Single cell biochemistry to visualize antigen presentation and drug resistance

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Chaperoning Antigen Presentation by MHC Class II Molecules and Their Role in Oncogenesis

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Tumor vaccine development aimed at stimulating the cellular immune response focuses mainly on MHC class I molecules. This is not surprising since most tumors do not express MHC class II or CD1 molecules. Nevertheless, the most successful targets for cancer immunotherapy, leukemia and melanoma, often do express MHC class II molecules, which leaves no obvious reason to ignore MHC class II molecules as a mediator in anticancer immune therapy. We review the current state of knowledge on the process of MHC class II-restricted antigen presentation and subsequently discuss the consequences of MHC class II expression on tumor surveillance and the induction of an efficient MHC class II mediated anti-tumor response in vivo and after vaccination.

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Introduction

The MHC class I pathway is the only known system able to present intracellular antigens to the immune system. $CD8^+$ cytotoxic T cells recognize the Major Histocompatibility Complex Class I-peptide (MHC class I-peptide) combination and subsequently eliminate cells presenting altered or non-self fragments. As a consequence, this mechanism efficiently clears cells with viruses or other intracellular pathogens. This could include tumor-causing viruses like human papilloma virus, Epstein-Barr virus (EBV) and hepatitis virus. Moreover, cells with mutated self-proteins will also present antigenic fragments of these proteins to $CD8^+$ cytotoxic T cells. Since various mutated proteins can cause cancer, the resulting tumors can be targets for immune surveillance by cytotoxic T cells (1). This, plus the fact that MHC class I molecules are expressed on virtually all cells, makes them the most intensively studied targets in the development of strategies for tumor vaccination (2).

Unlike MHC class I molecules, MHC class II molecules are expressed mainly in the hemapoietic system but can also be expressed in other cell types after stimulation by, for example, interferon γ. Due to this restricted tissue distribution, MHC class II molecules are less popular as mediators in tumor vaccination strategies. Interestingly, the tumors that are currently treated by various vaccination protocols are mainly leukemia and melanoma, tumors that often express MHC class II molecules. These tumors express the accessory panel of proteins necessary for successful loading of MHC class II molecules with antigenic peptides. Since the proteases expressed in tumors may be different from those expressed in normal hemapoietic cells, different fragments of a normal antigen can

