proficient DCs. To

investigate impact of CLIP, independent of DO overexpression, some of non-treated DCs could be first pulsed with CLIP peptide and then loaded with specific peptides to stimulate T cells for subsequent transcriptome analysis. However, silencing of DM alters the loaded peptide on APCs. To address the impact of DM independent of peptide, some T cells could be stimulated with bSAgs instead of peptide. To have a more thorough understanding, it would be desirable to look at the DM and CLIP influence in different MHC context. In this view, DCs and naïve CD4 T cells could be prepared from 3A9 (recognize HEL in the context of I-A^k), HNT (recognize HA in the context of I-A^d) and OT-II (recognize OVA in the context of I-A^b) TCR transgenic mice.

The role of DM and CLIP in T_h differentiation is also supported by clinical data. In cancers where tumor cells act like APCs and express MHC II molecules, high levels of CLIP expression on myeloid leukemia blast cells predicted poor survival of patient and associated with decreased DM expression (Chamuleau et al., 2004). Cells from pre-B acute lymphoblastic leukemia patients with ETV6–AML1 translocation showed low CLIP expression and significantly higher DM expression, revealing good prognosis with late relapse in comparison to translocation negative patients (Jastaniah, Alessandri, Reid, & Schultz, 2006). Expression of DM by tumor cells was associates with a T_h1 profile and predicts improved survival in breast carcinoma patients (Oldford et al., 2006). Also, down-modulation of CLIP enhanced the immunogenicity of myeloid leukemic blasts resulting in increased $CD4^+$ T cell responses in acute myeloid leukemia (AML) patients (van Luijn et al., 2010). In fact, upregulation of CLIP is an immune escape mechanism of AML (van Luijn, Chamuleau, Ossenkoppele, van de Loosdrecht, & Marieke van Ham, 2012). van Luijn et al. co-cultured $CD4^+$ T cells of acute myeloid leukemia (AML) patients with their autologous $CLIP^-$ and $CLIP^+$ primary leukemic blasts and observed increased DR and IFN-γ expression in $CD4^+$ T cells stimulated with $CLIP^-$ leukemic blasts, which indicated an activation and polarization of the $CD4^+$ T cells toward T_h1 cells (van Luijn et al., 2011). In addition, CLIP- leukemic blasts induced greater outgrowth of effector memory $CD4^+$ T

cells that were associated with better leukemia-specific reactivity than with CLIP+ leukemic blasts. Therefore DM, by removing CLIP and directing immune response toward T_h1, can play an important role in anti-tumor immune response and immunotherapy.

DMY and conformational isomers

DM not only selects high affinity peptides for MHC II molecules, but also modulates binding of finest conformational isomer (type A conformer) among a set of MHC-peptide complexes (Lovitch & Unanue, 2005). Conformational isomers (conformers) of a peptide-MHC complex can be generated from exogenous or autologous proteins in endosomal compartments and the less stable conformers – type B, are edited by DM and are not displayed on the surface of APCs (Z Pu et al., 2004). Because type B T cells fail to recognize the peptide-MHC conformer generated upon processing of native protein in presence of DM, the self-reactive type B T cells might escape negative selection and constitute a significant component of the naïve T-cell repertoire (Lovitch et al., 2005). Type B conformers could be generated and presented on cell surface and early endosomes by proteolysis of self-proteins, due to absence of DM or lack of its activity in these cell compartments (Lovitch et al., 2005). Alternatively, the type B conformer can be generated from intracellular processing of the native protein, if such processing occurs in the absence of DM or if DM is inhibited (Lovitch & Unanue, 2005). In non-obese diabetic (NOD) mice, a substantial number of autoreactive CD4 T cells are type B T cells that escape thymic deletion and cause autoimmunity (Mohan et al., 2010).

We showed that expression of DMY on DCs can prevent presentation of type B conformers and therefore hinders stimulation and proliferation of type B T cells. The type B T cells are probably not useful in peptide-based tumor vaccines because APCs in tumor microenvironment will not display type B conformers. However, as described below, by taking an opposite approach and expression of type B conformers on tumor resident DCs we could turn these type B T cells against the tumor.

Due to mechanisms of central and peripheral tolerance toward self-antigens, tumor-specific T cells are absent from the immune repertoire or, at best, only have weak reactivity with tumor antigens. This helps tumors to avoid immunosurveillance and is one of the

major challenges of immunotherapy (Casucci et al., 2012). While highly self-reactive type A T cells are deleted from repertoire, high affinity self-reactive type B T cells are available in repertoire because they have never been exposed to their specific ligands (**Figure 4.1**). We can exploit these available T cells for immunotherapy by exposing their ligands on tumor-resident APCs. In this approach, we could present type B conformers on tumor-resident DCs by inactivating DM. DCs with inactivated DM present both type A and B conformers from internalized fragments of tumor. Presentation of type B conformers on tumor-resident DCs will stimulate highly specific type B T cells that could efficiently fight tumor and compensates the absence of high-affinity type A T cells (**Figure 4.1**). It has been shown that self-reactive T cells escape negative selection by forming unusual immunological synapse and thus may not be efficient for tumor therapy (D. A. Schubert et al., 2012). However, type B T cells escape negative selection because their specific ligand are not presented by thymic DCs, therefore type B T cells should be as efficient as other peripheral T cells for activation upon proper stimulation (Lovitch et al., 2005).

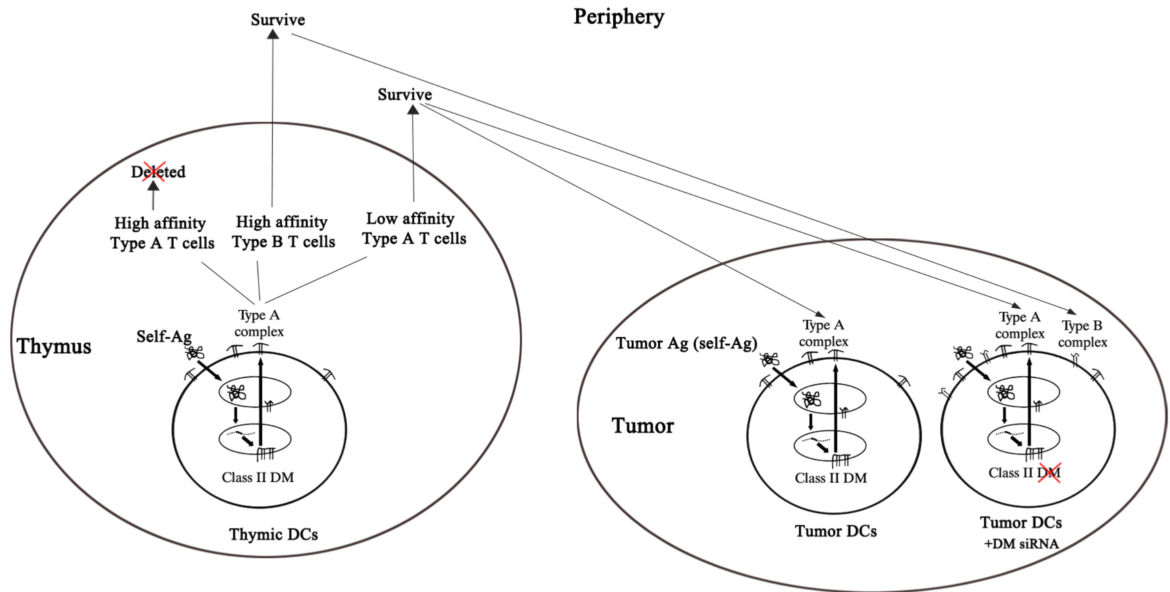

Figure 4.1. Model for the use of high-affinity type B T cells in immunotherapy: During negative selection in the thymus, due to activity of DM, DCs do not present type B conformers so, high-affinity type B T cell clones for self-antigens escape. Presentation of type A conformers by DCs leads to deletion of high-affinity type A T cell clones from T cell repertoire while low-affinity type A T cells survive negative selection. Tumor resident DCs present only type A conformers. Inactivation of DM enables them to present type B conformers and recruit high-affinity type B T cells to fight tumors.

