Interferon- γ inducible exchanges of 20S proteasome active site subunits: Why?

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Abstract — When cells are stimulated with the cytokines IFN- γ or TNF- α , the synthesis of three proteasome subunits LMP2 (β 1i), LMP7 (β 5i), and MECL-1 (β 2i) is induced. These subunits replace the three subunits delta (β 1), MB1 (β 5), and Z (β 2), which bear the catalytically active sites of the proteasome, during proteasome neosynthesis. The cytokine-induced exchanges of three active site subunits of a complex protease is unprecedented in biology and one may expect a strong functional driving force for this system to evolve. These cytokine-induced replacements of proteasome subunits are believed to favour the production of peptide ligands of major histocompatibility complex (MHC) class I molecules for the stimulation of cytotoxic T cells. Although the peptide production by constitutive proteasomes is able to maintain peptide-dependent MHC class I cell surface expression in the absence of LMP2 and LMP7, these subunits were recently shown to be pivotal for the generation or destruction of several unique epitopes. In this review we discuss the recent data on LMP2/LMP7/MECL-1-dependent epitope generation and the functions of each of these subunit exchanges. We propose that these subunit exchanges have evolved not only to optimize class I peptide loading but also to generate LMP2/LMP7/MECL-1-dependent epitopes in inflammatory sites which are not proteolytically generated in uninfamed tissues. This difference in epitope generation may serve to better stimulate T cells in the sites of an ongoing immune response and to avoid autoimmunity in uninfamed tissues.

proteasome / interferon- γ / antigen presentation / MHC class I

1. Introduction

The proteasome is by far the most important provider of peptide ligands for the presentation on major histocompatibility complex (MHC) class I molecules [1–3]. However, the proteasome executes a host of important cellular functions other than antigen processing and evolved much earlier than the specific immune system [4, 5]. It is therefore assumed that both the peptide binding groove of MHC class I molecules as well as the transporter associated with antigen processing (TAP) [6] adapted to the range of peptide products which can be produced by the proteasome [7]. In vitro, the 20S and 26S proteasomes produce peptides of 3–25 amino acids in length of which about 15% meet the strict length requirement of eight or nine amino acids for MHC class I ligands [8–11]. In order to bind with reasonable affinity to the cleft of class I proteins, the peptide ligands need to contain anchor residues which are situated at the C terminus and at some other position within the peptide sequence which varies among different MHC molecules and alleles [12]. The proteasome is responsible for generating the C-termini of

class I ligands but the binding pockets for P1 residues in proteasomes do not perfectly match the C-terminal anchor residues of MHC class I peptide ligands which are hydrophobic in mice and either hydrophobic or basic in humans. The proteasome contains three different active site subunits named β 5, β 2, and β 1 which according to mutagenesis experiments in yeast preferentially cleave C-terminal of hydrophobic, basic, and acidic residues [10, 13]. These three activities have been classified with fluorogenic peptides according to their P1 specificity as chymotrypsin-like, trypsin-like, and caspase-like, respectively. Since no class I molecules with acidic anchor residues have been described in mice or humans in spite of extensive investigations it appears that the caspase-like activity would not be able to generate class I ligands in mice and humans and may even destroy them by internally cleaving potential class I ligands.

It appears that the immune systems of mice and humans have invented a mechanism to adjust the specificity of the proteasome to the needs of MHC class I restricted antigen presentation. When the antiviral cytokines IFN- γ or TNF- α are produced by T cells during an acute immune response, the cytokine-stimulated cells in the inflammatory site will transcriptionally induce the synthesis of three extra proteasome subunits called LMP2 (β 1i), LMP7 (β 5i), and MECL-1 (β 2i). They are homologous to the three active site bearing subunits delta (β 1), MB1 (β 5),

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Abbreviations: CTL, cytotoxic T lymphocyte; IFN, interferon; MHC, major histocompatibility complex; TAP, transporter associated with antigen processing; TNF, tumor necrosis factor.

and Z ($\beta 2$) of the proteasome and due to a strong overproduction, the inducible subunits are incorporated into newly assembled proteasomes instead of the constitutively expressed subunits. The inducible subunits also possess the residues which are required for the peptidolytic activity like ^1Thr or ^{33}Lys and can thus be expected to be catalytically active. Although these subunit exchanges have been intensively investigated for the past 10 years, their precise function is still a matter of debate and largely unknown.

