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Hijacking a chaperone: manipulation of the MHC class II presentation pathway

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Novel antigen delivery systems are currently being developed by genetic manipulation of the MHC class II trafficking pathway. Specific targeting of endogenously synthesized antigens to the class II loading compartment can result in massively enhanced presentation of peptide epitopes. This emerging technology holds promise for a variety of clinical applications including vaccine development, cancer therapies and control of autoimmune diseases.

The dissection of the major histocompatibility complex (MHC) class II presentation pathway has paved the way for rational approaches to enhance immune responses. Antigen presentation by MHC class II molecules requires the coordinated action of accessory molecules in guiding MHC class II molecules to the correct site(s) for antigen loading, and in the generation and loading of antigenic peptides into the MHC class II groove[1]. A key player in escorting newly synthesized MHC class II molecules from the endoplasmic reticulum (ER) to the endosomal pathway is the invariant chain (Ii). In the ER, newly synthesized Ii polypeptide binds to the peptide-binding groove of MHC class II heterodimers and prevents aggregation with unfolded polypeptides. A signal consisting of a leucine–isoleucine or methionine–leucine motif within the cytoplasmic domain of Ii targets the MHCII–Ii complex to the endocytic pathway. After transport to the endosomal compartments, Ii is degraded by the cathepsins S, L and F – cysteine proteases that vary in their tissue and cellular distribution[2,3] – leaving a fragment of Ii, CLIP (class II invariant chain-derived peptide), bound to the MHC II groove. The MHC encoded HLA-DM (DM), or mouse homologue H2-M, heterodimer then catalyses the release of CLIP, allowing the groove to bind antigenic sequences. DM molecules can also further edit bound peptides to select for highly stable class II-peptide complexes. A modulator of DM, HLA-DO/H2-O, that is found predominantly in B cells, might further influence the profile of epitopes presented[4].

The conventional approach to introduce antigen into the class II pathway is via the exogenous route, for example as vaccine antigen preparations *in vivo* or by antigen loading onto antigen presenting cells (APCs) *in vitro*. However, loading APCs with antigens or defined peptide epitopes has limitations, in particular a limited occupancy of MHC class II molecules with processed antigenic epitopes and a short half-life of antigenic peptides. On the other hand, an ongoing encounter of endogenously synthesized antigens with MHC class II molecules within the endosomal loading compartment would be expected to enhance presentation of antigenic epitopes. A further disadvantage of using free peptide epitopes is that the resulting MHC class II–peptide complexes might differ antigenically from those generated by intracellular loading of peptides derived from the endogenous processing of antigen, presumably because of structural differences between the class II-peptide complexes[5]. Class II loading by genetic engineering has the potential to overcome this difficulty and produce essentially monospecific APC presenting a sole T-cell epitope. Various strategies have been explored over the past few years in an attempt to obtain more effective $CD4^+$ T-cell responses and thereby enhance downstream protective cytotoxic responses and/or the production of T-cell-dependent antibodies.

Manipulation of the class II presentation pathway

Targeting of full length antigens to the class II pathway

Endogenously synthesized antigens can be targeted to the endosomal system using signals associated with resident lysosomal membrane proteins such as the lysosomal-associated membrane protein, LAMP-1. This membrane protein, which is localized to the late endosomes and lysosomes, contains a tyrosine-based targeting signal in its cytoplasmic tail[6]. Wu and colleagues[7] constructed a chimeric cDNA encoding the E7 protein from the human papilloma virus 16 (HPV 16) fused to the transmembrane and cytoplasmic region of LAMP-1; the specific targeting of the chimeric protein to the endosomal and lysosomal compartments resulted in enhanced MHC class II presentation, as assessed by stimulation of E7(30–67 aa) peptide-specific T cells[7]. Furthermore, vaccinia virus containing the chimeric 16E7-LAMP-1 generated not only greater E7-specific T-cell responses, but also enhanced antibody titres and induction of cytotoxic T lymphocytes (CTLs) compared with constructs containing wild-type 16E7 (Ref. 7). Similarly the model antigen, hen egg lysozyme (HEL) was fused to different lysosomal hydrolases, enzymes that acquire the mannose-6-phosphate lysosomal address signal. APCs transfected with these constructs elicited T-cell activation, albeit at different efficiencies[8]. These studies demonstrate that the use of endosomal/lysosomal targeting signals can result in delivery of antigenic peptides to the MHC class II pathway, as a consequence of the intersection of the endosomal/lysosomal trafficking pathway with the class II presentation pathway.