To inactivate DM in tumor resident DCs, one can use targeted siRNA delivery approaches (J. Guo et al., 2011; Zhou et al., 2011). It could be a tumor-targeted liposomes carrying DC-targeted siRNA. After homing of these tumor resident DCs to local lymp nodes, they can present type B conformers to high affinity type B naïve T cells. This activates the type B conformers with high affinity for tumor peptides. The tumor-peptide vaccination strategy could be the traditional DCs-peptide vaccine, which presents both type A and B conformers to their already activated specific T cells (activated due to use of DM-siRNA). We can also load a tumor cell lysate on DCs with inactivated-DM. In this way, DCs present type B conformers of several tumor antigens to high affinity type B T cells that have already been activated by tumor resident DCs (DM-KO). This leaves less room

for immune editing mechanisms of tumor. The pros and cons of DM inactivation in DCs loaded with tumor-lysate should be evaluated in vivo; to compare the benefits of more amplification of type B T cells to the negative effects of DM inactivation on loading efficiency.

bSAgs potential for vaccination

Inactivation of antigen presentation chaperones may be used in other protocols aiming at the immunotherapy of tumors. As we have shown, knocking-down of DM significantly increases binding of peptide-dependent bSAgs through accumulation of CLIP at the cell surface of APCs. CLIP-mediated increase in bSAgs binding could compensate the negative effect of DM inactivation in peptide loading and T cell activation in DCs-vaccines targeting both type A and B tumor specific T cells (Kato et al., 2011). Moreover, it has long been realized that many tumor antigens are poorly immunogenic and CLIP-mediated bSAg binding has the potential to evolve as a novel tool in the DC vaccines (Radcliff et al., 2012). bSAgs have separate binding sites for MHC, TCR and costimulatory molecules (G Arad et al., 2000). The TCR binding site could be removed to avoid nonspecific T cell stimulation and fuse the MHC and costimulatory parts with peptides or tumor-antigens (Radcliff et al., 2012). This antigen or peptide-SAg hybrid (conjugate) could be bind to all MHC II^{+} APCs, internalized, processed and presented to T cells. In this way, we can make a universal tumor-vaccine with almost no limitation for MHC type of recipient. This could be a novel approach for *in vivo* targeting of professional APCs and DCs. In fact CLIP accumulation could be an advantage, because it considerably increases the binding of Ag-bSAg conjugates to DCs, though decrease the available peptide-binding groove. Another drawback is that CLIP accumulation, through loading of CLIP peptide, induces T_h2 polarization (Chaturvedi et al., 2000; Pezeshki et al., 2011; T. A. Rohn et al., 2004). Interestingly, we have shown that accumulation of CLIP through DO overexpression, does not skews T_h response toward T_h2. Therefore, for application of CLIP accumulation in peptide-bSAg hybrid vaccination, overexpression of DO would be a preference.

DO and Th polarization

Our results and those of others have shown that the accumulation of CLIP on the cell surface induces T_h2 polarization (Chaturvedi et al., 2000; Pezeshki et al., 2011; T. A. Rohn et al., 2004), while CLIP accumulation through DO overexpression did not skew T_h response. It has been reported that bSAgs could develop IL-10 producing inducible T_{reg} cells (iT_{reg}) (Taylor et al., 2010). Interestingly, we did not detect iT_{reg} or IL-10 producing T cells while evaluating DO impact on T_h response. For stimulation of naïve CD4 T cells we used SEB that CLIP accumulation has the least effect on its binding to MHC II. Cytokine intracellular staining results indicated less differentiated cells ($IL4^+$ /$IFN\text{-}\gamma^+$) in +DO condition and led us to investigate the difference in stimulation and proliferation of T cells. However, monitoring naïve CD4 T cell proliferation after stimulation with different concentration of SEB did not show any difference in carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. If the larger population of double negative cells ($IL4^-$ /$IFN\text{-}\gamma^-$) in DO overexpression condition is not due to their lower proliferation potential; then DO may slowdown or arrested the differentiation of proliferative T cells in their T_h0 stage of development. This is in line with inhibitory function of DO on DM; as we showed that DMY favors T_h1 polarization so, DO might by arresting T cells on T_h0 stage inhibit T_h1 skewing function of DM. It is fascinating how sole accumulation of CLIP, without involvement of DO, shifts T_h response toward T_h2, while DO-mediated CLIP accumulation does not polarize T_h response. DO may independent of CLIP and through an unknown mechanism arrest and delay differentiation of stimulated and proliferative T cells. In this way, DO may not only regulate function of DM but also autoregulate its own CLIP-accumulating function.

The function of DO in adaptive immune response is still unclear. It has been suggested that DO by inhibiting DM activity generates a tolergenic peptide repertoire (Denzin et al., 1997; Jensen, 1998). Recently it has been shown that DO prevents autoimmunity by

shaping MHC II self-peptide repertoire and promotion of T cell tolerance (W. Yi et al., 2010). However, DO may have more direct effects beside regulation of DM function in antigen presentation. Also it remains to be determined if DO may function as an inhibitor of unleashed T cell stimulation and differentiation by suspending proliferative T cells on their T_h0 stage of development. Accordingly, DO may prevent some autoreactive or pathogenic immune responses by delaying T_h subset decision making point, waiting for more certain and definite third signal of T_h differentiation and polarization. Therefore, make the T cell response and the subsequent immune response more focused and specific (**Figure 4.2.**).

