Chapter 11

Processing and Presentation of Glycoproteins in the MHC Class I and II Antigen Presentation Pathways

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Abstract

$CD8^+$ and $CD4^+$ T cells are stimulated by peptides bound to MHC class I and II respectively. The processing pathways for the generation of class I or class II binding peptides are specialized in surveying different intracellular compartments. While class I peptide loading occurs in the ER, class II loading occurs in the endocytic compartments. Many of the peptides generated in either compartment are derived from glycoproteins from normal or malignant cells or from intracellular or extracellular pathogens. In a given polypeptide there are potentially many T cell epitopes but only a few actually bind to the MHC molecules and generate a T cell response, while many others are cryptic. The carbohydrate moiety present in glycoproteins has been shown to influence the antigen processing pathway and generation of T cell epitopes in different ways. It can stabilize a certain three-dimensional conformation of the glycoprotein determining which sites are more accessible to proteases, it can hinder the access of proteases or block certain sites, it can target exogenous antigen to different antigen presenting cells and it can also be part of the epitope that will stimulate T cells. Changes in glycosylation patterns are common in malignancies, age and pathological mechanisms. These changes have the potential to alter the hierarchy of peptides that will bind to MHC molecules and induce a T cell response that would otherwise be cryptic, causing the onset of undesirable immune responses as is the case for autoimmune processes.

Introduction

T cell mediated responses are pivotal in the defense against infectious agents,[1,2] against tumors[3] and in the onset of autoimmune processes.[4,5] Many of the peptides that stimulate $CD8^+$ or $CD4^+$ T cells are derived from glycoproteins present in normal, virally infected or malignant cells as well as glycoproteins derived from extracellular pathogens. The presence of glycans in proteins may influence the recognition of epitopes by T cells directly or indirectly. Carbohydrates have a direct effect when the carbohydrate moiety is actually part of the epitope recognized by T cells.[6–12] Alternatively, carbohydrates have an indirect effect when the carbohydrate moiety influences the processing and generation of the peptides that will bind to the MHC.[13–17] N–linked glycans are large (approximately 30Å, similar to an immunoglobulin domain) and mobile,[18] so they can shield large areas of a protein surface (see, for example, the movie at http://www.biochem.arizona.edu/classes/bioc462/462a/NOTES/CARBO/mobility.html) and thus have a large effect on protein-protein interactions, including degradation by proteases. In this chapter we discuss what is currently known about the processing and

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presentation of glycoproteins in the MHC class I and class II antigen presentation pathways and how their carbohydrate moieties can contribute to the diversity of T cell responses. We also point out what is not known in this field and suggest directions for future work.

Where Does Glycosylation Occur?

Glycosylation is divided into N-linked and O-linked modification. N-linked glycosylation is the dominant form of glycosylation for secretory and membrane-bound proteins and occurs on certain Asn residues present in the motif Asn-X-Ser/Thr. A core $GlcNAc_2Man_9Glc_3$ structure is transferred en bloc to the Asn in the ER. This core glycan can then be modified extensively in the ER and as the protein moves through the Golgi apparatus. O-linked glycosylation occurs principally on Ser and Thr but also on Hydroxyproline and Hydroxylysine, present in collagen. O-glycosylation mainly occurs in the ER and Golgi but more recently it has been found that a number of cytosolic and nuclear proteins possess an O-GlcNAc modification.[19] Key things to bear in mind are that it is very difficult to predict from protein sequences alone if a protein will be glycosylated and how it will be glycosylated. Also, a range of different glycans may be present at a single glycosylation site in a protein.[20]

The MHC Class I Processing Pathway

Major Histocompatibility Complex (MHC) class I processing is one of the most intensively studied systems in cell biology and is described in detail in several excellent reviews.[21,22] We give only an outline of the class I processing pathway here, before focussing on how this pathway can be affected by glycosylation of the antigenic protein.

Outline of the Class I Processing Pathway (Fig. 1)

Assembled MHC class I molecules consist of three components: heavy chain which is a glycoprotein with a single transmembrane helix, the non-glycosylated soluble protein β2–microglobulin (β2m) and peptide. MHC class I is expressed on the surface of nearly every nucleated cell in the body. MHC class I binds peptides which are predominantly 8–10 residues in length with both their N- and C- termini buried within the peptide binding groove. These peptides are principally generated by degradation of proteins in the cytosol by the proteasome. The 26S proteasome in the cytosol adds ubiquitin to proteins, unfolds them and degrades them into peptides of 4–25 residues.[23] These peptides can be subsequently trimmed by other more recently discovered cytosolic proteases.[22] The Transporter associated with Antigen Processing (TAP), a member of the ATP Binding Cassette superfamily, transports peptides of 8–16 residues from the cytosol into the ER where the peptides are loaded onto the heavy chain:β2m complex. Loading of peptides onto class I occurs when the heavy chain: β2m complex is associated with TAP, calreticulin, ERp57 and the specialized TAP associated glycoprotein called tapasin. The formation of this peptide loading complex seems to be crucial for the assembly of class I with high affinity stable-binding peptide.[24,25] This may be because of some catalysis of dissociation of fast off-rate peptides, or because of trimming of extended peptides to an optimal length for occupying the class I binding groove.[26] After loading with peptide, class I can traffic through the Golgi apparatus to the cell surface, where it can be recognized by $CD8^+$ T cells.

How Does Glycosylation of the Protein Antigen Affect MHC Class I Processing?

Proteins for degradation by the proteasome may come from a number of sources. Conventionally they are fully folded cytosolic or nuclear proteins which may well have been O-glycosylated. Alternatively, in 2001 it was demonstrated by two groups that a major substrate for the proteasome was defective ribosomal products (DRiPs)—polypeptides which had just been produced by ribosomes and had been mistranslated or failed to fold.[27,28] DRiPs produced from genes encoding glycoproteins of the secretory pathway will not have been