* M. Marsman and I. Jordens contributed equally to this paper

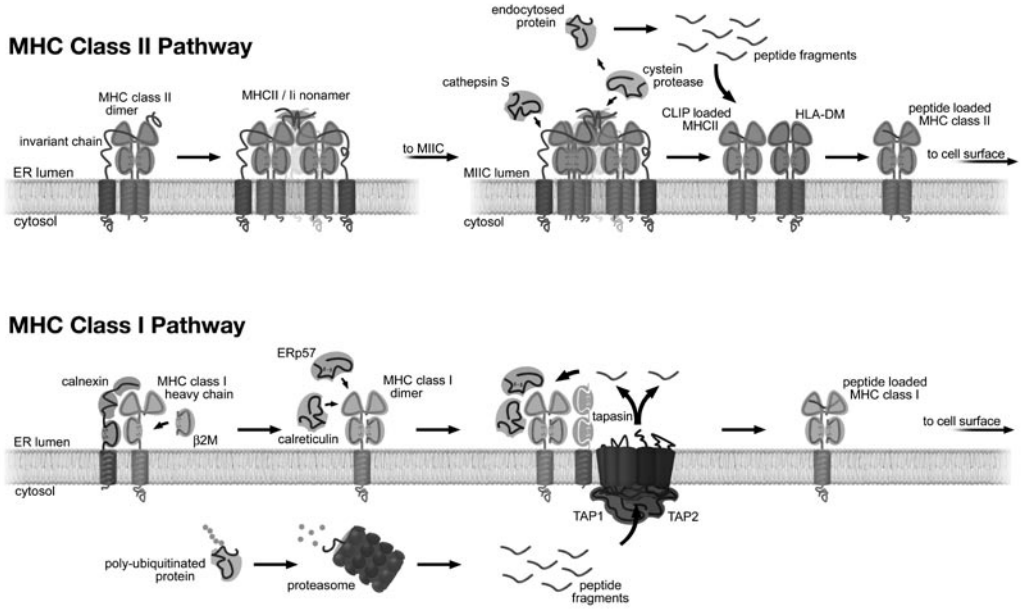

Figure 1. Peptide loading of MHC class II and MHC class I molecules. (**Top**) MHC class II molecules (MHCII) are assembled as dimers in the endoplasmic reticulum (ER) with help of the specialized chaperone invariant chain (Ii), which, in addition, occupies the peptide-binding groove (upper panel). Three of these MHC class II / Ii complexes together form a nonameric complex that is transported to the MHC class II-containing compartments (MIIC). Here, the invariant chain is degraded by cathepsins and proteases until only the part occupying the peptide-binding groove, which is called CLIP, is left. In these compartments, MHCII also encounters antigenic peptide fragments derived from proteins degraded in the endocytic track. CLIP is then exchanged for one of these fragments with help of the chaperone HLA-DM, and the peptide loaded MHCII is transported to the plasma membrane for presentation to the immune system. (**Bottom**) Peptide loading of MHC class I molecules follows a different route (lower panel). After assembly in the ER, and with the subsequent help of the chaperones calnexin, calreticulin and ERp57, the MHC class I molecules dock onto the ER-resident peptide transporter TAP. This process is facilitated by the specialized chaperone tapasin. TAP pumps antigenic peptides from cellular origin that are produced in the cytosol into the ER lumen. These can then bind the ER-retained MHC class I molecules. Peptide binding stabilizes the recipient molecules and allows their transport to the plasma membrane.

be generated and presented, rendering novel antigenic peptides and thus different T cells responses. The resulting $CD4^+$ T cell response may directly eliminate such tumor cells and/or stimulate surrounding $CD8^+$ or NK killer T cells to do so. Fact is that this part of the cellular immune response against tumors is still largely ignored.

Here, we first describe the system required for successful antigen presentation by MHC class II molecules and how this is regulated with the help of three dedicated chaperones. We then discuss the current state of knowledge on the relationship between MHC class II expression and cancer. Finally, the potential application of MHC class II-restricted antigen presentation in the development of tumor vaccination strategies will be discussed.

Multiple steps in MHC class II antigen presentation

A brief introduction in the process of antigen presentation

Although MHC class I and II molecules are very similar in structure and both present peptide fragments (**Figure 1**), they differ in almost all other aspects. The major difference is the source of antigens sampled by these molecules. MHC class I presents fragments from cytoplasmic or nuclear antigens. MHC class II molecules present fragments from proteins degraded in

the endocytic pathway (3, 4). This implies that many steps in the process of successful peptide loading of MHC class II molecules differ from that of MHC class I molecules (**Figure 1**). MHC class II molecules are composed of an α and β chain that assemble in the endoplasmic reticulum (ER) into an αβ heterodimer.

Subsequently, a third chain, the invariant chain or Ii, interacts with this heterodimer to form a heterotrimer. In fact, a trimer of this heterotrimer is formed, resulting in a nonameric complex (5-7). Ii acts as a sort of a pseudosubstrate by allowing a small segment (called CLIP for Class II-associated Ii peptide) to enter the peptide-binding groove of MHC class II. Moreover, Ii is necessary for transport out of the ER as illustrated in mice lacking Ii, which show a reduced surface expression of MHC class II (8, 9).

Whereas most proteins, including MHC class I, are transported via the Golgi directly to the plasma membrane, Ii targets MHC class II molecules from the trans-Golgi network to late endosomal structures, collectively called MIIC for "MHC class II-containing compartments" (10). In the MIIC, all the requirements for efficient peptide loading of MHC class II are concentrated. First, proteases that degrade Ii until only the CLIP fragment is left in the peptide binding groove of MHC class II molecules (11). Second, proteases, reductases and unfoldases that process antigenic fragments which have entered the cell by receptor-mediated or fluid-phase endocytosis (12). Finally, HLA-DM mediating the exchange of the CLIP fragment for fragments generated from the endocytosed antigens (13). The activity of HLA-DM, in turn, can be controlled by a chaperone-of-chaperones called HLA-DO (14-16). Thus, in these specialized MIIC, the unique combination of dedicated (endosomal) chaperones and proteolytic activity supports proper antigen loading of MHC class II molecules. Loaded MHC class II molecules are subsequently transported to the plasma membrane for presentation of endocytosed antigens to $CD4^+$ T cells (11, 17).

The invariant chain, a transporting pseudopeptide

As has been discussed, MHC class II molecules can encounter at least three different specialized chaperones during their existence. The formation of the MHC class II αβ heterodimer is the first step in the formation of the MHC class II complex in the ER and is assisted by various common chaperones such as BiP and PDI (18-21). Rapidly after this assembly step, the first dedicated chaperone Ii is co-assembled (5-7). Ii is usually expressed in molar excess over MHC class II αβ heterodimers and is retained in the ER. Whereas Ii supports exit of MHC class II αβ heterodimers from the ER, the reverse is also the case. MHC class II αβ heterodimers are required for release of Ii from the ER (7-9).

Ii is not as invariant as the name suggests since multiple splice variants exist that are usually co-expressed. The p31/p33, p35, p41 and p43 forms of Ii have been described in humans, whereas mice contain the p31 and p41 form (22-24). Ii is a type II transmembrane glycoprotein containing a short amino-terminal cytoplasmic domain, a single transmembrane domain, a domain that occupies the peptide-binding groove of MHC class II (called Class-II-associated Ii peptide or CLIP), and a carboxyl-terminal trimerization motif (**Figure 2**). Ii forms trimers that interact with dimers of MHC class IIαβ in the ER (5-7) (**Figure 1**). The interaction of Ii with MHC class II is necessary for proper folding and supports transport of MHC class II molecules (9, 25-27) from the ER to endosomal structures (8).