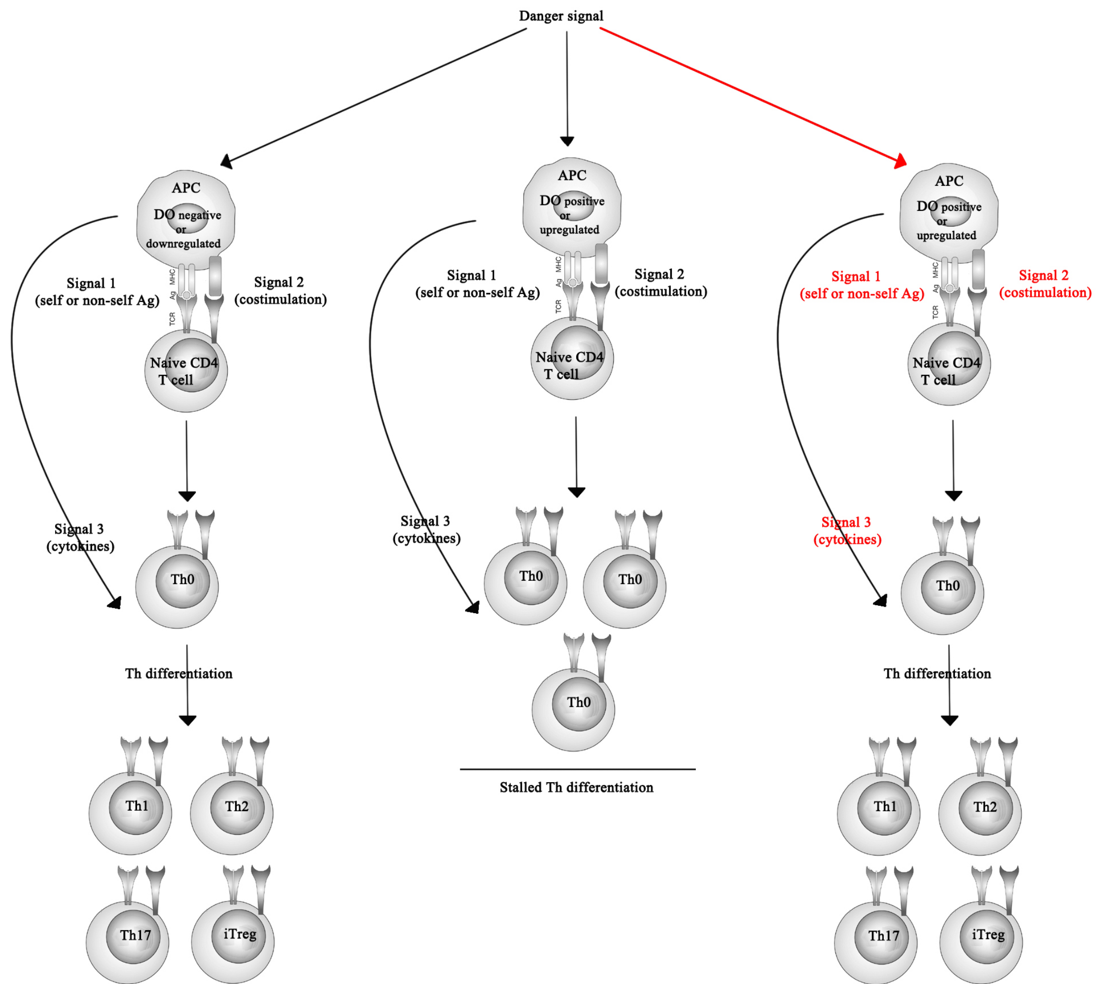

Figure 4.2. Model for effect of DO on T_h differentiation: In lymph nodes APCs present self and non-self antigens to naïve CD4 T cells as first signal of activation. However, to fully activate and differentiate T cells, APCs need to deliver second and third signals. Exogenous and endogenous danger signals, by upregulating costimulatory molecules and secretion of cytokines activate APCs and provide second and third signals. If APCs present self-antigens, this T cell activation and differentiation could lead to autoimmune response. It is possible that expression of DO by APCs delays in subset decision process and keep proliferating cells in T_h0 stage, waiting for more decisive and stronger signals. This decisive signal could be a stronger danger signal or any of three activation signals or a combination of them (text in red indicates stronger signal).

It is very interesting to understand how DMY and DO affect T_h polarization. To identify genes and pathways involved in differentiation of naïve CD4 T cells, under the influence of DMY, CLIP and DO we can perform a comprehensive transcriptome analyses of stimulated T cells. Stimulation of T cells should take place by both specific peptide and SAg, pulsed on APCs under various conditions. The different conditions of APCs would be overexpression of DMY and DO and also accumulation of CLIP on the cell surface. The accumulation of CLIP should take place through pulsing of CLIP peptide on APCs, independent of DM and DO overexpression. Formation of immunological synapse should be analyzed under these conditions to study their influence on peptide-MHC or SAg-MHC accumulation and also on transport of TCR–peptide-MHC or TCR-SAg-MHC complexes into central supramolecular activation cluster (cSMAC). For imaging on lipid bilayer and because of the technical limitation that DO instability would cause, we could use recombinant soluble DO that was developed based on The findings made in our laboratory (Deshaies et al., 2005; Yoon et al., 2012).

CLIP and bSAgs binding to MHC II

DM and DO, beside the direct impact on MHC II, might influence the conformation of MHC II molecules through CLIP and we showed that DO overexpression by accumulating CLIP on cell surface increases binding of bSAgs to MHC II molecules. Although it is not clear how CLIP increases binding of peptide-dependent bSAgs but we assume that unique trait of CLIP especially human CLIP and its affinity for different haplotype of MHC are involved in this observation. Kropshofer et al. have shown the self-release of human CLIP from DR molecules (H. Kropshofer, Vogt, Stern, Hammerling, & Hämmerling, 1995; T. Kropshofer et al., 1995). In endosomal pH, the N-terminal segment of human CLIP (81-89) that is protruding from binding groove, interacts with an effector site outside the binding-cleft of DR and catalyzes the release of its core segment (90-105) that resides in peptide-binding groove (H. Kropshofer, Vogt, Stern, Hammerling, & Hämmerling, 1995; T. Kropshofer et al., 1995). This contact by N-terminal, seems to prevent tight interactions of core segment with specificity pockets in the peptide-binding groove that normally occurs during maturation of long-lived MHC-peptide complexes (Kropshofer et al., 1995). These

data were supported by previous observations that treatment of DR–peptide complexes with acidic pH provoked release of CLIP, but only in longer types of CLIP (Urban, Chicz, & Strominger, 1994). Therefore, longer CLIPs are more susceptible to self- release. The self-release of CLIP explains why only less than 50% of DR molecules in DM-deficient cell lines carry CLIP (Riberdy et al., 1992; Sette et al., 1992). This is in contrast with the results obtained with H2-M deficient mice where surface IA^b molecules are almost exclusively loaded with CLIP (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). This inconsistency maybe due to shortness of the CLIP peptide (86-105) bound to $I\text{-}A^b$, which lacked most of the sequence required for self-removal (Riberdy et al., 1992; Rudensky et al., 1991). The difference between affinity and stability of DR-CLIP and $I\text{-}A^b$-CLIP complexes could be another reason. $I\text{-}A^b$-CLIP complex are SDS-stable at room temperature while DR-CLIP complexes are unstable (Miyazaki et al. 1996; W. D. Martin et al. 1996a; Riberdy et al., 1992; E. Mellins et al. 1990). The MHC type of H2-O KO mice that we used for SAgs binding experiments is $I\text{-}A^b$ and inconsistency between human and mice system could be due to shortness of murine CLIP and/or the different affinity of CLIP for DR and $I\text{-}A^b$. We may find the same effects on bSAgs binding as in human, by using SDS-unstable mice MHC II.

Perspectives

The *in vivo* confirmation of DMY potency should be tested in a preclinical murine tumor model. The B16-Ova melanoma model in C57BL/6 mice would be suitable for this purpose. Bone marrow derived DCs, expressing or not DMY, should be pulsed with Ova peptide and used to immunize mice subcutaneously with 7 days intervals. Tumor challenge would be at day 14 by subcutaneous injection of B16-Ova cancer cells. Tumor growth and survival of mice should be monitored overtime. Efficiency of antigen presentation could be monitored by exposure of irradiated splenocytes to OT-I and OT-II T cells on days 1 and 8.

Conclusion

Collectively, our data point out that DM is functional at cell surface level. DMY quantitaivelly improves the level of peptide loading on DCs. DMY is able to fine tune MHC-peptide conformers on cell membrabe and in physiological pH. We showed that DMY shifts T_h cell response toward T_h1. These findings show that DMY could quantitatively and qualitatively improves peptide-based-DC vaccines.

Our results also point out a non-appreciated function of MHC II antigen presentation pathway components. We showed the influence of DO on bSAgs binding that might has potential for application in vaccination approaches. The influence of DM and DO on T_h response deserves to be investigated in details for better understanding of their functions and their effects on immune response.

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CHAPTER .6 - Supplemental Article:

Evidence for a human leucocyte antigen-DM-induced structural change in human leucocyte antigen-DOβ

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IMMUNOLOGY ORIGINAL ARTICLE

Evidence for a human leucocyte antigen-DM-induced structural change in human leucocyte antigen-DOβ

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Summary

Human leucocyte antigen (HLA)-DO is a non-classical major histocompatibility complex class II molecule which modulates the function of HLA-DM and the loading of antigenic peptides on molecules such as HLA-DR. The bulk of HLA-DO associates with HLA-DM and this interaction is critical for HLA-DO egress from the endoplasmic reticulum. HLA-DM assists the early steps of HLA-DO maturation presumably through the stabilization of the interactions between the N-terminal regions of the α and β chains. To evaluate a possible role for HLA-DM in influencing the conformation of HLA-DO, we made use of a monoclonal antibody, Mags.DO5, that was raised against HLA-DO/DM complexes. Using transfected cells expressing mismatched heterodimers between HLA-DR and -DO chains, we found that the epitope for Mags.DO5 is located on the DOβ chain and that Mags.DO5 reactivity was increased upon cotransfection with HLA-DM. Our results suggest that HLA-DM influences the folding of HLA-DO in the endoplasmic reticulum. A mutant HLA-DO showing reduced capacity for endoplasmic reticulum egress was better recognized by Mags.DO5 in the presence of HLA-DM. On the other hand, an HLA-DO mutant capable of endoplasmic reticulum egress on its own was efficiently recognized by Mags.DO5, irrespective of the presence of HLA-DM. Taken together, our results suggest that HLA-DM acts as a private chaperone, directly assisting the folding of HLA-DO to promote egress from the endoplasmic reticulum.