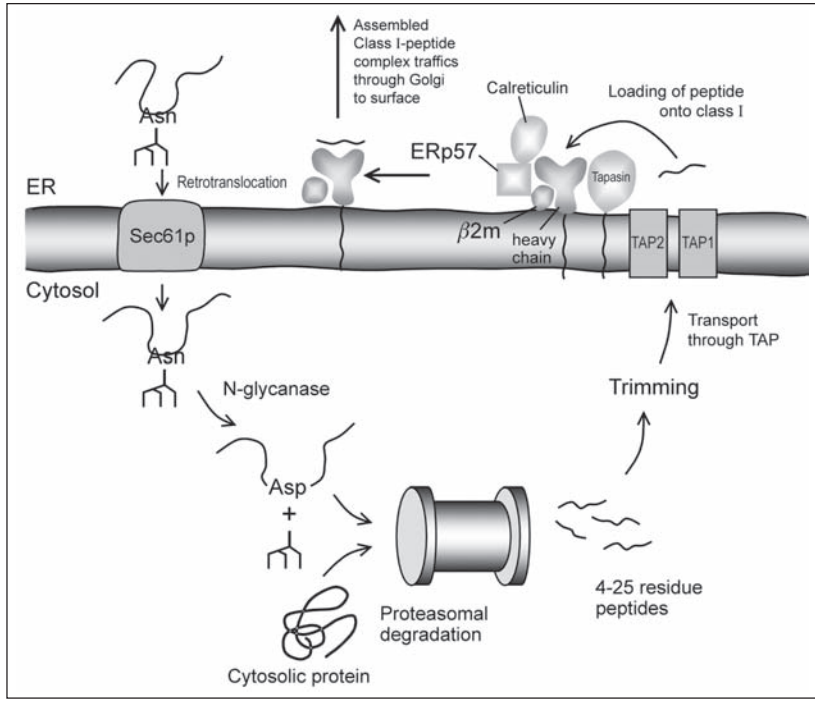

Figure 1. The MHC class I processing pathway.

transported to the ER and so will not be glycosylated. A third source of proteasomal substrates comes from proteins in the secretory pathway, which have been retrotranslocated from the ER to the cytosol via Sec61p,[16,29,30] as part of the ER-associated degradation pathway. A large proportion of proteins in the secretory pathway are glycosylated and when they are translocated back into the cytosol their N-glycans can be removed by N-glycanase.[31] This enzyme hydrolyses the bond between Asn and the first GlcNAc of the glycan, to leave a tell-tale Asp in place of the Asn that was originally translated.

How Significant Are Epitopes Deamidated by N-Glycanase?

The first CTL epitope found to be modified by deamidation came from the search for melanoma tumor antigens important in generating $CD8^+$ responses in cancer patients. There are two different approaches to identify tumor antigens. The genetic approach involves the transfection of pools of cDNA made from the tumor into COS cells that express an appropriate MHC molecule. A positive pool will be identified using $CD8^+$ T cells specific to the tumor.[32] The biochemical approach involves eluting peptides from the surface of the tumor.[33] The peptides are fractionated by HPLC and the fractions are tested for the ability to sensitize targets for lysis using tumor-specific $CD8^+$ T cells. The positive fraction is then sequenced by mass spectrometry. The epitope derived from a melanocyte differentiation antigen, tyrosinase, was identified using a genetic approach[34] as residues 368–376 of tyrosinase. On the other hand, the biochemical approach indicated that a mutated form of 368–376 was the epitope: Asp at position three was found instead of Asn. It was subsequently demonstrated that the unmutated tyrosinase had undergone a posttranslational modification that changed the genetically encoded Asn into Asp.[35] Although not formally proved, the likelihood was that this epitope was derived from an enzymatic reaction, given that this Asn was part of a glycosylation motif.

Later work has demonstrated that presentation of posttranslationally deamidated epitopes is not restricted to self/tumor antigens but is also important for viral glycoproteins. In an attempt to map epitopes from the Hepatitis C virus envelope glycoprotein E1 important in stimulating $CD8^+$ T cells in infected chimpanzees, Selby et al[36] generated an E1-specific CTL line and tested synthetic epitopes spanning the whole glycoprotein for CTL activity. One such peptide did stimulate CTL, 233-GNASRCWVA-241, but the peptide concentration required to sensitize target cells for lysis was surprisingly high (>200nM). Given that the Asn residue from this peptide was part of a known glycosylated site, they tested a deamidated version of the peptide, with the Asn at Asn-234 changed to Asp. With this deamidated peptide they found that as little as 0.001nM of peptide could now sensitize target cells for lysis using the same CTL. CTL against this epitope were still present in chimpanzees 5 years after infection. It was demonstrated that this viral glycoprotein had to be glycosylated, retrotranslocated into the cytosol and the deamidated processed peptide transported back via TAP into the ER.[36] The same posttranslational modification has been described for another TAP-dependent epitope from the HIV-1 envelope glycoprotein.[37]

Deamidation and the dominant source of proteasome substrates from glycoproteins were explored further in a study on Lymphocytic Choriomeningitis Virus (LCMV).[38] LCMV Armstrong glycoprotein encodes a peptide, GP92, which has the sequence 92-CSANNSHHYI-101. This peptide bound well to $H\text{-}2D^b$ but produced a weak immune response. It was suggested that this weak response was because Asn-95 was glycosylated in the mature viral protein. At the cell surface they found both the unglycosylated peptide (Asn-95), likely produced from DRiPs, and the deglycosylated peptide (Asp-95), produced after retrotranslocation and cleavage by N-glycanase, but not the N-glycosylated peptide (they analyzed the peptide with a single N-linked GlcNAc residue attached). All three forms of the peptide bound equally to $H\text{-}2D^b$ and could elicit CTL when mice were immunized with the respective peptides but the study was complicated by the cross-reactivity exhibited by these CTL. Presentation of the deglycosylated peptide provided further evidence for the significance of retrotranslocation in the generation of epitopes from glycoproteins. Presentation of the unglycosylated peptide is consistent with the DRiP hypothesis, although other possibilities, such as incomplete occupancy of the glycosylation site in the native viral protein, are also possible. The lack of presentation of the unglycosylated peptide derived from tyrosinase, as shown by mass spectrometry, with only the de-N-glycosylated peptide reaching the cell surface, even though both bind equally well to HLA-A2, suggests that, at least for this protein, epitope production from DRiPs is insignificant.[39]

All these examples mean that when searching for class I binding motifs or molecular mimics, it would be wise to consider the possibility that if the gene sequence of a secretory protein encodes Asn-X-Ser/Thr, Asp-X-Ser/Thr may actually be generated in the cytosol and be presented at the cell surface.

How Do Cytosolic Proteases Deal with the Glycosylation of Their Substrates?