Ii, however, not only functions as a mediator in transport. Early in assembly, the CLIP segment of Ii enters the MHC class II peptide-binding groove. This acts as a pseudopeptide, preventing premature loading of MHC class II with peptides that have entered the ER for binding to MHC class I molecules (28). In addition, peptide (or pseudopeptide) binding is required to pass the 'ER quality control system' for transport to the endocytic pathway (8, 9), a situation strongly resembling that of MHC class I molecules, which also require peptide binding for transport out of the ER (29, 30). After ER exit, the vast majority of MHC class II/Ii complexes enters the endocytic pathway (10), although a small percentage is transported directly to the plasma membrane via the secretory pathway (31), followed by rapid internalization and sorting into the endocytic pathway (32, 33).

The cytoplasmic domain of Ii contains two di-leucine motifs (**Figure 2**), which are necessary for sorting of MHC class II molecules to the MIIC and also for internalization from the plasma membrane (34-39). There is some debate about how the MHC class II complexes traffic to the MIIC. Some reports describe trafficking via early endosomes to MIIC (32, 40), but most show direct targeting from the trans-Golgi network to the MIIC (10, 41-43). In all cases, the MHC class

II complexes are targeted to the pre-lysosomal MIIC compartment.

Processing of the invariant chain

Once inside the endocytic pathway, Ii is degraded by various proteases in a processive manner. The Ii degradation is essential for transport of antigen-loaded MHC class II molecules from the MIIC to the plasma membrane. A 22 kD fragment (P22 LIP for leupeptin-induced protein), a 10 kD fragment (P10 SLIP for small LIP), and as has been described, the ~2.5 kD fragment CLIP have been defined as Ii degradation intermediates associated to MHC class II molecules (44). Although cathepsin B and D were originally claimed to be responsible for degradation of Ii, this concept was abandoned when it was observed that Ii degradation proceeded normally in mice lacking these proteases (45, 46). Instead, Ii degradation involves more specific proteases such as cathepsin S and L. Inhibition of cathepsin S impaired MHC class II antigen presentation and Ii degradation (47, 48). A marked tissue specific expression profile is observed for the cathepsins. In macrophages cathepsin S is up- and cathepsin L is down-regulated upon interferon γ stimulation (49). Where peripheral antigen presenting cells contain relatively high cathepsin S levels (49, 50), cathepsin L appears to be crucial for Ii degradation in cortical tissue endothelial cells (51, 52). Thus, cathepsin S and L are both important with partially overlapping activities for Ii degradation, although other (currently unknown) proteases will be involved in other steps of this degradation.

Further studies found cathepsin L tightly bound to the p41 form of invariant chain (53). By interacting with the thyroglobulin domain (TGD) of the invariant chain (**Figure 2**) proteolytic activity of cathepsin L is inhibited (54). On the other hand, cathepsin L is protected from premature destruction by binding to the p41 isoform (55). Collectively this may result in regulation of antigen processing and loading of MHC class II and could explain the enhanced antigen presentation observed for the p41 form (56), but this has to be further elucidated.

In conclusion, several proteases are required for one simple but crucial act, the removal of a transporting chaperone 1 to 3 hours after assembly of the MHC class II/Ii nonamer. This is critical for the exchange of remaining CLIP fragments for antigenic peptides and for the transport of MHC class II to the plasma membrane.

The invariant chain, more than just a MHC class II chaperone

Invariant chain does not merely function as a MHC class II chaperone preventing peptide loading in the ER, stimulating exit from the ER and modulating antigenic peptide loading, but it may have additional functions as well. The development from immature to mature B-cells is impaired in Ii-deficient mice. These B-cells arrest in an immature stage with low IgD and CD23 levels (57, 58). The N-terminal cytoplasmic domain of Ii is required for B-cell maturation, since expression of only this domain suffices to stimulate