Keywords: antigen presentation; antigen processing; B cells; human; major histocompatibility complex

Introduction

Major histocompatibility complex class II molecules (MHC II) present antigenic peptides to specific $CD4^+$ T lymphocytes.[1] Once synthesized in the endoplasmic reticulum (ER), classical MHC class II αβ heterodimers associate with the invariant chain (Ii). This chaperone stabilizes the MHC II and targets the complex to the proper endosomal/lysosomal compartments.[2–5] In the endocytic pathway, Ii is cleaved by proteases to generate the Class II-associated invariant chain peptide (CLIP) fragment which occupies the peptide-binding groove of the MHC II.[6] Exchange of CLIP for cognate antigenic peptides or polypeptides is catalysed by human leucocyte antigen (HLA) -DM (DM), a class II molecule found within specialized lysosomal structures named MHC class II compartments (MIICs).[7–10]

In contrast to the classical MHC II HLA-DR (DR), -DP and -DQ, DM is a non-classical MHC II molecule mainly because of its monomorphic nature.[11] The inability to present antigenic peptides and the intracellular localization of DM are in line with an indirect role in antigen presentation for this molecule.[8,10,12] Besides its activity on CLIP, DM shapes the final peptide repertoire displayed by a given MHC II isotype.[9,13–15] DM chaperones empty classical class II molecules, allowing binding of suitable peptides capable of filling pocket 1 and of making hydrogen bonds with the DRα backbone.[16–20]

DM and DR interact principally in acidic vesicles and membrane colocalization increases the efficiency of

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peptide exchange.[21] The mutual recognition would involve fine structural changes in both molecules. A DM-susceptible, flexible isoform of DR may arise from changes induced by inadequate peptides and from protonation of histidine α33 at low pH.[22,23] For DM, many experiments also suggest that protonation in the endocytic pathway results in minor, reversible structural changes exposing hydrophobic regions of the heterodimer.[24–26] Ullrich *et al.* used 8-anilino-1-naphthalenesulphonic acid (ANS), a fluorescent dye binding to hydrophobic protein patches, to demonstrate subtle pH-induced changes in purified DM and DR molecules.[25] Since the interaction of DM with DR reduces ANS binding to both molecules, it was postulated that the surface of contact is comprised of pH-sensitive regions on both proteins.[7,24,25] In line with this hypothesis and with the critical role of the peptide N-terminal region, Mellins and coworkers recently proposed a model for the DR–DM interaction based on elegant functional mutagenesis data.[27,28]

Another non-classical MHC-related molecule, HLA-DO (DO), was shown to accumulate in MIIC-like compartments.[29] DO was first described as a DM inhibitor since its overexpression in class II transactivator-transfected cells increased the fraction of classical class II molecules bound to the CLIP fragment.[30,31] However, mouse B cells proficient or deficient in H2-DO do not exhibit dramatic variations in the levels of CLIP bound to I-A^{b}.[32–34] It was proposed that H2-DO promotes the presentation of antigens internalized by membrane immunoglobulins by selectively inhibiting the activity of H2-DM in early endocytic compartments rich in proteins internalized by fluid-phase endocytosis.[33] This hypothesis is consistent with an active inhibitory role of DO *in vitro* at mildly acidic pH (early endosomes) and to a poor inhibitory potential at low pH (MIIC and lysosomal compartments).[33] Also, this model is consistent with results obtained using splenocytes from mice with a targeted mutation in the *H2-DOA* genes and which showed slightly increased efficiency in the presentation of soluble antigens.[33] Accordingly, antigen-presenting cells from H2-DO- or DO-overexpressing transgenic mice showed reduced presentation of exogenous antigens.[32,35] Others suggested that DO stabilizes DM and promotes stable MHC–peptide complexes when physiological conditions are met.[36] Interestingly, the recent demonstrations of DO expression in specific DC subsets argue against a specific role in B cells in the context of the surface immunoglobulin antigen uptake.[37–39] Still, it is now accepted that H2-DO and DO 'modulate' MHC class II antigen processing.[32,34,36,40]

In human B lymphocytes, as opposed to their mouse counterpart, the majority of DO molecules was found associated with DM[30,36,39,41] and this association allows DO to egress the ER.[29,42] The lack of a recognizable di-basic ER retention motif (RXR or KKXX) suggests that the assembled DOαβ heterodimer is improperly folded in the absence of DM and most probably unstable. Misfolding of newly synthesized proteins is known to induce retention and aggregation in the ER.[43] So far, the mode of interaction between DO and DM remains poorly characterized. We have recently shown that DM binds the DOα1 domain, probably by stabilizing the N-terminal regions of DO to allow ER egress.[44] In the present study, using a conformation-dependent DO-specific antibody, we confirm that DM binding impacts on the conformation of DO.

Materials and methods

Plasmids and complementary DNAs

RSV.3DR$_{18}$/DOβ, pBSDOα.9, RSV.5neo DRα, pBudCE4-A, pBud DOαβ, pBud DM, pBud DRβ and RSV.5gptDN1 have been previously described.[44–46] The DOβ complementary DNA (cDNA) from the 721.45 cell line.[47] was excised with *Bam*HI and the 1·3-kilobase fragment was cloned into the RSV.5neo plasmid to generate RSV.5neo DOβ. Then, RSV.5neo DR$_{18}$/DOβ (cDOβ) was generated by inserting the *Mlu*I–*Eco*RV fragment of RSV.3 DR$_{18}$/DOβ into RSV.5neo DOβ. The DR$_{18}$/DOα chimeric cDNA (cDOα) was made by overlap extension polymerase chain reaction (PCR) as described.[45] using the DRα cDNA cloned in the *Bam*HI site of pBlueScript (Stratagene, La Jolla, CA), and RSV.5gptDN1 (DOα) as templates. The sequences of the oligonucleotides used for PCR are available upon request. The *Sal*I–*Pvu*II fragment encompassing the junction between DR and DO was subcloned into RSV.5gptDN1. The nucleotide sequence was confirmed by DNA sequencing. A 2-kilobase pair *Bam*HI fragment was either cloned into the *Bgl*II site of pBudCE4-A DRβ to generate pBudDR$_{18}$/DOα $^{+}$DRβ or cloned into SRαpuro to generate SRαpuroDR$_{18}$/DOα.

The DM variant devoid of its YxxL motif (DMY) was generated by mutating the tyrosine 230 in the DMβ cytoplasmic region. PCR overlap reactions were performed on pBS 1-DMβ.1.[45] and the products were mixed in a final PCR using flanking primers. This PCR product was digested with *Sac*I and *Hin*dIII, cloned into pBS 1-DMβ.1 and sequenced (pBSDMβY230A). A *Sal*I–*Xba*I fragment was subcloned into pBud DMα[46] to generate pBud DMY.

Antibodies

Monoclonal antibodies (mAbs) L243 (DRα-specific), XD5.117 (XD5; DRβ-specific), MAP.DM1 (DM-specific) as well as the rabbit sera against DOα, calnexin and DMβ have been described previously.[45,46,48,49] Anti-actin (immunoglobulin G1; IgG1) is specific for the N-terminal of the molecule (Chemicon International, Temecula, CA). HKC5 is an IgG1 mouse mAb specific for the cytoplasmic tail of DOβ[49] Mags.DO.5 mAb was generated from mice

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immunized with purified DO/DM complexes and was described previously.[50] Secondary antibodies were the Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR), peroxidase- and biotin-coupled goat anti-rabbit antibody (Bio/Can Scientific, Mississauga, Canada) and Texas red-coupled streptavidin (Jackson ImmunoResearch, West Grove, PA). All these antibodies were titrated and used at saturating concentrations.