There is very little work addressing the proteolysis of glycosylated substrates in the cytosol. These glycosylated substrates are likely to be principally O-glycosylated since N-glycanase would remove most N-glycans. It is unlikely that a large glycan would be able to enter the proteasome since the size of the pore of the proteasome is 13Å[40] and so this may limit access to the proteasome of peptides even with glycans as small as monosaccharides attached. Proteasome degradation of the transcription factor Sp1 has been shown to be inhibited by the presence of O-GlcNAc at multiple sites.[41] This glycosylation was reduced when there was glucose starvation and Sp1 became susceptible to rapid proteasomal degradation. This result needs to be confirmed with proteasomes in vitro to establish whether O-glycosylation causes the block in degradation and does not merely correlate with it.

How Does Glycosylation Affect the Generation of Class I Epitopes by Proteolysis in the ER?

Degradation of longer polypeptides in the ER, without retrotranslocation to the cytosol, can also generate epitopes that bind to class I.[42,43] We have explored the influence of glycans on this degradation using Influenza nucleoprotein (NP) targeted to the ER with a signal sequence.[17] This causes NP to be glycosylated at two sites and abolishes presentation of the immunodominant D^b- or K^k-restricted epitopes in TAP-deficient cells. However, blocking glycosylation with tunicamycin or by mutating out the glycosylation sites from NP restored presentation of these epitopes. Further analysis of the rate of degradation of the different forms of NP led to the conclusion that N-glycosylation could abolish the proteolytic generation of antigenic peptide in the ER independent of the effect of glycosylation on the stability of the protein.

How Well Does TAP Transport Glycosylated Peptides?

Our group has shown that addition of an O-GlcNAc residue had almost no effect on the strength of binding to TAP, measured by the extent to which the glycopeptide competed with the transport of another peptide.[44] However, when the efficiency of transport of the glycopeptide into the ER was measured, transport of the glycosylated peptide was still possible but was reduced by approximately 40% compared to the unglycosylated peptide. TAP-transport of glycopeptides is consistent with the work of Gromme et al who have shown that peptides containing side-chains with an extended size of 70Å, equivalent to the size of a 21-mer peptide, can still be efficiently transported by TAP.[45] Despite this result it would still be worth testing whether peptides with glycans larger than monosaccharides can be efficiently transported by TAP.

Does Glycosylation Affect Other Steps in the MHC Class I Pathway?

After assembly of MHC class I with peptide, it exits the ER and traffics through the Golgi apparatus to the cell surface. The influence of peptide glycosylation on the peptide loading complex or trafficking through the secretory pathway has not yet been investigated.

The MHC Class II Processing Pathway

MHC Class II molecules are formed by two non-covalently associated transmembrane proteins, α and β. In the mouse the class II molecules are I-E and I-A and in humans HLA-DR, DQ and DP. Class II is expressed mostly by professional antigen presenting cells (APCs): B cells, macrophages and dendritic cells. Class II molecules evolved to present peptides derived from proteins located in the lysosomal/endosomal compartments. These could be resident membrane and soluble proteins as well as derived from exogenous antigen that can be concentrated in such compartments by different internalization mechanisms.[46,47] While class I, whose peptide binding groove is closed at each end, binds the N- and C-termini of the peptide, the peptide binding groove of class II is open at each end. As a result, class II molecules have the ability to bind to much larger peptides than MHC class I, but most of the peptides eluted from class II molecules range in size from 12 to 19 residues.[48] After synthesis and translocation into the ER, the α and β chains associate with a third transmembrane glycoprotein, the invariant chain Ii (Figure 2). Ii has three major functions: (i) it acts as a chaperone aiding in the correct folding of MHC class II molecules, (ii) one part of Ii, CLIP (the class II-associated Ii peptide), binds to the peptide binding groove and impedes the binding of peptides and polypeptides to the class II in the ER, and (iii) it has a targeting signal which will direct the class II molecule to endocytic compartments. A nonameric complex of $(\alpha\beta Ii)_3$ is formed and is exported from the ER but cannot yet bind to peptide ligands since the CLIP region occupies the binding groove. Two to three hours are necessary between transit through the Golgi and expression of class II as mature molecules at the cell surface.[49] During this period intersection of this nonameric complex with the endocytic pathway enables mixing of endocytosed antigen and the loading of class II with stable peptides. The invariant chain is removed by the combined action of

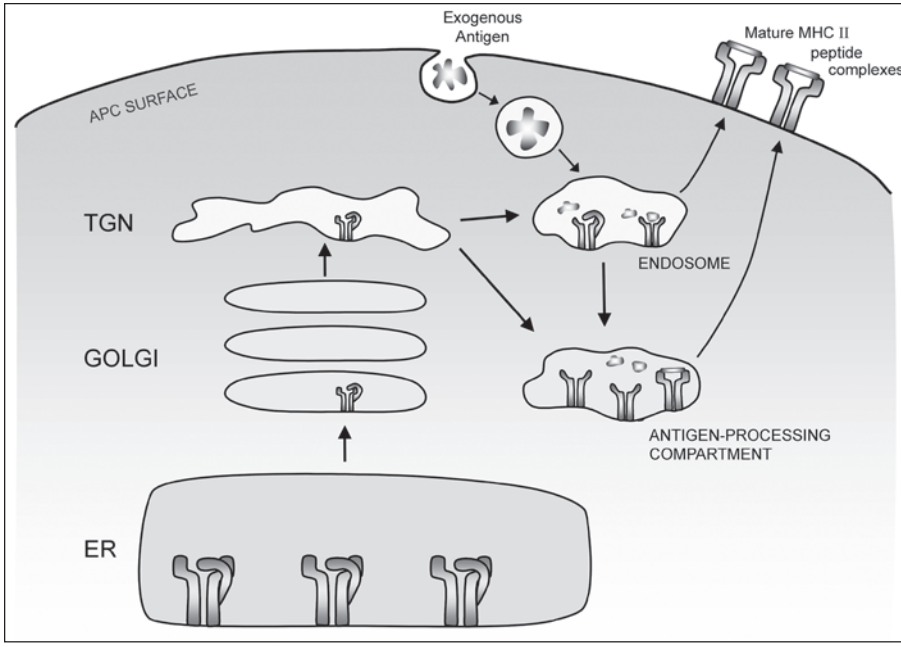

Figure 2. The MHC class II processing pathway.