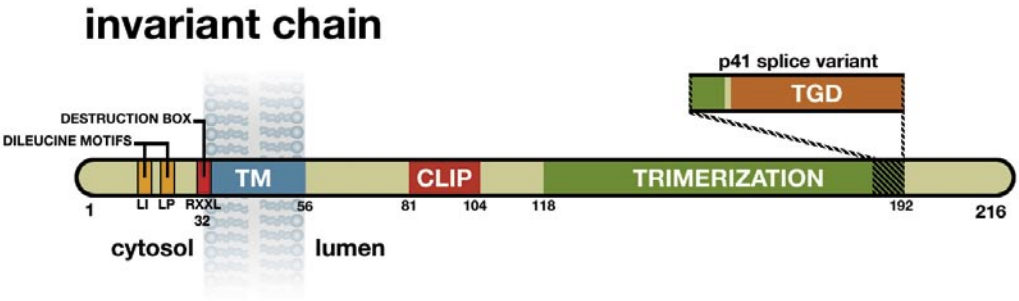

Figure 2. Structural overview of the p31 isoform of the human invariant chain. The invariant chain is a type II transmembrane glycoprotein containing a short amino-terminal cytoplasmic domain, a single transmembrane domain (TM), a domain that occupies the peptide-binding groove of MHC class II (called Class-II-associated Ii peptide or CLIP), and a carboxyl-terminal trimerization motif. The cytoplasmic domain contains two di-leucine motifs that are essential for sorting and intracellular trafficking. A destruction box motif (RXXL) near the transmembrane region appears to be involved in nuclear NF-κB signaling. Also depicted is the p41 isoform that harbors an additional 64 amino acid Thyroglobulin domain (TGD) known to regulate cathepsin L activity.

B-cell maturation (59). The Ii cytosolic domain diffuses from MIIC into the nucleus, where it is supposed to activate NF-κB signaling which then results in B-cell maturation (60). A destruction box RXXL motif (**Figure 2**) in the released cytosolic domain of Ii appears to be important in down-regulation of the signaling cascade, by inducing degradation of the signaling peptide. Mutations in this domain block degradation of the cytosolic Ii fragment, inducing NF-κB activation. This process resembles that found for various integral membrane proteins where a short-lived soluble fragment is released after proteolysis that migrates to the nucleus to induce transcription, a process named RIP for Regulated Intramembrane Proteolysis (61).

HLA-DM, the editor for antigenic peptide loading of MHC class II molecules

The need for additional chaperones in the loading of MHC class II molecules with peptides was not obvious. Only after a thorough analysis of B cell lines with deletions in the MHC locus at chromosome 6, it became apparent that additional proteins were required for successful peptide loading of MHC class II molecules. The genes were subsequently identified as HLA-DMA and HLA-DMB that assemble into HLA-DM (H2-M in mice), a nonpolymorphic type I membrane protein with high similarity in sequence and structure to MHC class II molecules (62-66). HLA-DM and MHC class II genes probably arose by gene duplications of a shared ancestor gene. After assembly in the ER, HLA-DM is transported to MIIC through a tyrosine-based targeting signal in the cytoplasmic tail of HLA-DMB (67, 68). Although HLA-DM accumulates in MIIC, it probably recycles via the plasma membrane by efficient re-internalization mediated by the tyrosine-motif in the HLA-DMB tail (69, 70).

HLA-DM deficient cells and mice express surface MHC class II molecules loaded with the Ii-degradation fragment CLIP instead of antigenic peptides (63, 64, 71-75). Thus, although some spontaneous exchange of CLIP for antigenic fragments can occur in the acidic MIIC (76), efficient exchange requires HLA-DM to release CLIP and low affinity peptides, while allowing high affinity peptides to remain associated (77-83). Further *in vitro* experiments showed that the interaction between HLA-DM and MHC class II molecules and the 'activity' of HLA-DM were facilitated by acidic pH, as found in the MIIC (84-87). HLA-DM appears to stabilize MHC class II molecules devoid of peptide to allow binding of high-affinity peptides and, at the same time, as a true chaperone, prevents the aggregation of 'empty' MHC class II molecules (86, 88).

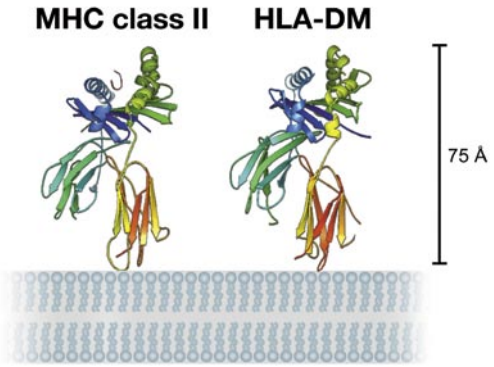

Figure 3. The structure of HLA-DM shows high structural identity to MHC class II molecules. The structures of a MHC class II molecule (in this case HLA-DR3) and HLA-DM are projected above the membrane. The transmembrane regions of both molecules are undefined and not depicted. Note the absence of an open peptide-binding groove in HLA-DM, which prohibits peptide binding.

The structure of HLA-DM reveals a molecule with a high structural identity to MHC class II molecules (**Figure 3**). One major difference is the absence of an MHC class II peptide-binding groove in HLA-DM, which renders it unable to bind peptides (and the invariant chain) (89, 90). A co-crystal of HLA-DM and MHC class II molecules has not been generated, but mutational studies have revealed areas in the top part (peptide-binding groove) of MHC class II and HLA-DM as interacting segments (91, 92). MHC class II molecules are highly polymorphic and different MHC class II alleles present different fragments from the same antigen. Still, Ii as well as HLA-DM are non-polymorphic and interact with all polymorphic MHC class II alleles (93). As a consequence, the binding affinity of CLIP for MHC class II molecules differs for the different MHC class II haplotypes, possibly resulting in a different dependency on HLA-DM (94). Whether this results in differences in peptide loading of MHC class II remains unclear.