Cell lines and transfections

HeLa DRα $^{+}DR_{18}$/DOβ (DRα/cDOβ), HeLa DO and HeLa DM.5 were previously described.[45,46] Raji and HeLa cells were kindly provided by Dr R.P. Sékaly. HEK293T cells were obtained from Dr Eric Cohen. Cells were cultured in Dulbecco's modified Eagles's minimal essential medium (DMEM), 10% fetal bovine serum. HeLa cells were cotransfected by the calcium phosphate precipitation method.[51] using 2–20 μg of each DNA or transfected with Fugene6 (Roche Diagnostics, Laval, Canada) using 1 μg of each DNA.[45] HeLa DM.5 cells were transfected with Fugene6. HeLa cDO were sorted on magnetic beads (Invitrogen, Toronto, Canada) after staining with Mags.DO5. For transient expression, HEK293T cells were transfected by the calcium phosphate precipitation method using 2 μg of each DNA. Cells were analysed 2 days post-transfection.

Flow cytometry

Cells were harvested using trypsin, washed and stained for surface expression. For intracellular staining, cells were treated with formaldehyde for 20 min, then with 50 mM NH_4Cl for 15 min and permeabilized with phosphate-buffered saline/bovine serum albumin containing 0·05% saponin.[45] Cells were analysed on a FACSCalibur (Becton Dickinson, San José, CA).

Fluorescence microscopy

HeLa cells were plated on coverslips in 24-well plates and cultured for 2 days before intracellular staining as described for flow cytometry analysis. Cells were analysed by fluorescence microscopy on a Zeiss axioplan 2 imaging microscope. Photographs were taken with a Sony DXC-390P digital camera.

Immunoprecipitation, Western blotting and endoglycosidase H treatment

Cells (10^7) were trypsinized, washed in phosphate-buffered saline and lysed into Triton-X100.[45] Post-nuclear supernatants were prepared and analysed directly on immunoblots or used for immunoprecipitations overnight at 4° using primary antibodies bound to protein-G coupled to sepharose 4B (GE Lifesciences, Piscataway, NJ). Samples were analysed on Western blots.[45] For EndoH treatment, lysates containing 2×10^5 cells were directly digested with Endoglycosidase H for 30 min at 37° (New England Biolabs, Pickering, Canada) and resuspended in reducing loading buffer. Samples were boiled and analysed by Western blotting.

Adenovirus production and HeLa cells infection

Production of recombinant adenoviruses (Ad) was as described.[52] AdDO encode both DOα and DOβ. A mutant AdDO was obtained by the same strategy using a DOβ cDNA with substitutions at positions V184H, V186K and E187K (AdDO VVE). The empty adenoviral vector (Ad0) is a replication-deficient recombinant adenovirus derived from serotype 5 with the deletion of E1 and E3 regions.[53]

For adenovirus transduction, HeLa cells were distributed in flat-bottom, 24-well plates at $2{\cdot}5 \times 10^5$ cells/well in 250 μl DMEM without serum. Viruses were added at a multiplicity of infection of 50 and incubated at 37° in the presence of 5% CO_2 for 3 hr. Complete medium was added and cells were cultivated for 2 days.

Results

Mags.DO5 binds the HLA-DOβ chain

In DM-negative cells, transfected DO was shown to be retained in the ER.[29] We postulated that DM assists the folding of the DOαβ heterodimer, allowing its maturation. To gain insights into a possible impact of DM expression on the structure of DO, we made use of the Mags.DO5 mAb obtained following immunization of mice with DO/DM complexes purified from a human B-cell line.[50] Given our recent demonstrations that DM binds to the α chain of DO[44] and that Mags.DO5 recognizes the DO/DM complex,[50] we hypothesized that this mAb binds to the DOβ chain. This was verified using mixed DR/DO pairs expressed in class II-negative HeLa cells.[54] These mixed heterodimers (DRα/cDOβ and cDOα/DRβ) overcome ER retention and were made possible by exchanging the first 18 amino acids of DO for the corresponding sequence of DR (Fig. 1a,b). The cell surface expression of DRα/cDOβ and cDOα/DRβ was controlled using DR-specific antibodies against the α and β chains, respectively (Fig. 1c). Mags.DO5 recognized the DRα/cDOβ mixed pair expressed on HeLa cells but not its cDOα/DRβ counterpart. Although we cannot entirely rule out that the DOα chain somewhat contributes to the efficiency of antibody binding, these results suggested that Mags.DO5 is specific for an epitope located on the DOβ chain. In support of this, although Mags.DO5 does not

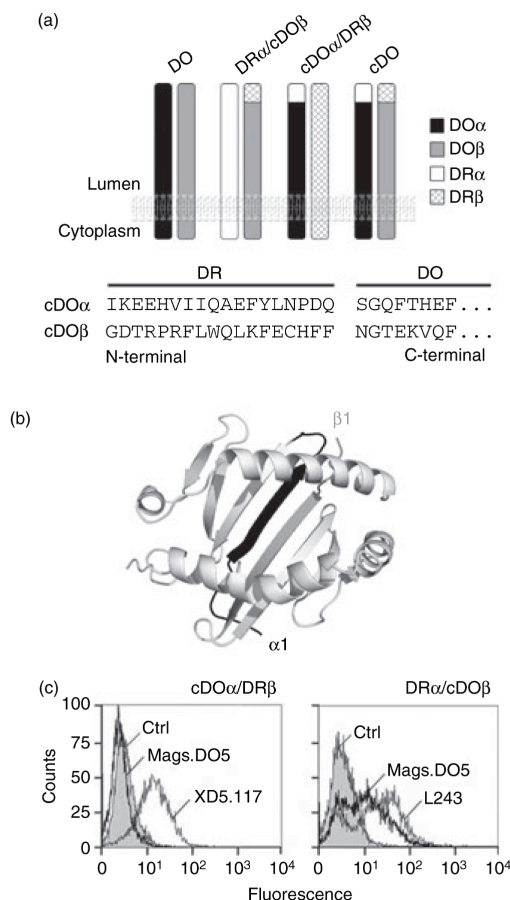

Figure 1. Mags.DO5 epitope is located on the β chain of DO (a) Schematic representation of the various DO constructs used in this study. The predicted amino acid sequence of the N-terminal region of mature cDO chains is shown. (b) The position of the DR α (black) and β (dark grey) regions grafted on cDO chains are highlighted on a top view of human leucocyte antigen-DR. α1 and β1 indicate the position of the N terminus of each domain. (c) Flow cytometry analysis of HeLa cells stably expressing mixed cDOα/DRβ or DRα/cDOβ pairs and stained for cell surface expression using DO-specific Mags.DO5 (bold line), DRβ-specific XD5.117 (thin line; left panel) or DRα-specific L243 (thin line; right panel). Filled histograms represent control staining using HeLa cells incubated only with the Alexa-488-coupled secondary GAM antibody.

bind DR, we observed some competition between this mAb and an anti-serum made against the DRα/cDOβ mixed pair (data not shown).

Mags.DO5 recognizes a conformational epitope

Mags.DO5 does not recognize denatured DOβ (data not shown) suggesting that the mAb recognizes a conformational epitope. This was confirmed by flow cytometry on permeabilized cells. Mags.DO5 did not recognize the DOα chain when expressed independently. However,

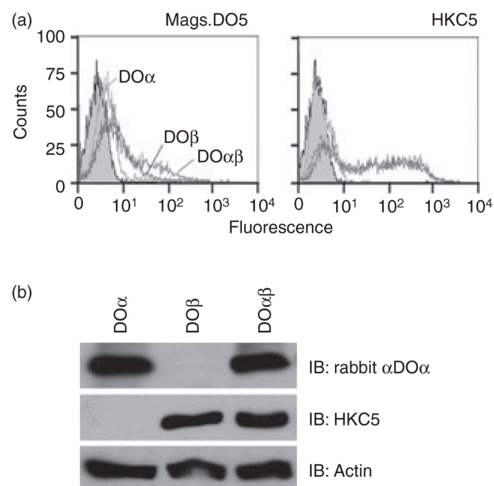

Figure 2. Mags.DO5 monoclonal antibody (mAb) is conformational (a) HEK293T cells were transiently transfected with either DOα alone (light grey), DOβ alone (dark) or DOα +DOβ (grey) complementary DNAs and stained with Mags.DO5 and HKC5 after permeabilization. Cells were analysed by flow cytometry. Filled histograms represent control cells stained with the GAM-Alexa-488 secondary antibody. This experiment was done a minimum of 10 times with similar results. (b) Cell lysates were prepared and analysed on immunoblots for the expression of DOα (upper panel), DOβ (middle panel) and actin (lower panel).

slight reactivity to the free DOβ chain was detected and this reactivity increased slightly by the expression of DOα and DOβ together (Fig. 2a). The equivalent binding of HKC5, a mAb specific for a linear epitope on the DOβ cytoplasmic tail,[49] confirms that the observed increased binding of Mags.DO5 in the DOA/DOB transfectant was not caused by an accumulation of β chain in the presence of DOα (Fig. 2). Semi-quantitative Western blotting with HKC5 confirmed that both transfectants express similar levels of DOβ chain (Fig. 2b). Collectively, these results support the notion that Mags.DO5 recognizes a conformational epitope located on DOβ. The conformation change may be subtle as some antibody reactivity is observed on the isolated β chain.