proteases and acidic pH, with the help of another protein, HLA-DM (human) or H-2M (mouse). DM not only exchanges CLIP for cognate peptide but also exchanges low-stability peptides (with a high off-rate) for peptides that bind class II molecules more stably.[50] In B cells another molecule, HLA-DO/H-2O, participates in this editing process affecting the repertoire of peptides presented by class II molecules by a mechanism which is as yet unknown.[50] The precise trafficking pathway used by class II αβ–Ii complexes to access peptide-rich antigen processing compartments is a matter of debate, but class II can be found throughout the endocytic system.[51] A special compartment referred as the MHC class II compartment (MIIC) has been isolated and identified as the class II loading compartment.[52] It is likely that this pre-lysosomal/lysosomal antigen-processing compartment is the major compartment for peptide loading, since it is the most denaturing to foreign antigens, is the most proteolytic, and contains the highest concentration of the CLIP-removing accessory molecule HLA-DM. Given that class II molecules are detected throughout the endocytic system, peptide loading can occur in different compartments.[53] The balance between the availability of the peptide as unfolding and proteolysis of the antigen is initiated and the availability of a class II molecule to capture this peptide will determine the compartment in which class II is loaded. The open ends of the class II MHC peptide-binding groove are well suited to the capture of unfolded and extended antigen domains and this may be essential to avoid overdigestion and destruction of T cell epitopes.[54] A stable complex of peptide:class II will be exported to the cell surface and stimulate the cognate $CD4^+$ T cells.

How Does Glycosylation Affect Class II Processing?

Glycans could protect proteins from degradation by proteases, either by hindering access of proteases to a given T cell determinant or perhaps by inducing conformational changes in the protein's three-dimensional structure.[55–57] In some cases the formation of epitopes is constrained by heavy glycosylation of the protein. Protein deglycosylation may increase susceptibility to

proteases. It is interesting to note that the priming of the T cell response for the tumor antigen mucin was much more efficient when its glycosylation was reduced or removed.[58] For influenza virus hemagglutinin (HA), glycosylation has been shown to hinder the generation of $CD4^+$ T cell epitopes either from HA that had been taken up exogenously by APC[14] or from HA endogenously expressed by the APC.[13] Gelder et al[59] conducted an extensive analysis of HA responses in immunized volunteers. Using synthetic peptides he tried to establish a correlation between the strength with which certain peptides would bind to class II and the ability to generate a $CD4^+$ response. Many of the peptides that did bind well to class II but did not generate $CD4^+$ responses corresponded to glycosylated regions of HA.[59] In the case of the tumor antigen MUC 1, glycosylation seems to target and localize this heavily glycosylated protein in early endosomes and no antigen processing occurs, possibly because the antigen recycles back to the cell surface and is released before being processed for presentation. On the other hand, deglycosylated mucin traffics to late endosomes or the class II loading compartment and gets processed rapidly.[60] However, for other T cell determinants the glycosylation of the protein was essential for the generation of the epitope, even though the epitope itself was unglycosylated.[14,15] Here the presence of the N-glycans was more likely to alter the pathway of glycoprotein processing. The discovery that the major protease involved in the processing of a microbial antigen presented by class II is an asparaginyl endopeptidase provided direct evidence that glycosylation can have a major role in antigen processing.[61] Asparaginyl endopeptidase was isolated from lysosomes of a human B cell line but is also present in other APCs. The glycosylation of an Asn from a domain of tetanus toxin protein blocked cleavage by asparaginyl endopeptidase and thus prevented the generation of a class II epitope from this protein.

There are some examples in the literature that show N-glycans to be essential for T cell recognition, but since the T cell determinants have not been identified, the mechanism by which glycosylation is important is not clear. Dudler et al[62] demonstrated that clones derived from allergic patients were specific for a glycosylated form of the bee venom allergen phospholipase A_2 (PLA_2). These clones were not stimulated by full-length or truncated recombinant PLA_2 produced in *E.coli*, nor by a set of overlapping peptides. The authors argue that a glycosylated epitope was being recognized by the T cell clones but no formal evidence was provided for this, so a role for the carbohydrate moiety in influencing antigen processing cannot be excluded. Also, a deamidated form of the peptide was not tested. Interestingly, treatment of PLA_2 with mannosidase also abolished its recognition. This indicates that if the carbohydrate moiety were part of the epitope at least 5 sugar residues were being recognized by the TCR. This seems unlikely according to the crystal structure of glycopeptide:MHC complexes (see below). Mannosidase treatment also reduced $CD4^+$ T cell stimulation by a *Mycobacterium tuberculosis* antigen complex.[63] It is important to note here that this also could be caused by reduced uptake of deglycosylated antigen by APCs by, for example, the mannose receptor[65] or differential targeting of the antigen into a different endocytic loading compartment through a lectin interaction (not yet identified) where the antigen processing environment is different.

The importance of glycans in recognition by $CD4^+$ clones specific for two different tumor antigens has been reported. We isolated three different anti-tumor clones from mice that had been previously vaccinated against the colon carcinoma, CT26. These clones were stimulated by the ER-resident precursor of gp70, the glycoprotein of the endogenous murine leukemia virus, which is gp90. The recognition of gp90 was both glycosylation- and conformation-dependent. As with PLA_2, synthetic peptides spanning the whole of gp90 failed to stimulate the clones, raising the question as to whether the class II epitope itself was glycosylated. In this case we also tested the deamidated forms of peptides, which failed to stimulate the clones.[64] For the melanoma antigen tyrosinase, the stimulation of $CD4^+$ T cells isolated from melanoma patients was also dependent on glycosylation of tyrosinase.[66] Again, the epitope could not be identified from a set of overlapping synthetic peptides but point mutations in the glycoprotein indicated that four out of seven potential glycosylation sites were important for the recognition of tyrosinase by the clone, suggesting an important role for the

N-glycans in affecting presentation of tyrosinase. For both gp90 and tyrosinase it seems that glycosylation might have both a direct and an indirect effect on processing and presentation, where the epitope itself is probably glycosylated (given that overlapping synthetic peptides were not stimulatory) and the generation of this glycopeptide epitope is dependent on the three-dimensional conformation of the protein. It is also possible that none of the synthetic peptides was optimal and that glycans have only an indirect effect. Given the difficulty in synthesizing a whole range of different peptides when the stimulatory epitope cannot at least be mapped to a certain region of the protein, it seems that the only solution would be to directly identify the peptides biochemically. This would involve pulsing APCs with the glycosylated protein, eluting peptides from the restricting class II molecule, and sequencing the immunogenic peptide.