2. LMP2 versus delta: a coherent picture

The most consistent data and conceptions exist for the replacement of subunit delta by LMP2 because it has been unanimously reported by several laboratories that the overexpression either of mutated or wildtype LMP2 reduces the caspase-like activity of the proteasome almost to background levels [9, 14–16]. Conversely, the 20S proteasomes from spleen cells of LMP2^{-/-} mice displayed an enhancement in the caspase-like activity compared to wild type mice [17]. Incorporation of LMP2 led to a slight increase in the chymotrypsin-like activity which is consistent with the labeling of LMP2 but not delta (also named ‘Y’) by active site inhibitors selectively inactivating the chymotrypsin-like activity of the proteasome [18]. The data is in perfect agreement with structural considerations which are based on the three-dimensional structure of 20S proteasomes from *S. cerevisiae* that has been resolved at high resolution by X-ray crystallography [19]. A sequence alignment of the $\beta 1$ subunits of yeast (PRE3) and humans (delta) as well as LMP2 ($\beta 1i$) suggests that the exchange of LMP2 for delta renders the P1 pocket of the $\beta 1$ subunit such that hydrophobic rather than acidic residues can be accommodated. As outlined above, this change in substrate specificity can be expected to favour the generation of MHC ligands for murine and human class I molecules. Interestingly, chickens, which do express MHC class I molecules that bind peptide ligands with an acidic residue at the C-terminus, lack MHC encoded proteasome subunits like LMP2 or LMP7, suggesting that these subunits evolved as a consequence of the restrictive peptide specificity of murine and human class I molecules [20]. Accordingly, one may expect that the MHC class I cell surface expression would be reduced in the absence of LMP2, but such a phenomenon was not observed in spleen cells of LMP2^{-/-} mice indicating that the fairly high class I expression of B cells and T cells can be sustained with peptide pools from LMP2-deficient proteasomes [17]. Also the class I-restricted antiviral response was not generally impeded in LMP2^{-/-} mice. The frequency of cytotoxic T cells (CTLs) specific for a nucleoprotein epitope of Sendai virus was the same in infected LMP2^{-/-} and wild type mice whereas the frequency of influenza nucleoprotein specific CTLs was

reduced in LMP2-deficient mice suggesting that the lack of LMP2 affects antigen presentation in an epitope-specific manner.

3. LMP7 versus MB1: a structural requirement?

If the LMP2/delta exchange takes care of silencing the caspase-like activity, what is left for the other two exchanges of LMP7 for MB1 and MECL-1 for Z? As a matter of fact, the function of these two exchanges are poorly understood and the in vitro data obtained with fluorogenic peptides are contradictory. The overexpression of LMP7 in transfected HeLa cells yielded a 36% and 22% increase in the chymotrypsin-like and trypsin-like activity, respectively [14], but a discordant result was obtained in the analysis of proteasomes purified from the spleen of LMP7^{-/-} mice where LMP7 deficiency led to a three-fold enhancement of the chymotrypsin-like activity [21]. The reason why different systems produce such different results is not clear but this may be linked to species differences between mouse and human or to the different proteasome subunit compositions in the utilized tissues. In our transfection experiments, the overexpression of LMP7 in mouse fibroblasts [9] and human T2 cells [15] did not cause a significant change in the hydrolysis of fluorogenic substrates used to monitor the chymotrypsin-like, the trypsin-like, and the caspase-like activity. Consistent with our results, inhibitor studies using either ^3H -lactacystin [22] or ^{125}I -NLVS [18] labeled LMP7 and MB1 with comparable efficiency which suggests that both subunits contribute to the chymotrypsin-like activity to a similar extent. Moreover, based on crystal structure neither the exchanges of LMP7 for MB1 nor that of MECL-1 for Z was predicted to alter the binding characteristic of the P1 pocket [19].