Coexpression of DM with DO enhances Mags.DO5 reactivity

Although Mags.DO5 was generated following immunization of mice with DM/DO complexes, the mAb is specific for DO because it does not recognize DM[50] (data not shown). However, cotransfection of DM and DO together into 293T cells clearly showed that Mags.DO5 reacted more strongly to DO when complexed with DM (Fig. 3a). These differences were not the result of differences in DOβ expression levels because HKC5 staining was identical in the absence and presence of DM (Fig. 3a). The

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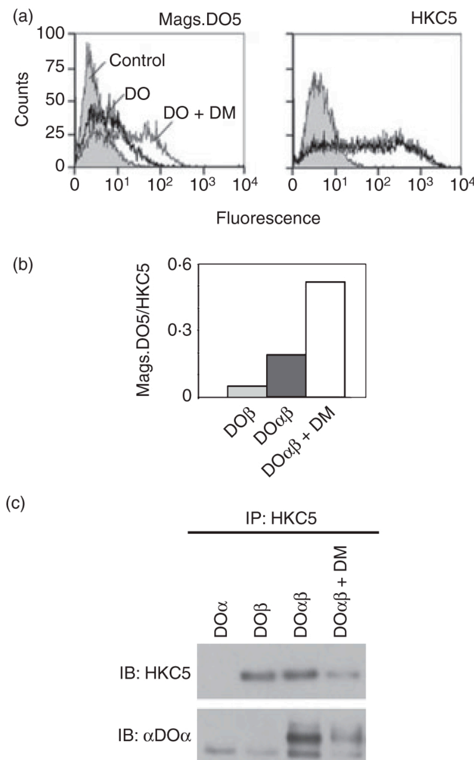

Figure 3. Coexpression of DM increases Mags.DO5 reactivity (a) HEK293T cells were transfected with either DO (thick line) or DO $^{+}$DM (thin line) cDNAs, fixed, permeabilized and stained after 48 hr with Mags.DO5 (left panel) and HKC5 (right panel) monoclonal antibodies (mAbs). (b) HEK293T cells were transiently transfected with either DOβ alone, DOαDOβ or DOαDOβ^{+} DM complementary DNAs (cDNAs) and stained with Mags.DO5 and HKC5 before flow cytometry analysis. The *y*-axis represents the ratio between the mean fluorescence values obtained for the two mAbs. Similar results were obtained in three other independent experiments. (c) HEK293T cells were transiently transfected with either DOα alone, DOβ alone, DOαDOβ or DOαDOβ $^{+}$DM cDNAs. After 48 hr, cells were lysed and immunoprecipitation was performed using the DOβ-specific HKC5 mAb. Samples were analysed on immunoblots by probing with HKC5 or the DOα-specific rabbit antiserum.

positive impact of DM is most apparent when the mean fluorescence values obtained by flow cytometry are expressed as a ratio between Mags.DO5 and HKC5 (Fig. 3b). Importantly, DOβ and DOα were produced from a bi-cistronic vector to minimize transfection-dependent variations in the relative expression of the two chains. Indeed, immunoprecipitation of DOβ revealed that αβ heterodimers were formed, even in the absence of DM (Fig. 3c). The increased Mags.DO5 reactivity in the presence of DM was also observed in transfected HeLa cells (see below).

DO conformation change occurs in the ER

The possibility remained that the effect of DM was indirect and simply the consequence of its ability to target DO to acidic compartments. Indeed, Liljedahl *et al.* demonstrated that DO undergoes a conformation change at low pH.[33] To evaluate if the Mags.DO5 reactivity pattern described above depended on accumulation of DO/DM complexes in acidic vesicles, we transiently expressed DO with wild-type DM or with a DM variant (DMY) devoid of its YxxL lysosomal sorting signal.[44] DMY interacts with DO and the complex accumulates at the plasma membrane (Fig. 4a). Cells expressing DO with DM or DMY were permeabilized and stained with Mags.DO5 (Fig. 4b,c). The results showed that the increase in the reactivity of Mags.DO5 was independent of DO localization to intracellular acidic compartments.

To test if DM interaction with DO in the ER promoted the formation of the Mags.DO5 epitope, we examined mAb reactivity for a DO molecule that bears a triple mutation in the β2 domain (DO VVE) (Fig. 5a). These mutations, originally described in DR, were shown to affect the interaction with DM.[27] When introduced separately into DOβ, these mutations did not affect the binding to DM. However, when introduced together, these three mutations affected the integrity of DO and strongly impaired ER egress (Deshaies and Thibodeau, unpublished data). The mutant β chain was transduced together with DOA into HeLa cells either expressing DM or not.

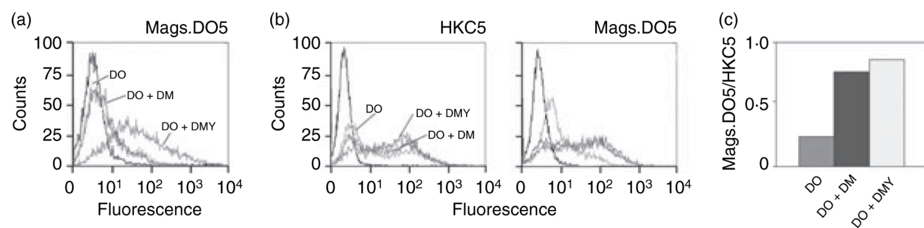

Figure 4. DM-induced conformation change in DO occurs in endoplasmic reticulum (ER) (a) HEK293T cells were transfected with DO alone, DO and DM wild-type (wt) or DO and DMY. After 48 hr, cell surface DO was stained using Mags.DO5. The mean fluorescence values were 119 and 194 for DM and DMY, respectively (not shown). (b) Cells were permeabilized and stained with HKC5 (left panel) or Mags.DO5 (right panel). (c) Mean fluorescence values obtained in (b) were plotted as the Mags.DO5 over HKC5 ratio. This experiment is representative of three independent experiments.

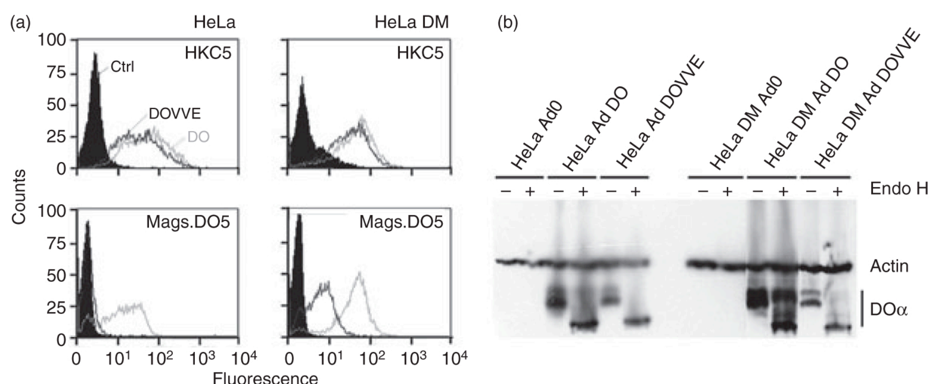

Figure 5. DM affects the conformation of a transport-incompetent DO mutant. (a) HeLa (left panels) and HeLa DM (right panels) cells were transduced with AdDO (thin line) or AdDO VVE (thick line) adenoviral constructs. Cells were fixed, permeabilized and stained with HKC5 (upper panels) or Mags.DO5 (lower panels). (b) Cells were lysed and half of the post-nuclear supernatants was treated with EndoH. Samples were analysed on immunoblots using first the DOα-specific rabbit antiserum and then the control actin-specific probe. Similar results were obtained in an independent experiment.