Glycopeptides As T Cell Epitopes

Although the experiments described above showed that glycosylation affects the generation of class I and class II epitopes, they did not clearly demonstrate that the epitope recognized by T cells was itself glycosylated. For a long time it was generally believed that only unmodified peptides could bind to MHC class I and II molecules. The immune response to polysaccharides was T cell-independent and monosaccharides or polysaccharides could not compete or block binding of peptides to class II molecules.[67] Despite this, studies with conjugation of haptens (e.g., trinitrophenyl or fluorescein derivatives) to polypeptides had clearly demonstrated that T cell responses could be primed against the haptenated peptide and subsequently generate hapten-specific responses and responses to the unmodified peptide.[68] This provided a foundation for studies to test if glycopeptides would also affect T cell responses, given that glycosylation is a physiologically relevant modification (for a review of T cell responses to other posttranslational modifications see Kastrup et al[69]).

Initial studies demonstrating that glycopeptides could bind to MHC molecules were done using synthetic peptides. Ishioka et al[6] synthesized a series of glycopeptides, in which a GlcNAc N-linked to an Asn residue was placed at various positions throughout the sequence of a model T cell epitope, ovalbumin. The glycopeptides that bound stably to the MHC class II I-A^d were used to immunize mice. The results showed that in most instances the glycopeptide was less immunogenic than its non-glycosylated analog, but that a detectable glycopeptide-specific response could be obtained. The substitution of GlcNAc by a structurally related analog ablated T cell recognition, indicating that the carbohydrate moiety was important .[6] Harding et al[7] did the same set of experiments using different glycopeptides with affinity for a different murine class II molecule, I-A^k. Mice were immunized with a peptide from hen egg lysozyme (HEL) that had the disaccharide galabiose linked to the amino terminus. In vitro assays to analyze the fine specificity of glycopeptide-specific hybridomas from the immunized mice indicated that the T cells were not sensitive to small changes in the carbohydrate moiety, but they were sensitive to its location in the peptide sequence and changes to the peptide sequence itself. This indicated that the carbohydrate moiety was influencing T cell recognition by changing the conformation of the peptide backbone.[7] When the disaccharide was attached to the middle of the same HEL-derived peptide and used to immunize mice a very different result was obtained. In this case the fine structure of the sugar moiety was important in creating the T cell determinant and sugar substitutions stopped recognition by the TCR.[8] Two of these galabiose-specific T cells were analyzed further regarding their TCR fine specificity for the glycopeptide. Two different glycopeptides were engineered: the hydroxyl group at the C-6 position was removed, either from the Gal distal to the anchoring amino acid, or from the proximal Gal. Removal of the hydroxyl group from the proximal Gal was tolerated but not from the distal Gal. Although the proximal hydroxyl group could be removed the substitution of the proximal Gal by Glc was not tolerated. This indicated that the outer Gal was in direct contact with the TCR but the proximal Gal was also important in maintaining T cell recognition. The identity of the peptide side-chains was also essential for T cell recognition.[70]

Otvos et al[71] had isolated T cell clones specific for an epitope on the rabies virus glycoprotein 29-VVEDEGCTNLSGF-41, and tested the glycosylated version of this peptide for T–cell stimulatory activity. The rationale for these studies came from the fact that different strains of rabies-related viruses can have mutations that favor differential glycosylation patterns and this specific peptide contained a glycosylation motif. While β-N-glycosylation (GlcNAc) of the sequence fully abolished the peptide's ability to bind to class II and to stimulate a $CD4^+$ specific hybridoma, incorporation of an α-linked GalNAc did not.

While the previous studies used glycopeptides in which the peptide in itself was immunogenic in mice, another group tested the same approach but using a non-immunogenic self peptide known to bind well to the murine class II I-E^k.[72] The aim of this study was to test whether the glycosylation of a non-immunogenic peptide could make it immunogenic. The carbohydrate chosen was the Tn antigen, a small mucin type O-linked α–D-GalNAc that is expressed in 90% of carcinomas.[73] Unfortunately analysis of bulk T cell responses from mice immunized with the glycopeptide showed that changing residue 72 to Thr or Ser, in order to to allow O-linkage of the Tn antigen, made the peptide very immunogenic by itself. The same group synthesized glycopeptides of higher complexity and demonstrated that while glycopeptides containing a trisaccharide unit could still bind well to MHC class II they were not as immunogenic as peptides containing one or two sugar units.[12] Using bulk T cell cultures from immunized mice they could demonstrate that T cells primed with glycopeptides having one or two sugar units were highly specific for the identity of the glycan group. The important conclusion as to whether a self non-immunogenic peptide could be converted into an immunogenic one could not be made, given that all immunogenic glycopeptides had one amino acid change from the wild-type sequence. The analysis of the fine specificity of these Tn-peptides was done after generating clones of T cell-hybridomas from these bulk cultures derived from immunized mice.[74] Although partial cross-reactivity was observed in some hybridomas, several of the hybridomas were highly specific. For example, 17 out of 19 hybridomas that were specific for the glycopeptide 67-VITAFTEGLK-76 with the α–D-GalNAc group attached to Thr-72 did not respond to the same peptide with α–D-GlcNAc attached to the same position. The only difference between these sugar residues is the orientation of the hydroxyl group located at the fourth carbon atom in the carbohydrate ring structure, indicating that the glycan is recognized by the TCR.[74] Interestingly, although these two glycopeptides generate completely different responses from the TCR, molecular modelling indicates that the conformation of the glycopeptide relative to the TCR is identical.[75]

All the studies described above involved testing glycopeptides as epitopes for $CD4^+$ T cells. For epitopes recognized by $CD8^+$ T cells the candidate glycosylation was the cytosolic addition of O-GlcNAc sugar residues. O-GlcNAc addition to cytosolic proteins has been identified by Wells et al.[19] This modification is thought to be important in signal transduction pathways since phosphorylation/O-GlcNAc additions occur in the same residue in a given protein in an alternate manner. O-GlcNAc addition is thought to "protect" sites from being phosphorylated.[19] It was therefore conceivable that some peptides that bind to class I would have this sugar residue. To test if an O-GlcNAc addition could generate a new T cell epitope, Haurum et al[9] added this sugar residue to peptides related to the Sendai virus nucleoprotein 324-FAPGNYPAL-332 that can bind to both MHC class I D^b and K^b. Those glycopeptides that bound well to both class I molecules were used to immunize mice. Glycopeptide-specific $CD8^+$ T cell clones could be isolated that were highly specific for the O-GlcNAc since they could not be stimulated by the same peptide without an O-GalNAc attached to it.[9]

From these studies with synthetic glycopeptides some important conclusions could be made:

i) glycosylation of epitopes can inhibit, have no effect on or in rare cases enhance binding to MHC molecules,
ii) glycopeptide-specific T cell responses can be elicited,
iii) peptides linked to monosaccharides or dissacharides are most likely to generate a glycopeptide-specific response.

iv) Depending on where the sugar is located, the TCR can directly recognize the sugar residue and some amino acids in the peptide backbone, or it recognizes the peptide backbone under a different conformation induced by the carbohydrate moiety. Therefore the potential for glycosylation to contribute directly to epitope diversity is enormous, but do naturally processed glycopeptide epitopes exist?