Therefore, it is surprising that T lymphocytes from LMP7^{-/-} mice displayed a two-fold reduction in class I cell surface expression [23]. This reduction could be overcome by exogenous loading of synthetic peptide ligands for the respective class I molecules indicating that insufficient amounts of appropriate peptides were produced in the absence of LMP7. In addition, the stimulation of HY-specific T cells was reduced when LMP7^{-/-} splenocytes were used as antigen-presenting cells but since the presentation of other antigens was shown to be independent of LMP7 [24] the effect of this subunit may also be epitope specific. With respect to the function of LMP7, an interesting study on the presentation of an influenza matrix epitope (M1) was recently published: cells which lack LMP2 and LMP7 were shown to present the M1 epitope inefficiently and this defect could be corrected by expression of a catalytically inactive form of LMP7 [25]. Similarly, the in vitro processing of an epitope from hepatitis B virus core protein was shown to be dependent on LMP7 and again a catalytically inactive T1A mutant of

LMP7 was able to promote epitope generation [26]. These findings suggest that the effect of LMP7 on epitope generation may rely not only on its catalytic activity but also on structural alterations of the proteasome.

One consequence of an LMP2/7-mediated change in proteasome conformation could be that the association with regulators of the proteasome is affected. We had previously investigated whether the peptide hydrolysing activity of purified immunoproteasomes (i.e., proteasomes containing LMP2, LMP7, and MECL-1) would be stimulated by the IFN- γ inducible proteasome regulator PA28 α/β to a greater extent than proteasomes lacking these subunits. However, we could not obtain convincing evidence for a preferential activation of immunoproteasomes by PA28 α/β [9] and also in a study by Ustrel et al. it depended on the utilized fluorogenic substrate whether the PA28 α/β -mediated enhancement was greater for constitutive proteasomes or for immunoproteasomes [27]. Proteasome activity is influenced both by PA28 α/β as well as active site subunit exchanges and these effects may influence each other in an unpredictable manner. Therefore, we have reinvestigated the association of PA28 with proteasomes in a novel binding assay which is based on the binding of proteasome regulators to immunoprecipitated 20S proteasomes from IFN- γ stimulated- or unstimulated cells in fresh lysates. These experiments showed that PA28 α/β preferentially associates with 'immunoproteasomes' containing LMP2, LMP7, and MECL-1 suggesting that these subunits may alter the structure of the proteasome such that the binding of PA28 α/β is favoured and we are currently trying to obtain further evidence for this preferential association by different experimental approaches.

4. MECL-1 versus Z, a species difference between mice and men?

The subunit β 2 (called Z in the human and MC14 or LMP9 in the mouse) can be replaced by the IFN- γ inducible subunit MECL-1. Unlike LMP2 and LMP7, the subunit MECL-1 is not encoded in the MHC locus and this may be the reason why the discovery of this third subunit exchange lagged behind by 5 years [28–30]. To date there are no MECL-1 deficient mice or cell lines available and therefore the *in vivo* function of this third exchange is still poorly characterized. The inactivation of the β 2 subunit in yeast resulted in a selective loss of the trypsin-like activity [13]. Also the overexpression of a catalytically inactive T1A mutant of MECL-1 in LMP2/LMP7 double transfected mouse fibroblasts led to a complete downregulation of the trypsin-like activity [31] indicating that β 2 is the principal subunit in charge of this activity in budding yeast and mice. When cells are treated with IFN- γ for 3 days we usually see a complete exchange of LMP2 for delta and LMP7 for MB1 but, curiously, only

half of the MC14 subunits become substituted by MECL-1 both, in human and murine cell lines. Also in the aforementioned MECL-1(T1A) mouse transfectants there was only a 50% replacement of MC14 by MECL-1(T1A) and it is unclear how this partial replacement could lead to a complete silencing of the trypsin like activity.

We investigated if the cell surface expression of an MHC class I molecule like HLA-A68, which requires peptide ligands with basic C-termini, could still be maintained if LMP2 and a T1A mutant of MECL-1 were overexpressed in a HLA-A68⁺ human lung carcinoma cell line. In proteasomes from this transfectant the subunit Z was replaced by MECL-1(T1A) to an extent of 50%. In spite of this replacement we did not measure a significant reduction in the trypsin-like activity of purified 20S proteasomes from this transfectant although the caspase-like activity was markedly reduced as expected for a complete replacement of delta by LMP2. Moreover, the HLA-A68 cell surface expression was not altered in this transfectant. The outcome of this experiment is in contrast to the results obtained in mouse transfectants and may indicate that in the human proteasome subunits other than β 2 can maintain the trypsin-like activity and provide peptide ligands for class I molecules which need basic C-termini in their peptide ligands.