HLA-DO, the chaperone of chaperones

As described, efficient loading of MHC class II with specific antigenic peptides is tightly regulated by the

chaperones Ii and HLA-DM. More recently, attention has shifted to another MHC class II look-a-like, also encoded in the MHC locus. Two genes encoding for HLA-DOA and HLA-DOB were identified that assemble into a HLA-DO (or H2-O the murine homologue of HLA-DO) heterodimer (95, 96). Like HLA-DM, HLA-DO has a very high sequence identity to HLA-DR molecules, which suggests that they arose from recent gene duplication (97). HLA-DO is also a nonpolymorphic heterodimer with lysosomal targeting sequences located in the cytoplasmic tail of HLA-DOB (69). Unlike HLA-DMB, HLA-DOB contains two putative targeting signals, a di-leucine motif and a tyrosine-based motif (69).

Whereas HLA-DM is always co-expressed with MHC class II molecules in APCs, HLA-DO is only expressed on a subset of thymic medullary epithelium and in immature B cells (95, 98, 99). Moreover, both HLA-DO and HLA-DM are rapidly down-regulated upon activation of B cells (100). A stable interaction is formed between HLA-DO and HLA-DM, and targets HLA-DO to the MIIC (16). Upon deletion of its targeting signals, HLA-DO is still targeted to the MIIC via HLA-DM (69). Subsequently, the HLA-DM/DO heterotetramer recycles between MIIC and the plasma membrane, although it accumulates in MIIC (69).

HLA-DO acts as a negative regulator of HLA-DM since MHC class II molecules loaded with the CLIP fragment appeared at the plasma membrane in response to ectopic expression of HLA-DO or overexpression of H2-O in transgenic mice (14, 15, 101, 102). Mice deficient for H2-O have only mild phenotypes, including an increase in antibody titer in plasma, suggesting that B cell proliferation is less tightly controlled (103, 104). Further studies revealed that HLA-DO altered the pH sensitivity of HLA-DM in supporting peptide loading of MHC class II molecules *in vitro*. Apparently, HLA-DO acts as a pH sensor restricting HLA-DM activity to more acidic (late endosomal) structures (101). The function of HLA-DO is not fully understood, the assumption is that the activity of B cells should be tightly controlled, implying that antigenic peptide loading of MHC class II should primarily occur with antigens recognized by surface immunoglobulins. The activity of HLA-DO skews peptide loading to the late endosomal structures where B cell receptor-mediated antigens are processed. HLA-DO may thus play a critical role in controlling B cell activation by regulating the activity of HLA-DM.

From the MIIC to the plasma membrane

Obviously, proper loading with antigenic peptides in the MIIC is not sufficient for successful MHC class II antigen presentation. Therefore, MHC class II molecules first have to be transported to the plasma membrane. Transport of MHC class II has been studied using combinations of electron microscopy and real-time imaging of GFP-tagged MHC class II molecules. MIIC move along microtubules from the Golgi area around the microtubule organizing center (MTOC) toward the plasma membrane (105). This transport is similar to lysosomal transport and occurs in a bi-directional manner and in a stop-and-go fashion, mediated by the alternate activities of the dynein/dynactin and kinesin motor proteins (106). How the motor protein activities are controlled is largely unclear, but the dynein/dynactin-mediated transport toward the minus-end involves at least the activity of the small GTPase Rab7 and its effector protein RILP (107).

Finally, the MIIC reaches the end of the microtubule at the cortical actin cytoskeleton just underneath the plasma membrane. How the last step occurs is unclear, but ultimately the MIIC fuses with the plasma membrane, as shown by electron microscopy (105, 108). At the plasma membrane, part of the intracellular content (the internal vesicles in a multivesicular body) is secreted in the form of so-called exosomes (108, 109). This is probably a small fraction because otherwise many internally residing proteins like HLA-DM and the tetraspans would be depleted from cells within 1 to 2 hours (which is the turnover time of MIIC in most cells (10)). The majority of the internal structures of the MIIC probably fuse back to the plasma membrane, followed by rapid internalization of the late endocytic MIIC proteins via their internalization signals (69, 70). Subsequently, these proteins are transported back to the MIIC through the endocytic pathway. Since only a fraction of the MHC class II molecules can be internalized, MHC class II accumulates at the plasma membrane (110).

Interestingly, surface MHC class II molecules do not behave identically in all cell types. In fact, the half-life of MHC class II molecules differs considerably among different cell types. It is relatively long in melanoma cells and B cells compared to primary monocytes and dendritic cells. The half-life in dendritic cells increases (up to 100 h) after activation, which is possibly due to an increase in stable MHC class II-peptide complexes (111, 112).