DO VVE showed a reduced capacity to egress the ER and to acquire complex EndoH-resistant carbohydrates in both the presence and absence of DM (Fig. 5b). Although the ER-retained DO VVE was expressed at levels similar to wild-type DO, this mutant was not efficiently recognized by Mags.DO5 in the absence of DM. Coexpression of DM partially restored Mags.DO5 reactivity, supporting the idea that assembly of DM with DO in the ER generates the epitope recognized by Mags.DO5. Moreover, cells transfected in the presence of brefeldin A exhibited a block in the ER exit of DO/DM complexes but the treatment did not prevent DM from increasing Mags.DO5 reactivity (data not shown).

Relationship between folding of DO and the reactivity of Mags.DO5

We postulated that Mags.DO5 would recognize properly folded DO molecules independent of the presence of DM. To test this hypothesis, we stably expressed a chimeric DO (cDO) in which the first 18 amino acids of both DOα and β chains were substituted for those of the corresponding DR chains (Fig. 1). As opposed to wild-type DO which colocalizes with the ER marker calnexin, the cDO heterodimer was able to egress the ER in DM-negative HeLa cells and to accumulate in Lamp-1$^+$ vesicles (Fig. 6a and data not shown). In contrast to wild-type DO, the conformation-dependent Mags.DO5 mAb recognized cDO as efficiently as HKC5 in stably transfected HeLa cells (Fig. 6b). Such strong Mags.DO5 staining is reminiscent of the pattern observed for DO in DM$^+$ cells (Figs 3 and 5).

Since Mags.DO5 reacted strongly with cDO in the absence of DM, we postulated that the Mags.DO5 staining on correctly folded cDO molecules was optimal and could not be improved by coexpressing DM. We first ascertained that the modifications imparted to the DO molecule through the fusion of the first 18 amino acids of DR would still allow a strong interaction with DM. Both DM and DO were transiently transfected in 293T cells and DO was immunoprecipitated from detergent lysates. Western blot analysis showed that DM was efficiently coimmunoprecipitated with cDO in both CHAPS and Triton X-100. This suggests that the cDO/DM interaction is representative of the one observed for DO. This is different from the association of DM/DO with DR, which dissociates from DM upon lysis of Raji cells in Triton X-100 (Fig. 6c). However, as expected, coexpression of DM did not increase the reactivity of Mags.DO5 to cDO (Fig. 6d). Consequently, the Mags.DO5 mAb recognizes properly folded DO molecules.

DM binding discloses the Mags.DO5 epitope on DO

Braunstein and Germain carefully titrated the mAb concentration to determine if avidity or affinity of a class II-specific mAb could explain the differential staining observed between transfectants expressing mismatched MHC heterodimers.[55] The rationale behind such experiments is that at limiting concentrations of the mAb, antigenic molecules of different intrinsic affinities toward the mAb will show divergent staining intensities, even if expressed at similar levels. On the other hand, in these conditions, antigens with the same affinity will show similar binding (read out by flow cytometry as mean fluorescence value) even if expressed differentially. We used a similar approach to determine if the enhanced reactivity of Mags.DO5 for DO in the presence of DM was the result of a difference in affinity or avidity.

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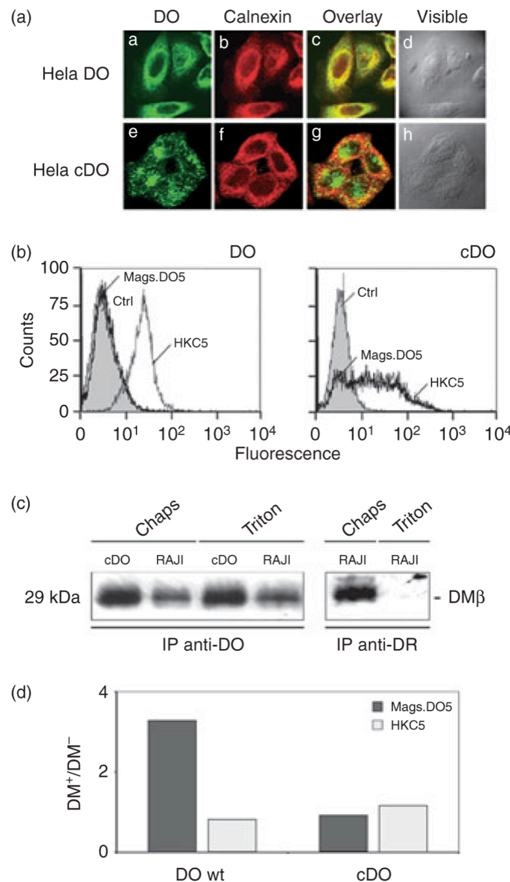

Figure 6. Transport-competent cDO molecules reveal the Mags.DO5 epitope independent of DM (a) Immunofluorescence microscopy analysis of cDO subcellular localization. HeLa DM-negative cells were stably transfected with either wild-type (wt) DO (a–d) or cDO chimera (e–h), permeabilized and incubated with Mag.DO5 and rabbit anti-calnexin followed by Alexa 488-labelled goat anti-mouse antibodies (a,e), biotinylated goat anti-rabbit antibody and Texas-red conjugated streptavidin (b,f). (c) and (g) show the merge of wt DO or cDO images with those obtained for calnexin. (d) and (h) show the cells in visible light. (b) Flow cytometry analysis of HeLa cells stably transfected with DO (left panel) or cDO (right panel). Cells were permeabilized and stained with Mags.DO5 or HKC5. (c) HEK293T cells were transfected with cDO and DM, lysed in Chaps or Triton X-100 (Triton) and DO was immunoprecipitated with the DOα-specific rabbit antiserum. Samples were analysed on immunoblots using the DMβ-specific rabbit antiserum. Control Raji cells were lysed in the same conditions and immunoprecipitation was performed for DO or for DR using XD5. (d) HEK293T cells were transiently transfected with DO or cDO in the absence or presence of DM. After 48 hr, cells were permeabilized and stained with Mags.DO5 and HKC5. Mean fluorescence values obtained for DM$^+$ and DM$^-$ cells were plotted as a ratio. These ratios are representative of at least two other experiments.

DO with or without DM was transfected into 293T cells and stained with titrated amounts of Mags.DO5 after permeabilization (Fig. 7). At low or saturating concentrations of mAb, the staining was always more efficient on cells coexpressing DM, suggesting that the affinity of the mAb is different for DO or DO/DM complexes. As expected, the control HKC5 showed similar staining patterns independent of DM expression. These results suggest that in the absence of DM, the Mags.DO5 affinity is weak for DO. Upon DM binding, DO most probably goes through a conformation change that fully unveils the epitope.

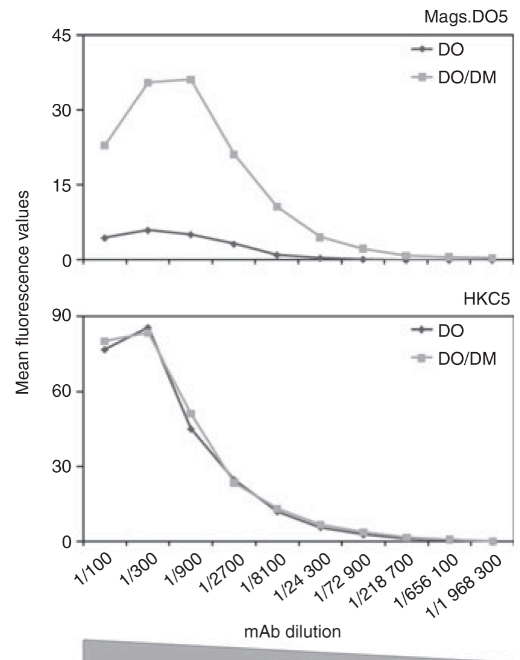

Figure 7. Coexpression of DM reveals the Mags.DO5 epitope on DO molecules. HEK293T cells transiently expressing DO or DO $^+$DM were permeabilized 48 hr post-transfection and stained with serial dilutions of the Mags.DO5 (top panel) or HKC5 (lower panel) monoclonal antibodies. These experiments were repeated three times with similar results.