Naturally Processed Glycopeptide Epitopes

Naturally processed glycopeptides have been identified by mass spectrometry in a pool of peptides bound to human MHC class I and class II alleles.[44,76–78] Using mass spectrometry, a peptide with an N-GlcNAc modification was identified from HLA-DR1 and a peptide with an N-GlcNAc-GlcNAc modification was identified from DQ8.[76,77] Dustin et al[79] showed that a significant percentage of peptides (0.75%) eluted from HLA-DR from human cell lines contained mannose 6-phosphate. Phosphorylation of mannose residues to generate Man-6-P is carried on the outer mannose residues of the N-glycan and targets proteins to lysosomes, so this MHC-bound glycan should have at least 9 sugar residues. Several attempts to sequence peptides containing Man-6-P were not successful, but one of the peptides could be identified, after 15 cycles of microsequencing, as deriving from lysosomal acid lipase. There was no direct identification of the N-glycan (probably lost during the cycles of microsequencing) but the Asn was thought to be located outside the class II binding groove.[79]

To identify naturally processed glycopeptides bound to class I, peptides were extracted from normal human spleen, fractionated by reverse phase HPLC and eluted fractions were subjected to galactosyltransferase-mediated labelling with [^{3}H]-Gal, which will specifically label terminal O–β–GlcNAc residues.[80] Approximately 0.1% of peptides were positively labelled. As an alternative approach, peptides were eluted from human class I molecules and subjected to a series of lectin affinity columns. As opposed to Concanavalin A or *Arachis hypogea* lectin that did not retain any significant fraction of the peptides, the wheat germ agglutinin (WGA) column (specific for terminal GlcNAc residues) retained approximately 1% of the peptides. Sequencing by Edman degradation of the pool of peptides bound to the WGA column showed an increased amount of the canonical anchor motif of the appropriate MHC I as well as Ser and Thr which might be expected for peptides with O-GlcNAc addition.[44,78] Although these reports show that naturally processed glycopeptides can be eluted from surface class I and II molecules, there have been no reports of naturally occurring TCRs specific for these glycopeptides.

The first example of a naturally glycosylated T cell determinant came from studies of collagen-induced arthritis in mice.[10] The immunodominant epitope was located within a cyanogen bromide-cleaved fragment of rat type II collagen (CII), containing residues 256–270. Further analysis of this fragment through tryptic digestion and RPHPLC separation showed that naturally glycosylated peptides could bind to class II and stimulate the CII-specific T cells. Mass spectrometry of this CII fragment revealed that it was variably glycosylated, containing up to five sugar units. It was later demonstrated that most of the CII-specific hybridomas recognized the form of peptide with Gal on residue 264.[81] Although T cells with specificity for the deglycosylated form were also detected, the presence of the carbohydrate moiety played a clear role in the development of arthritis, since deglycosylated CII peptide induced arthritis with a later onset, lower incidence and milder symptoms.[10]

There is only one example of a naturally occurring glycopeptide-specific class I restricted T cell response but it provides evidence that inducing responses to glycopeptides could have an important role in future tumor immunotherapy protocols. This T cell response was found by Zhao and Cheung[82] after immunizing mice with irradiated EL4 lymphoma cells. The paper is complicated by the fact that they were not able to determine the molecular identity of the immunogenic glycopeptide, associating its presentation on various tumor cell-lines with the presence of the glycolipid disialoganglioside GD_2. They showed that the response is $CD8^+$ T cell-dependent, αβ TCR-dependent, TAP-dependent, H-2^b-restricted and required GD_2 expression. Antibodies to GD_2 or H-$2K^b$/H-$2D^b$ could prevent lysis but soluble GD_2 could not.

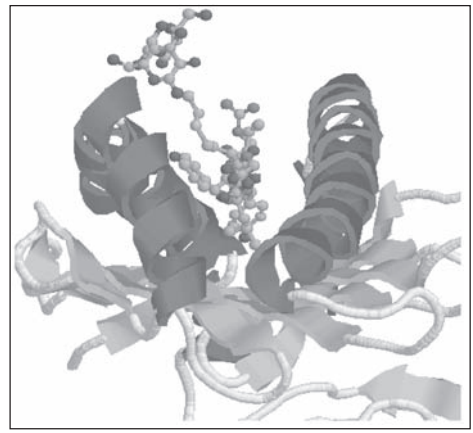

Figure 3. The crystal structure of RGY8-6H-Gal_2 bound to H-2K^b (taken from Entrez Structure file 1KBG).

It is unlikely that class I could directly present a glycolipid to T cells (although CD1 molecules can do this) and so we interpret their results to mean that the altered glycolipid glycosylation also led to altered glycosylation of certain proteins and thus generated one or several new immunogenic glycopeptide epitopes. Unfortunately the authors did not try to inhibit lysis separately with antibodies to H-2K^b or H-2D^b to see if the immune response were directed to more than one glycopeptide epitope. The presence of GD_2 on a wide range of human tumors suggests that this GD_2-associated immune response would be a productive area for further investigation.

Crystal Structures of MHC-Glycopeptide Complexes

Three crystal structures of glycopeptides bound to MHC molecules have been determined, all published in the same issue of Immunity in 1999. Speir et al[83] solved the structure of H-2K^b bound to a modified version of the Vesicular Stomatitis Virus nucleoprotein peptide RGYVYQGL (RGY8), where the disaccharide Gal–α(1,4)Gal–β is connected with a homocysteine linker to position 6 of the peptide (RGY8-6H-Gal_2). The structure of the peptide, heavy chain and β2m is nearly identical for RGY8-6H-Gal_2-K^b as for the previously determined structure of RGY8-K^b.[84] This is consistent with the peptide binding affinity to K^b being unaltered by the glycan modification.[85] It indicates that the carbohydrate specificity of the T cells depends on direct recognition of the glycan and not on a conformational change in the bound peptide induced by the glycan, as had been suggested by Harding et al for class II.[7] The glycan extended 12Å above the peptide backbone (Figure 3), presenting a substantial solvent-exposed structure and resting next to the α2-helix of K^b. The conformation of the glycan was very similar to that of the disaccharide when free in solution.[86] The exposure of the glycan above the peptide backbone in this structure may be the maximum that is consistent with an αβ TCR-mediated glycan-specific and peptide-specific response. Peptides linked to trisaccharides produced a γδ rather than an αβ T cell response[85] and even disaccharides on peptides bound to D^b are poorly immunogenic, possibly because they project further up towards the TCR as the peptide mainchain of D^b peptides arches up towards the TCR too. The γδ TCR response to a glycan occurred independently of whether the glycan is presented by an MHC-bound peptide or on a glycolipid.[85]

We have solved the structures of two O-GlcNAc substituted derivatives of the Sendai virus nucleoprotein peptide FAPGNYPAL bound to H-2D^b.[87] The results with FAPS(O-GlcNAc)NYPAL produced similar results to those of Speir et al:[83] the structure of heavy chain, β2m and the peptide backbone was essentially unchanged by the glycan modification.