The endosomal targeting signals of Ii, located on the cytoplasmic N-terminus[9], have been used in another approach to try to maximize the delivery of the antigen to the class II loading compartments. The Ii sorting signal directs the MHCII–Ii complex to the endocytic compartments. As Ii can independently be exported from the ER (Ref. 10), Ii fusion proteins might follow the same route separately from MHC

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molecules. Endogenously synthesized chimeric Ii molecules containing residues 1–80 of Ii, which includes the cytoplasmic sorting signal and transmembrane domain, fused to the model antigens chicken ovalbumin (OVA) or HEL resulted in activation of class II restricted OVA- or HEL-specific T-cell hybridomas[11]. By contrast, the OVA-specific T-cell hybridoma failed to respond to class II^+ cells expressing endogenous native OVA. As a result of the coordinated trafficking of Ii and class II molecules, Ii fusions would be expected to be superior to fusions with endosome/lysosome resident proteins in presenting antigen; however, a detailed comparison has not been carried out and a careful study, using stable transfected APCs where the levels of the different fusion proteins are similar, would be worthwhile.

Fusions with Ii(1–80) have also been used to identify novel tumour-specific antigens. Class II positive cells were transfected with an Ii(1–80) fusion library (generated from cDNAs from a melanoma tumour) and the Ii-fusion library clones were assessed for their ability to activate $CD4^+$ T cells obtained from metastatic lesions (tumour infiltrating lymphocytes) from a patient with melanoma[12]. A mutated form of CDC27, a protein involved in cell-cycle regulation, was identified as a novel melanoma antigen[12]. Such an approach could readily be used to identify immunodominant antigens recognized by $CD4^+$ T cells not only in tumours but also in autoimmune or infectious diseases. Some antigens that are detected by existing T-cell responses will, under physiological conditions, be targeted to endocytic processing compartments[13]. The use of Ii-fusion libraries has the advantage that it allows cloning of antigenic epitopes independent of whether the protein sequence contains an endosomal sorting signal, for example in the case of partial sequences or sequences of multicomponent complexes where only one component bears an endosomal sorting signal.

Targeting of endogenous epitopes to the class II pathway

Antigenic sequences have been added as C-terminal extensions to Ii. It is likely that C-terminal modifications will not perturb the structure of Ii, considering that Ii41 and Ii31 isoforms differ by a sequence inserted at the C-terminus[14]. Therefore, epitopes added as C-terminal extensions of Ii could be transported to the loading compartment either as part of the class II–Ii complex or independently as an Ii fusion protein. Once in the endosomal compartment, the T-cell epitope needs to be released from the Ii sequences. The processing of the C-terminus of Ii occurs early in the Ii degradation pathway[15]. In some studies the chimeric Ii have included a cathepsin cleavage site adjacent to the antigenic epitope, so that a single cleavage will release the C-terminal antigenic peptide[16,17]. Other studies indicate that the additional cleavage site is not required for presentation of a C-terminal appended antigen, presumably because endosomal degradation is the natural fate of Ii (Ref. 18).