A fraction of the MHC class II molecules can be internalized (110, 113) and recycled back to the plasma membrane. In monocytes, the reappearance of MHC class II at the plasma membrane is controlled by interleukin 10 (114). Treatment with IL-10 results in a strong reduction of cell surface MHC class II molecules, possibly by affecting the Rab7 pathway, which, in turn, controls dynein motor-mediated MIIC transport (our unpublished results). This is the first example of regulation of MHC class II responses by manipulation of the last step in intracellular transport of MHC class II molecules to the cell surface.

An alternative route of MHC class II molecules from the MIIC to the plasma membrane has been proposed for dendritic cells (115-117). Upon activation of DCs, the MIIC appears to alter its morphology, resulting in the formation of long tubular structures extending into the periphery. Live imaging of these cells revealed that these class II-positive structures, similar to the conventional MIIC, move in a microtubule-dependent manner (116). Probably in response to the gross alteration of the cytoskeleton of activated dendritic cells, the MHC class II molecules move more in the direction of the contact site with a specific T cell. Surprisingly, no accumulation of GFP-tagged MHC class II molecules was observed in the 'immunological synapse' between the DC and the T cell (116). The exact function of this directed transport of MIIC-derived tubules after DC activation still has to be revealed.

Thus two modes of transport of MHC class II molecules from MIIC to the plasma membrane have been reported: direct transport and fusion of MIIC with the plasma membrane and the formation of tubular structures. In both cases, transport requires motor-based microtubule transport likely to be mediated by dynein/dynactin and kinesin motor proteins, with the small GTPase Rab7 as one of the controllers of this transport step.

Interfering with antigen presentation by MHC class II molecules

Promoting antigen presentation

The pathway of antigen presentation by MHC class II molecules which has been outlined above shows that it requires a 'multi-enzyme' process involving various chaperones, acidic pH and proteases at different stages of biosynthesis. Obviously, affecting one or more of these enzymes can positively or negatively influence antigen presentation. For instance, MHC class II antigen presentation can be improved when antigens are more efficiently acquired and targeted to MIIC. Macrophages and monocytes, in contrast to B cells, are able to internalize large volumes. This implies that many antigenic fragments have to compete for access to MHC class II molecules in the MIIC. More selective uptake of antigen using surface immunoglobulins in B cells (118-120), Fc-receptors on macrophages and DCs (121, 122) or mannose-receptors on DCs (123) will strongly improve antigen presentation by MHC class II molecules (124). Cells may also alter the conditions for antigen presentation in MIIC. Best studied are DCs that acidify the MIIC upon activation (125), and B cells that down-regulate HLA-DO upon activation (100). In both cases, antigen presentation by MHC class II molecules is more efficient.

Inhibiting antigen presentation

If activation of MHC class II antigen presentation is an option, the reverse is almost certainly true as well. For instance, Th2 cell activity controlling immune responses can inhibit class II antigen presentation. Another means of inhibiting MHC class II responses is by interfering with endosomal proteases, as was first shown by using leupeptin. Leupeptin is a protease inhibitor that inhibits complete degradation of Ii (11, 126). Since Ii degradation is a prerequisite for transport of MHC class II molecules to the cell surface, these inhibitors are negative regulators of class II presentation (11, 127, 128). Naturally occurring protease inhibitors exist as well. Cystatin is a reversible inhibitor of cysteine proteases like the cathepsins. Cystatin family members are expressed in a tissue-specific manner and can modulate cathepsin activities and thereby inhibit antigen presentation by MHC class II molecules (129), although this is still somewhat controversial.

Pathogens are known to use a similar system to inhibit MHC class II presentation, as has been reported for two filarial nematodes, *Onchocerca volvulus* and *Acanthocheilonema viteae* (130, 131). Both nematodes produce cystatin-like molecules that have an immunosuppressive activity by inhibiting cathepsin S and L. Moreover, Bm-CPI-2 is a cystatin homologue secreted by the parasite *Brugia malayi* that also interferes with MHC class II processing by inhibiting multiple cysteine proteases (132).

Intracellularly growing bacteria use different strategies to prevent presentation by MHC class II molecules.

These pathogens include *Salmonella Thyphimurium*, *Mycobacterium Tuberculosis* and *Mycobacterium Leprae*, which usually reside in the endo/phagosomal pathway. Some of these pathogens are able to modulate the endo/lysosomal compartments. *Salmonella* injects several effector proteins into the host cytosol, which prevents fusion of the phagosome with mature lysosomes (133). It has been found that one of the bacterial effectors is a PI3P phosphatase (134) that has been implicated in inhibition of the formation of internal structures within the MIIC (135) and antigen presentation by MHC class II molecules (136). Thus, *Salmonella* might interfere with MHC class II presentation in multiple ways. It escapes the degradation in the mature lysosomes, thereby limiting the amount of *Salmonella*-derived antigens. Secondly, by preventing the formation of internal membranes, *Salmonella* might reduce the peptide loading efficiency by preventing efficient interactions between MHC class II and HLA-DM (W. Zwart in preparation). Nature has thus developed a complicated system to allow presentation of antigenic fragments generated in the endosomal track. It has also developed multiple ways to manipulate this process, exploited not only by pathogens, but also by tumors, as will be discussed in the following text.