Discussi n

In transfected HeLa cells and in mouse B lymphocytes, under the experimental conditions tested so far, DO or H2-DO molecules did not egress the ER on their own.[29] In the present study, we sought to determine if DM affects the quaternary structure of DO upon formation of the complex in the ER. Interestingly, Liljedahl *et al.* showed by immunoprecipitation that DOα and β chains interact in DM-negative cells.[29] As the primary sequence of the α or β chain do not display known ER retention signals, we postulated that the conformation of DO was not optimal and that the heterodimer was most likely retained by ER chaperones. Consequently, DO does not acquire post-translational modifications in the form of complex Endo H-resistant sugars.[29]

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Early after their synthesis in the ER, MHC II α and β chains must associate with Ii to avoid aggregation and premature degradation. Although the exact sequence of events is still a matter of debate, formation of the nonameric complex with Ii prevents aggregation of MHC II.[56] Quality control chaperones like BiP, GRP94, calnexin, calreticulin and ERp72 have been implicated in the ER retention of class II molecules in the absence of Ii.[1] Although binding to these proteins has yet to be demonstrated, we can assume that DO will probably be under the control of similar checkpoints. The association of DO with such public chaperones probably persists until DM intervenes to optimize folding into a more stable, lower energy conformation and to generate a transport-competent multi-unit complex. The fact that the Mags.DO5 staining increases upon coexpression of DM indicated that a conformation change occurs on DO. Although this had been suggested previously from the fact that DM allows DO egress from the ER,[29,42] our results demonstrate for the first time that DM acts on the conformation of DO. We ruled out by flow cytometry that DM simply increases the steady-state amount of DO because the staining with the HKC5 mAb did not increase compared to Mags.DO5. Also, immunoprecipitation experiments revealed similar amounts of DO in DM-positive or -negative cells. The exact nature of the conformation change has not been determined. The membrane distal domains of DO are more hydrophobic than classical MHC II and may need to be shielded by DM, even more so knowing that the groove is empty. Also, the DM may be involved in the oxidation of DO.[37,57] We have tested on DO the reactivity of increasing concentrations of Mags.DO5 and HKC5 in DM^+ and DM^- HEK293T cells (Fig. 7). Although DM clearly impacts on the staining of DO at limiting Mags.DO5 concentrations, such titration experiments may somehow be difficult to interpret in terms of antibody affinity or avidity as we have not tested in parallel a DM^+ control cell line expressing various levels of DO. Still, coupled to our immunoblot and immunoprecipitation data showing equivalent amounts of DOβ in DM^+ and DM^- HEK293T cells (Figs 2b and 3c), these results are in line with an increased affinity of Mags.DO5 for DO/DM complexes as compared to free DO.

Three complementary results allow us to conclude that the conformational change on DO takes place early during maturation and is not merely the consequence of DM-induced transport to the Golgi or the endocytic pathway. First, brefeldin A did not prevent the stronger recognition of DO by Mags.DO5 in the presence of DM (data not shown). Second, point mutations in DO that clearly impair ER egress did not prevent the ability of DM to increase Mags.DO5 binding. Finally, diverting the flow of DO/DM complexes to the plasma membrane instead of the lysosomal compartments by mutating the YxxL motif of DM did not perturb Mags.DO5 recognition. Altogether, the data strongly suggested that the DM-induced conformation change on DO occurs as early as in the ER and they rule out an indirect role of DM in increasing Mags.DO5 reactivity following glycan modification in the Golgi or protonation in acidic vesicles.

Studies on mixed pairs suggested a *cis*-coevolution of α and β chain polymorphisms favouring pairing of molecules from a given haplotype.[58] A role for Ii in facilitating ER egress of mismatched heterodimers has been described.[59] Although DO behaves very much like a mismatched pair, Ii does not seem to affect its sorting.[29] Our results show that DM acts as a chaperone and may give some clues as to the mechanism by which it allows DO to egress the ER. We have recently discovered a point mutation that resulted in some DO egress independent of DM.[44] We proposed that this DOαP11V mutation might improve the folding of the N-terminal regions of DOαβ and reduce the need for DM. This contention is supported by the demonstration that cDO reactivity with Mags.DO5 is maximal and independent of DM. In line with this hypothesis, using the Mags.DO5 conformational mAb, we demonstrated here that DM affects the conformation of DO.

Mags.DO5 was made by immunizing mice with purified DO/DM complexes and we found that the epitope recognized by this mAb is located on the DOβ chain. The possibility that DM contributes to the epitope is unlikely considering that the Mags.DO5 reactivity toward cDO was not increased in the presence of DM (Fig. 6) The fact that amino acid changes in the N-terminal regions of DOα and β improved Mags.DO5 reactivity may indicate that the epitope is located in the membrane-distal β1 domains. Accordingly, DM improved Mags.DO5 binding on the DO VVE mutant, which most likely suffers from a severe conformation defect in its β2 domain. The DM-induced conformation change may be somewhat drastic but still, it could not override the negative impact of the DO VVE mutations on ER egress.

A chaperone role for DM in the ER was suggested by a study showing that in the absence of invariant chain, DM can improve the transport of MHC II.[60] However, the conformation change described here is more reminiscent of the appearance of the 16.23 epitope on DR3 molecules having encountered DM. Verreck *et al.* showed that this effect on the fine structure of the DR3 was independent of CLIP removal or of the peptide editing capabilities of DM.[61] Also, the well-characterized stabilization of peptide-free MHC II molecules points to a chaperone role of DM that is not directly related to its catalytic activity.[17] In this respect, DM would recognize DO just like any other empty MHC II.[62]

Whether DO affects the structure of DM remains to be established. The inhibition of DM by DO is certainly in line with a reciprocal effect of the two molecules on their respective fine structures. This could also explain the

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apparent increase in H2-DM half-life in the presence of H2-DO.[35] Comparing the crystal structures of isolated versus associated DO and DM would shed light on the interplay between the two chaperones.

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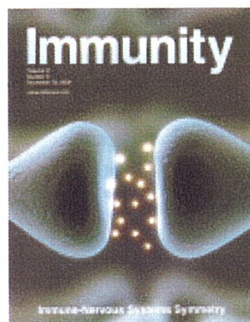

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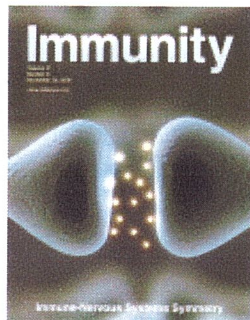

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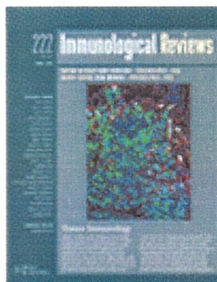

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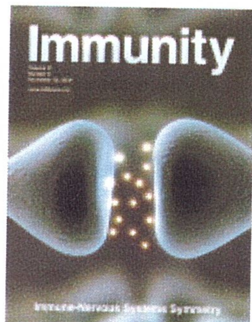

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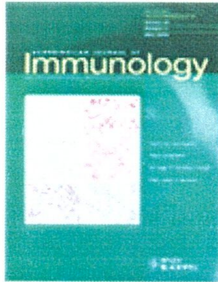

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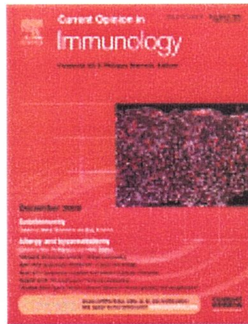

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