Epitopes attached to the C-terminus of Ii are efficiently presented to T cells both *in vitro* and *in vivo*. Nakano and colleagues[17] showed that an Ii fusion could present the epitope *in vivo*, as adenovirus delivery of the Ii peptide fusion constructs to the thymic epithelium influenced thymic selection. In another study, a major antigenic epitope from HEL(52–61) attached to the C-terminus of Ii stimulated T-cell hybridomas[18]. A maximal response was obtained using L cells transfected with Ii–HEL as APCs. Addition of native HEL to the Ii–HEL transfected L cells resulted in only poor activation of a T-cell hybridoma that recognized a different HEL epitope. This result demonstrated that the HEL(52–61) epitope from endogenously sythesized Ii–HEL fusion protein out-competes peptides from processed native HEL protein. In addition, Ii-HEL transfected L cells were able to elicit a primary T-cell response, demonstrated by *in vitro* stimulation of antigen-specific T cells from transgenic mice and by a strong HEL-specific T-cell response *in vivo* after immunization with dendritic cells (DCs) transfected with Ii–HEL (Ref. 18). The requirements for costimulation have not been defined in this system. It might be that a high density of MHC–peptide on the cell surface can activate naive T cells without the requirement for costimulatory signals, but this issue needs to be further investigated.

Replacing CLIP

An epitope attached to the C-terminus of Ii will be released in the loading compartment for competitive binding to the class II groove. This delivery strategy does not require that the epitope is well defined, because the peptide will be processed by the natural pathway. If, on the other hand, the class II epitope is well mapped, the class II binding region of Ii (81–104 aa), which includes the groove binding site (CLIP) and flanking promiscuous binding sequences[19], can be replaced directly with an antigenic epitope. Ii-fusion proteins containing antigenic sequences with high affinity for the MHC class II groove could even out-compete binding of endogenous Ii. This so-called 'CLIP replacement' approach exploits the natural assembly pathway of class II–Ii complexes to obtain loading of defined T-helper epitopes within the ER, in an environment where other class II peptide epitopes cannot compete for binding. Fig. 1 depicts the events associated with transport and processing of Ii fusion proteins.

Replacement of the groove-binding segment of Ii by antigenic sequences yields recombinant Ii chains that assemble with class II molecules[16,19,20]. These experiments demonstrate that CLIP is not specifically required to assemble transport-competent MHC class II–Ii complexes in the ER. In this strategy, the epitope replacement sequences should be inserted to preserve the natural cleavage sites of Ii, so that maximal normal Ii processing is retained. Van Bergen and colleagues[21] directly analysed the class II bound peptides in $HLA\text{-}DR1^+$ human embryonic kidney 293 cells, transfected with an Ii construct encoding an epitope from influenza haemagglutinin. The results showed that a large percentage of the HLA-DR molecules were occupied with HA sequences, suggesting that the processing of the Ii chimerae occurs in a similar fashion to that of wild-type Ii. Furthermore, these MHC class II bound peptides were efficiently presented to HA-specific $CD4^+$ T-cell clones by a number of different APCs (Ref. 22).

CLIP replacement enhances peptide presentation

The efficiency of class II loading of CLIP substituted epitopes is also illustrated in a study that replaced CLIP with the well defined