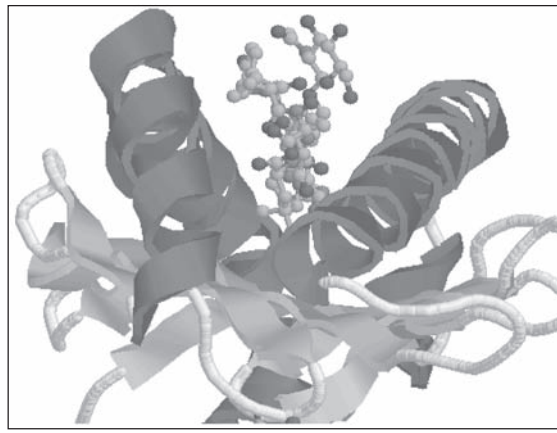

Figure 4. The crystal structure of FAPS(O-GlcNAc)NYPAL bound to H-2D^b (taken from Entrez Structure file 1QLF).

Again the glycan was highly exposed (Figure 4) with about half of the exposed surface area accounted for by the GlcNAc. The sidechain orientations in the glycopeptides were unaltered except for the Tyr at P6 which bends to stack with the sugar.

The structure of D^b in complex with FAPGS(O-GlcNAc)YPAL was, on the other hand, a whole new kettle of fish. In this case the glycan occurred where there would normally be an anchor residue Asn. The glycan cannot fit within the pocket usually filled by Asn and the peptide backbone was forced to rotate at P4-P7 by 180°, leaving the glycan exposed to solvent. The peptide backbone also has increased flexibility. This was the first structure of any MHC class I-peptide complex where a main anchor pocket was not filled but nevertheless the structure of the peptide binding groove was unaltered from a structure where all the anchor pockets are filled.[88] This has implications for any theory of peptide-induced conformational changes in class I which could regulate release of class I from the peptide loading complex, allowing export of class I to the cell surface.[89,90] P5 Asn normally forms two hydrogen bonds to Gln-97 in the pocket of D^b but in this structure two ordered water molecules formed these hydrogen bonds. Without a P5 anchor, the glycopeptide had enhanced mobility.

It should also be mentioned that Jensen et al modelled the structure of a hemoglobin peptide glycosylated with either Tn (α-D-GalNAc) or α-D-GlcNAc. The resulting structures were essentially identical, with only the difference in the position of the C4 hydroxy group of the sugar explaining the differential T cell reactivity against the two glycopeptides.[75]

How Does the T Cell Receptor Interact with MHC-Glycopeptide Complexes?

A crystal structure of an MHC-glycopeptide-TCR complex is to be eagerly awaited but at present one can only model how glycosylation would affect the interaction with the TCR. Our group has modelled the interaction of the TCR with the two D^b-glycopeptide complexes whose structures are described above.[87] This suggested that each glycan fits into a cavity between CDRα and CDRβ of the TCR and showed how conserved CDR3β residues could make hydrogen bonds with sugar hydroxyls to ensure the specificity for GlcNAc over GalNAc substitution of the peptide. With the glycan between CDR3α and CDR3β, the tips of the CDR3 loops can still scrutinize the exposed side-chains of the peptide. In each Db-glycopeptide complex the electron density indicated that the glycan had a set of distinct conformations which was more variable than for previously reported amino acid side-chains. However, oscillation between these glycan conformations was not detected by nuclear magnetic resonance

relaxation rate studies. Mobility of the glycan may have an impact on the thermodynamics and kinetics or TCR binding, because of the entropic cost of restricting the glycan to a single conformation and it would be interesting to test this using surface plasmon resonance.

Sequencing of many CDR3 sequences from TCRs that recognize glycopeptides has found an abundance of small polar amino acids (such as Asn, Ser, Glu) which may be ideal for hydrogen bonding with sugar hydroxyls. Aromatic residues were also common in these CDR3 regions and may be involved in hydrophobic and stacking interactions with the sugar.[91,87]

Glycosylation in Autoimmune and Anti-Tumor T Cell Responses

In a given polypeptide there are potentially many T cell epitopes but only a few actually bind to the MHC molecules and generate a T cell response: a phenomenon known as immunodominance. Whether the influence of carbohydrates on T cell recognition is direct or indirect, changes in glycosylation patterns of certain antigens have the potential to change the hierarchy of dominant/cryptic determinants from a given antigen. During some autoimmune processes and viral infections, T cells specific for epitopes that are no longer cryptic have an important role.[4,92,93] This comes from the fact that if epitopes are cryptic during T cell development of their cognate TCRs, these T cells may escape thymic deletion and be exported to the periphery. With time or some pathological process this epitope may be exposed and elicit an undesired T cell response that can trigger an autoimmune process. As discussed before, deglycosylation can certainly uncover cryptic epitopes generated in the ER[17] but whether natural changes in the glycosylation pattern play a role in regulating hierarchy of dominant/cryptic has not been investigated. Nevertheless the best known example of a naturally occurring glycopeptide-specific T cell response comes from the epitope from collagen that is involved in experimental arthritis. Posttranslational modifications of CII can be influenced by factors such as age and hormones[11] so it is possible that collagen specific epitopes presented to T cells could change with these events too. Indeed it has been demonstrated that in patients with rheumatoid arthritis the glycosylation patterns in IgG changes.[94] The analysis of self-peptides bound to the type I diabetes associated class II molecule HLA-DQ8 identified a naturally processed glycopeptide derived from HLA-DQ1 that had two N-GlcNAc units. This peptide was 13 amino acids long and the disaccharide was attached to Asn81 75-IVIKRSNSTAATN-87. Interestingly, the unglycosylated version of this peptide could not compete for binding to the class II molecule indicating that the glycan moiety contributed to the binding energy of the glycopeptide to class II.[77] Also of interest is the fact that the $CD4^+$ T cell epitope in patients with celiac disease is a deamidated epitope.[95] Although this epitope was not generated through N-glycanase activity because it was not part of a glycosylation motif, this example certainly shows that deamidation can generate epitopes important in autoimmune processes. This has been discussed in a recent paper by Manoury et al[96] This paper shows that myelin basic protein contains a major processing site for asparaginyl endopeptidase so the generation of a dominant $CD4^+$ epitope only occurs if this site is not cleaved. As discussed by the author, with time Asn residues in myelin basic protein may suffer spontaneous deamidation, destroying the cleavage site for asparaginyl endopeptidase and as a result the dominant epitope can be generated and initiate an undesirable T cell response. Many deamidated $CD8^+$ epitopes generated through N-glycanase activity in the cytosol have been identified. Although deamidated $CD4^+$ epitopes certainly exist[95,97] this deamidation is not caused by deglycosylation but by a natural deamidation reaction. It would be of great interest to determine if N-glycanase activity is also present in MHC class II loading compartments and if this has any importance in the generation of $CD4^+$ deamidated epitopes.