MHC class II molecules in oncogenesis

Immune system involved in tumor surveillance

Various tumors down-regulate MHC class I expression by inactivating transcription of the MHC locus (137). Specific MHC class I alleles can be down-regulated in rarer cases as well (138-140). The observation that down-regulation of MHC class I expression correlates with an aggressive or more advanced tumor phenotype (141) suggests that the immune system is involved in controlling tumor outgrowth. Still, the exact role of the immune system in tumor surveillance is not fully understood.

Transplantation patients that receive immunosuppressive drugs rarely develop tumors other than leukemia after use of cyclosporine for 10 to 12 years. Also, patients with genetic defects in components of the MHC system (CIITA, TAP) do not show a higher tumor incidence but merely a higher susceptibility to bacterial and viral infections (142-144).

Whereas MHC class I and II molecules may be not highly important in active immune surveillance to various tumors, there are a number of notorious exceptions. As has been mentioned, leukemias appear with high incidence in immunosuppressed patients (145, 146). Furthermore, melanomas and renal cell carcinomas are known to spontaneously regress in some patients and it is thought that this is due to tumor recognition by the immune system (147, 148). Especially for melanoma, many tumor-specific CTLs have been isolated, often recognizing melanoma proteins presented in the context of MHC class I, or activated with the help of MHC class II molecules that present tumor-specific antigens (149-157). It is, therefore, not surprising that most attempts to use immunotherapy for tumor eradication concentrate not only on the virally induced tumors, but also on leukemia (using minor histocompatibility antigens), melanoma (using dendritic cells pulsed with melanoma extracts or long antigenic peptides) and renal cell carcinomas, with some impressive successes (2, 158-160). Of note is that usually only MHC class I-restricted responses are considered in these therapies.

MHC class II expression and tumor development

Why are MHC class II-restricted responses usually not considered in tumor development? One reason could be that most tumors do not express MHC class II molecules. Exceptions are -obviously- B cell leukemias like chronic lymphocytic leukemia, Burkitt lymphoma, EBV-induced B cell Non-Hodgkin Lymphoma, follicular lymphoma, and Kahler's disease (161). In addition, melanoma can express MHC class II molecules (162), although often in a rather heterologous manner, and Glioma type 1 tumors also constitutively express MHC class II (163). Furthermore, interferon γ and possibly other factors can induce MHC class II expression on Glioma type 2 and many other tumors, including cervix tumors (164) and bladder cancer (165). In most cases, not only MHC class II molecules are expressed but also the accessory proteins required for efficient transport (Ii) and peptide loading (HLA-DM) (166).

Various studies report on a correlation between expression of MHC class II molecules and prognosis (167-170), but whether this is the result of an anti-tumor immune response or simply because MHC class II expression is a marker for another differentiation state of the tumor (and thus different growth and invading properties) remains unclear.

MHC class II-restricted presentation of exogenous antigens can easily be observed *ex vivo* by EBV-trans-

formed B cells (39) and melanoma cells (101), implying that the MHC class II system 'works' correctly and efficiently in these tumors. In addition, MHC class II molecules can present 'tumor-specific antigens' like Epstein Barr viral (EBV) antigen EBNA-1 (171) and melanoma-specific antigens like tyrosinase and gp100 (172, 173). Still although class II presentation is occurring, an efficient host response is obviously lacking when a tumor appears and selective outgrowth of tumor-specific $CD4^+$ T cells has not been reported in patients, even though these T cells can be expanded *in vitro*. Apparently tumor factors prevent expansion of these cells.

Interestingly, some tumors that express MHC class II also express inhibitors for the MHC class II antigen presentation process. Most notably is the inhibitor for HLA-DM, HLA-DO, in B cell leukemia. Whether this again reflects a differentiation difference (HLA-DO is best expressed in immature B cells) or an active attempt to inhibit antigen presentation by MHC class II is unclear. Other tumors secrete inhibitory cytokines like IL-10 to suppress MHC class II and other responses, which is particularly clear for melanomas (174) and EBV-induced B cell tumors (175). The EBV genome encodes a homologous protein for IL-10 (BCRF1; (176, 177) that may also inhibit T cell responses, although this is not fully established. Other tumors, like neuroblastoma, actively down-regulate expression of MHC class II by silencing the CTIIA promoter (178).