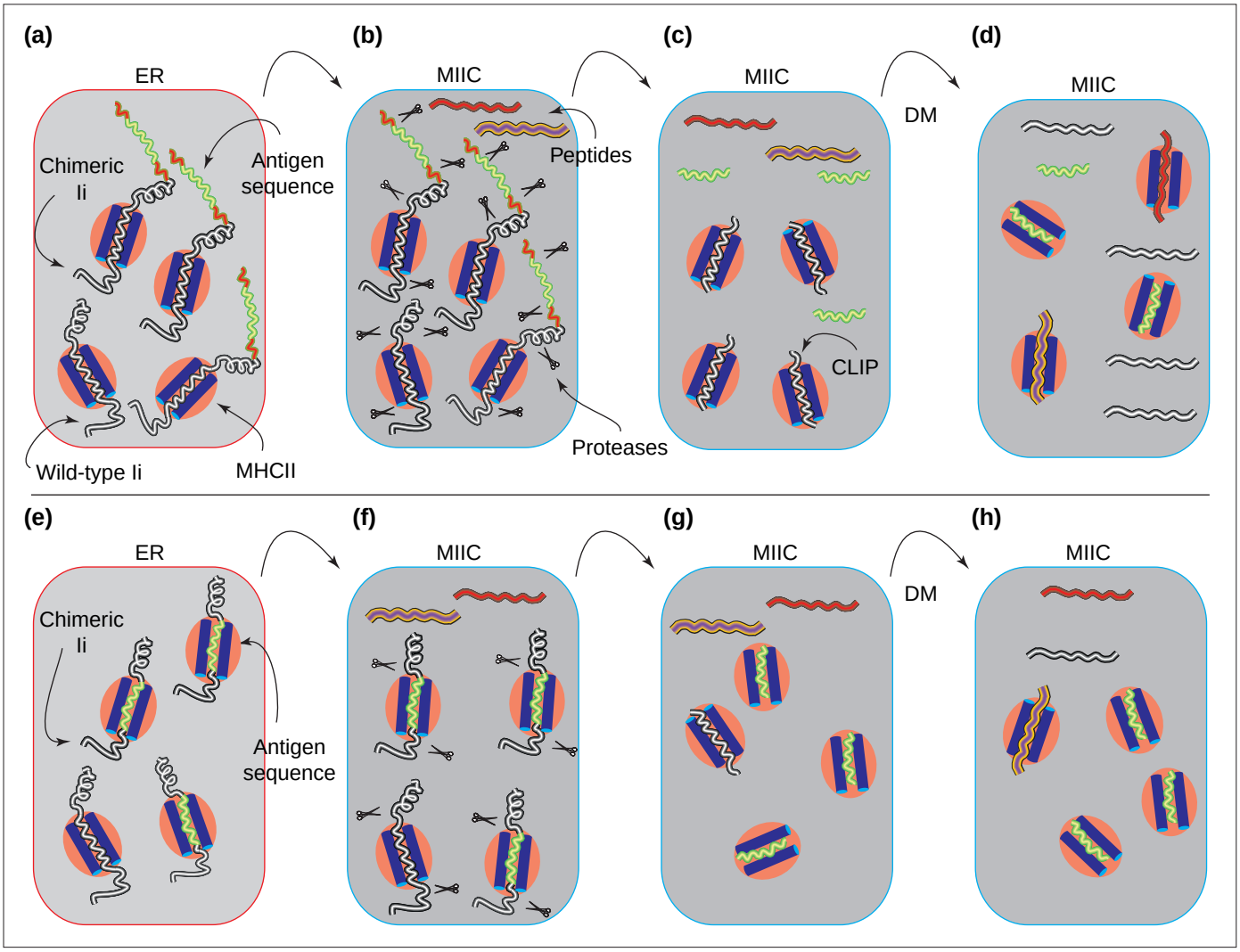

Fig. 1. *Strategies for hijacking a chaperone. (a–d) Sequences encoding an antigenic peptide are attached to the C-terminus of the invariant chain. A gene that encodes a chimeric invariant chain (Ii) with an antigen sequence attached to the C-terminus is introduced into antigen presenting cells. (a) Chimeric and wild-type Ii bind to the peptide binding groove of MHC class II molecules (MHC II) in the endoplasmic reticulum (ER) and are transported to the MHC class II peptide loading compartment (MIIC). In that compartment, exposed regions of Ii are fragmented by proteases (b) and the MHC II-binding peptide from the antigen (green–yellow) is liberated and represents an abundant species in the MIIC, whereas the class II invariant chain-derived peptide (CLIP) remains bound to MHC class II molecules (c). The CLIP–MHC II complexes that remain are acted on by HLA-DM (DM), and peptides in the MIIC are substituted for CLIP (d). The abundance of the antigenic peptide is such that a high proportion of the MHC II molecules are loaded with the MHC II-binding antigen peptide (d). (e–h) Sequences encoding an antigenic peptide are substituted for the CLIP segment of the invariant chain. In this case the antigenic sequence is bound by the MHC II groove in the ER (e). If the antigenic sequence binds with high affinity to MHC II, then the chimeric Ii might compete efficiently with wild-type Ii for MHC II binding. When transported to the MIIC the remainder of the Ii is degraded by proteases (f) leaving MHC II bound to antigenic peptide or CLIP (g). The action of DM will result in other peptides substituting for CLIP in the MIIC groove, but some studies have suggested that high-affinity peptides remain bound to MHC II (h).*