In many instances undesirable autoimmune responses can be desirable anti-tumor responses. For example many of the $CD8^+$ T cell responses in patients with melanoma are directed to melanocyte associated antigens such as tyrosinase and gp100.[98] Given the history of differential glycosylation patterns in different tumors and with the progression of a tumor[99,100] it is likely that generation of T cell epitopes will change as the malignant process evolves. As previously

mentioned, the generation of tumor-specific $CD4^+$ T cell responses to two different tumor antigens, gp90 and tyrosinase is dependent on the presence of N- glycans.[64,66] Another tumor antigen that has been extensively studied for its therapeutic potential is mucin. In particular, a member of the mucin family, MUC 1, which is a transmembrane glycoprotein expressed at the apical surface of normal glandular epithelia of many organs and is expressed in some hematopoietic cells (activated T cells, plasma cells, B cells). During malignant processes different things happen to mucin: the distribution of mucin is not polarized as it is on normal epithelial cells, its expression is greatly increased and the glycosylation pattern changes so that there are fewer sites glycosylated and the glycans are much shorter. As a result of this, new epitopes are exposed both on the polypeptide backbone and on the carbohydrate chains. The Tn antigen, for example, is exposed as a result of a blockage in mucin O-glycan elongation, a characteristic feature of human carcinomas. Immune responses against MUC 1 have been detected in cancer patients and this stimulated a great deal of research to explore the potential of this antigen in vaccine models.[101,102] Several pre-clinical trial experiments demonstrated that an effective cellular anti-tumor response could be obtained by vaccination against deglycosylated MUC1. The results from phase I trials showed a small increase in MUC 1-specific CTL in the peripheral blood in response to vaccination with deglycosylated MUC 1 peptide[103] but in most cases vaccination was associated with a predominant antibody response.[104]

Conclusions and Perspectives

As well pointed out by McAdam et al,[97] "The frequency and biological importance of posttranslationally modified T cell epitopes in immunology may well be underappreciated." There is little doubt that the importance of glycopeptide-specific responses found when studying collagen-induced arthritis and bee venom models can be extended to a wide number of conditions. Any hunt for an antigenic epitope, having identified a T cell response, is difficult, but it is many times harder if this epitope is glycosylated because of difficulties in analysis and synthesis of candidate epitopes. It is dispiriting to grow up $5x10^{10}$ cells (50 litres of cells if they are at 10^6 per ml) and to isolate only 15ng glycopeptide for analysis (Haurum and Elliott, unpublished results). This makes the study of glycopeptide-specific immune responses an area where the developments in sensitivity from nanoflow HPLC and electrospray ionization mass spectrometry will be especially crucial. These methods have already proved themselves in the identification of phosphorylated peptides presented by class I,[105] with detection limits down to 2 attomole, or in one case peptides present on the cell surface at an average of less than one copy per cell.[106] Fragmentation of the peptide with a secondary mass spectrometer and database searching can allow identification of the modified peptide sequence. The initiation of Glycomics projects, including a Consortium for Functional Glycomics, (http://glycomics.scripps.edu), with the long-term goal of understanding the glycosylation of every glycoprotein in the proteome, will also provide an enormous stimulus to this field.[107] Microarrays observing the change in expression of glycosyltransferases and carbohydrate binding proteins in malignant cells, for example, are promised. 2D-gel profiles of proteins that have a given glycosylation pattern, e.g., O-GlcNAc residues, will also be possible.[19,20] Also, neural networks which can scan sequences for likely O-GlcNAc modification are being developed.[108] This will give an enormous boost to looking for candidate glycopeptides as antigens in various conditions, notably cancer. Also, the ability to obtain various glycosylated peptides, a challenge to synthesize even for experienced organic chemists, will increase, which has been in many cases a limiting factor. The consortium also aims to generate mice with genes for glycosyltransferases and carbohydrate binding proteins knocked out. Transplantation of cells from wild-type mice to these knockout mice could generate a T cell-mediated immune response that could lead to the identification of glycopeptides presented by MHC molecules on wild-type cells but which these mutant mice have not become tolerant to. A knockout mouse for α-mannosidase II has already been generated by Chui et al[109] and interestingly enough it develops, by a yet unknown mechanism, a systemic autoimmune disease similar to human systemic lupus erythematosus.

From the examples in the literature it seems that bulky N-glycans can have a major influence on both class I and class II antigen processing but are less likely to be directly recognized by T cells. There are many examples of N-glycosylation leading to generation of deamidated epitopes which are seen by $CD8^+$ T cells but no examples of recognition of $CD4^+$ epitopes deamidated through N-glycanase activity. This could be simply a result of more research being done on $CD8^+$ epitopes or alternatively, we would like to speculate, a reflection of more efficient N-glycanase cleavage in the cytosol (where most class I epitopes are generated) than in the MHC class II loading compartment. Although glycosidases present in the endosomal/lysosomal compartment have been identified,[110] efficient glycan trimming and removal may not happen where and when the loading onto class II occurs. It would be of major interest to determine in which endocytic compartments the generation of N-glycosylation dependent epitopes occurs and analyze this loading compartment for the presence of different glycosidases.

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