Patients selectively lacking expression of MHC class II molecules (and not MHC class I) exist. These bare lymphocyte syndrome patients usually have genetic defects in the transcription machinery regulating expression of MHC class II molecules and its accessory proteins HLA-DM and the invariant chain (179). Defects have been reported for the transcription factor CIITA (143). Although these patients are prone to many infections, no increased rate of tumor formation has been reported. Similarly, no increased tumor incidence is observed in mice deficient for MHC class II molecules, DM or Ii. These observations suggest that class II plays no active role in the tumor formation.

However, this does not exclude a relevant role of MHC class II molecules in anti-tumor responses. First, MHC class II molecules are essential for the induction of proper CTL responses (180). These are most likely not the MHC class II molecules expressed on tumors but merely MHC class II molecules expressed on professional antigen-presenting cells, like DCs. These cells probably internalize apoptotic bodies or necrotic debris from tumor cells and present fragments to the $CD4^+$ T cells, which then stimulate cytotoxic T cell proliferation (180). State-of-the-art tumor vaccination strategies therefore include -beside antigens for MHC class I molecules- antigens for presentation by MHC class II molecules. These antigens can be targeted into the MHC class II pathway using Fc-receptor-mediated uptake (181), via the mannose receptor (182), or (although this pathway is more undefined) used directly in the form of exosomes (109). The stimulation of both the MHC class I and MHC class II pathways may ensure a better stimulation of tumor-specific cytotoxic T cells, and thus a better anti-tumor response.

Toward MHC class II-restricted tumor immunotherapy

Like MHC class I molecules, MHC class II molecules also require specific antigens as targets for immunotherapy. Three types of targets are available:

1) Viral antigens expressed in virally-induced tumors. An obvious candidate is EBV in B-cell non-Hodgkins lymphoma. Especially the EBV coat proteins (183) and the EBV protein EBNA-1 (184) should reveal good fragments for MHC class II molecules. Whether v-IL10 expressed by EBV prevents an efficient response is unclear (185). If so, a combination of vaccination with EBV protein or peptide antigen with simultaneous neutralization of v-IL10 could mount an efficient MHC class II-restricted anti-tumor response.

2) Tumor-specific proteins. These can be mutated proteins or proteins expressed in a strong tissue-specific manner. Examples of the first are mutated growth factor receptors that could become constitutively activated and can lead to constitutive cell growth and tumorigenesis, such as mutated EGF receptor and the ERB2 (neu) receptor (186, 187). These receptors are degraded in the endosomal pathway generating peptides for MHC class II molecules. However, no CTLs specific for these mutated antigens have been identified yet. Examples of normal antigens expressed in a tissue-specific manner are tyrosinase and gp100, two proteins expressed in cells of the melanocytic lineage (188). These antigens are currently tested as antigens for MHC class I-restricted tumor therapy against melanoma (189-192). Since these antigens are mostly degraded in the lysosomal pathway, strong MHC class II responses could be expected. Indeed, MHC class II-

restricted T cells against some of these antigens can be isolated and may be used to eradicate MHC class II expressing melanoma (172, 193). Obviously, tissue specific antigens are also present in B cells tumors. These include CD20, a cell surface protein that is currently targeted in antibody-based immune therapy against B cell non-Hodgkin's lymphoma (194). In principle, fragments of CD20 presented in the context of MHC class II molecules could be used for cancer therapy as well.

3) Minor histocompatibility antigens. Minor histocompatibility antigens were discovered following transplant rejections of fully HLA-matched individuals (195). A similar response occurred in leukemia patients following bone marrow transplantation. The resulting graft-versus-host response improved the anti-tumor response and minor histocompatibility antigens are now tested as a mode of tumor immunotherapy. The minor histocompatibility antigens were originally identified as MHC class I presented peptides from proteins with single amino acid variations between donor and acceptor (196). Such antigens have recently been identified for MHC class II molecules as well, yet they have not been tested in a tumor setting (197).

Concluding remarks

Most attention in immunotherapy of cancer has been on MHC class I molecules, but the concepts with respect to the source of targets (antigens) is very similar for MHC class II molecules. In fact, there is no good reason to ignore MHC class II molecules. Using antigen presentation by MHC class II may even have a number of profound advantages. First of all, any therapy is more selective since MHC class II molecules are only expressed on a restricted number of tissues. In addition, delivery systems for antigenic fragments to MHC class II molecules are considerably more simple compared to MHC class I. Antigens do not have to be delivered into the cytoplasm but simply opsonized to enter the endocytic track for efficient loading onto MHC class II. Finally, whereas MHC class I molecules interact only with short peptides that do not allow further modifications, MHC class II molecules bind peptides in a different way, as peptides associated to MHC class II molecules extent out of the peptide-binding groove (198). Modifications can safely be introduced in these extensions without affecting the immunogenicity. Yet, the pharmacodynamics and stability of the peptides can be strongly affected. However, these modifications do allow further improvement in targeting the peptides to the correct cells by using, for example, the mannose receptor on DCs (199), and may be critical for mounting an efficient and successful immune response against tumors.

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