class II I-E α-chain epitope (pEα). Transgenic mice expressing this Ii chimera, in a wild-type Ii deficient background, had about 95% of the total class II molecules occupied by this single peptide[23]. The almost homogeneous class II presentation of the pEα epitope, even in the presence of H-2M, strongly suggests an abundant occupancy by this CLIP substituted epitope during assembly in the ER. Studies using the Ii-pEα transgene on an Ii, H-2M double-knockout background, provided a powerful approach to address an outstanding issue on the role of self-peptides in positive selection; these studies led to the conclusion that a diverse population of self-peptides is required for the positive selection of a functional T-cell repertoire[23]. In a different approach, the same pEα has been covalently linked to a class II β-chain, and a high percentage of class II molecules were found to be occupied by pEα (Refs 24, 25). Thus, covalent attachment of epitopes to class II molecules also allows enhanced presentation of a single peptide.

Fujii and colleagues[16] directly compared the stimulation of T-cell clones by class II positive L cell transfectants expressing either an Ii

with CLIP replaced by a streptococcal antigen or an Ii fusion protein with the same antigen attached to the C-terminus. Stable transfectants expressed similar levels of intracellular recombinant Ii. The CLIP substituted Ii was considerably more effective than the Ii appended antigen in stimulating $CD4^+$ T cells, demonstrating that CLIP substitution is a highly effective strategy for presentation of epitopes.

One limitation of certain epitopes in generating T-cell responses is poor presentation as a result of low affinity for MHC class II molecules. CLIP replacement offers the potential to constrain low-affinity sequences for MHC presentation. For example, a mutation of Ala to Thr in the matrix protein from influenza A virus (MAT) strongly reduced the affinity for binding to DR1 (Ref. 19). However, an Ii-MAT_{Thr} construct elicits an IL-2 response of MAT-specific T cells that is not detected by MAT_{Thr} peptide loaded APCs (Ref. 36). Low affinity Ii-fusion proteins to generate a primary immune response might be useful; it is possible that presentation of the naturally processed low affinity peptide could be sufficient for a secondary response.

The sterical and structural requirements for assembly of Ii-fusion proteins with CLIP substitutions to MHC class II molecules in the ER have yet to be defined. There might well be structural limitations to the nature of CLIP replacement sequences as some Ii-fusions do not appear to properly assemble to MHC class II molecules (N. Koch, unpublished). Thus it is important to examine Ii fusion constructs for their ability to appropriately interact and assembly with class II molecules.

Clinical applications of recombinant Ii

Endosomal targeting of endogenous antigens could be incorporated into the design of vaccines to induce effective antigen-specific $CD4^+$ T-cell responses. Ii constructs could be delivered by recombinant virus or via transfected APCs as both strategies are effective in murine systems[7,17,18]. DCs would be favoured as the APC; however, to maximize antigen presentation it will be important to engineer DCs prior to their maturation.

Directing tumour-antigen presentation

It is now clear that potent $CD4^+$ T-cell responses are critical for generating immune responses against tumours in mice and humans[26,27]. Thus, targeting of antigens to the endosomal pathway for class II presentation has exciting possibilities in generating useful CTL antitumour responses. The principle has recently been demonstrated by tagging the nuclear/cytoplasmic human papilloma virus antigen E7 with the lysosomal targeting domain of LAMP-1. The recombinant vaccinia virus expressing the E7 fusion protein, but not the wild-type E7, provided *in vivo* protection when challenged with an $E7^+$ tumour and furthermore caused regression of established tumours[28,29]. These studies also clearly demonstrate that cytosolic tumour antigens do not reach the class II loading compartment and that retargeting such cystolic tumour antigen to the endosomal pathway dramatically improves the *in vivo* efficacy of recombinant viral vaccines. The E7–LAMP fusion protein in these studies is probably rapidly degraded in lysosomes, so it seems reasonable that engineering Ii would enhance class II presentation even further.

Melanoma-associated genes (MAGE) are activated in many different human tumours; for example, MAGE-3 is expressed in the majority of metastatic melanomas. A melanoma cell line that expresses HLA-DR13 and MAGE-3 was unable to stimulate a human $CD4^+$ T-cell clone, unless it was pulsed with peptide, indicating that endogenously synthesized MAGE-3 does not reach the class II presentation pathway[30]. By contrast, class II positive melanoma cells expressing an Ii(1–80 aa)/MAGE-3 fusion protein stimulated the MAGE specific T-cell clone[30].

Ii constructs have also been shown to introduce antigenic peptides into the MHC class I processing pathway[31]. It is likely that a small percentage of the newly synthesized Ii-fusion proteins can access the conventional class I processing pathway. Thus, it might be possible to design chimeric Ii molecules bearing both T helper and CTL epitopes to improve the efficacy of antitumour vaccination. Alternatively, a class II-targeting vaccine using Ii-fusion proteins could be used in conjugation with vaccines that give enhanced MHC class I presentation, such as fusions with heat shock protein 70 (Ref. 32). Such a combination might result in synergistic activation of multiple arms of the immune system.

For clinical use, addition of large antigenic sequences to the C-terminus of Ii has the advantage over CLIP replacement as a single Ii construct could be prepared for use in the outbred population. CLIP replacement would require class II allele-specific epitopes. On the other hand, CLIP replacement of Ii has the advantage over the covalent attachment of epitopes to class II molecules as the latter strategy requires not only the allele specific epitope but also the appropriate MHC class II β-chain construct for each epitope.

Modulating autoimmunity

Engineered APCs could be used to induce tolerance to autoantigens to modulate autoimmune responses. There is currently considerable interest in the ability of lymphoid-derived $CD8\alpha^+$ DCs to induce tolerance to $CD4^+$ T cells[33]. Isolated $CD8\alpha^+$ DCs could be transfected with recombinant Ii constructs encoding defined autoepitopes, and used as a cellular vaccine to induce tolerance to autoreactive T cells. Alternatively, blocking CD28–B7 signalling of engineered myeloid-derived DCs could induce tolerance to defined autoepitopes.

DNA vaccines

Finally, recombinant Ii constructs could well be amenable as DNA vaccines. A DNA carrier system consisting of covalently linked poly-L-lysine and either mannose or Ig could be used to target the Ii gene specifically to DCs via the mannose or Fc receptors, respectively. A similar strategy has previously been used to target genes successfully to hepatocytes via the asialoglycoprotein receptor[34]. Incorporation of immunostimulatory GpC motifs in the vector DNA (Ref. 35) would ensure activation of DCs and expression of costimulatory molecules. An Ii DNA carrier system would thereby target the construct to the APC and directly prime naive Th cells, overcoming the requirement for local APC recruitment to the site of antigen.

Concluding remarks

The development of an engineered antigen-delivery system superbly illustrates how our basic knowledge of membrane transport pathways can be exploited to develop more effective immunization strategies. It is now clear that effective *in vivo* $CD4^+$ T-cell responses are generated to endogenous synthesized antigens targeted to the class II loading compartment. There is considerable potential to extend these findings in the development of vaccines; in particular, to incorporate both class I and II restricted epitopes on the same polypeptide to generated effective $CD8^+$ responses to tumour antigens.

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