5. Are LMP2, LMP7, and MECL-1 incorporated in a concerted manner? A conflict of 'optimal loading' versus 'optimal diversity'

Two concepts have been discussed as rationale for the subunit exchanges: the first we call the 'optimal loading' argument implying that the subunit exchanges produce in total a better suited spectrum of peptide ligands for the loading and stabilization of MHC class I molecules on the cell surface. The second argument is that of 'optimal diversity' indicating that the diversity of peptide ligands which can be produced from a given viral protein is greater if different populations of proteasomes coexist in a cell after IFN- γ stimulation. In order to judge about these two concepts it is important to know whether the IFN- γ inducible subunits can independently incorporate into newly formed proteasomes or not. When we investigated the incorporation of MECL-1 into proteasomes of T2 cells which expressed either LMP2 or LMP7 or both, we found that the incorporation of MECL-1 into purified 20S proteasomes is strictly and mutually dependent on the presence of LMP2 but not on LMP7 [32]. In pulse chase experiments we and others saw that LMP7 accelerated maturation of proteasomes containing precursors of LMP2 and MECL-1 [33] but for unknown reasons this LMP7-dependent acceleration did not lead to a change in the steady state proteasome composition. This finding is supported by the analysis of proteasome composition of splenocytes from LMP7^{-/-} mice by means of NEPHGE/

PAGE which demonstrates that the quantity of LMP2 and MECL-1 incorporation was not changed as compared to wild type mice [21]. In agreement with these data, the level of LMP7 in proteasomes from lymphoblasts of LMP2^{-/-} mice is the same as in the wildtype control but the incorporation of MECL-1 does not occur in LMP2-deficient mice. In conclusion, it appears that after IFN- γ induction there is an increase in the diversity of proteasome populations in that proteasomes containing either LMP7 alone, or the two subunits MECL-1 and LMP2 together, or all three subunits together will emerge in the cell after IFN- γ stimulation. The interdependent incorporation of LMP2 (β 1i) and MECL-1 (β 2i) finds its pendant in the subunit topology of the proteasome where they are next neighbours in the β -ring and separated from LMP7 (β 5i) by two β -type subunits (β 3 and β 4) of the same ring [34]. In sum, there is an IFN- γ mediated increase in the diversity of proteasome populations and consequently of peptide products but it is not as extensive as it could be.

6. Why did the subunits LMP2, LMP7, and MECL-1 evolve to be cytokine-inducible?

A conceptional concern with the optimal loading hypothesis is that it remains unclear why LMP2, LMP7, and MECL-1 are expressed in an inducible rather than a constitutive manner if better ligands for class I molecules are made by immunoproteasomes. An obvious argument would be that the immunoproteasomes do not fulfil all the housekeeping functions of the proteasome system in the same way as constitutive proteasomes. The stable overexpression of LMP2, LMP7, and MECL-1 in mouse fibroblasts or human T2 cells did not result in a deficiency of cellular growth or viability but this does not rule out that maybe during embryonal development or in other tissues immunoproteasomes are insufficient to maintain the required proteolytic functions. We have investigated this issue by producing LMP7-transgenic mice which express the wildtype LMP7 gene under control of the ubiquitin promoter which was reported to allow expression during development and in virtually all mouse tissues at high level [35]. Indeed, we observed enhanced LMP7 expression in virtually all tissues and our preliminary analysis of LMP7 expression in two heterozygous founder mice shows a 50% replacement of MB1 by LMP7 even in brain, which normally is devoid of immunosubunits. However, according to our preliminary analysis of these mice, an obvious phenotype was not noted, indicating that at least a partial replacement of MB-1 by LMP7 is not harmful.

Further support for the conception that a substantial replacement of active site proteasome subunits is tolerated came from an *in vivo* experiment which we have recently performed. Although many researchers have documented the extent of proteasome subunit exchanges in cytokine treated cell lines, at least to our knowledge it has never

been investigated to what extent this exchange actually occurs *in vivo* during an infectious process. We have isolated proteasomes from livers of normal mice which were infected with the lymphocytic choriomeningitis virus which is known to induce a vigorous specific cytotoxic immune response in the liver and other organs. On day eight post infection, when the CTL response is maximal, we obtained the remarkable result that the subunit exchange of LMP2 for delta and LMP7 for MB1 had occurred to a degree of about 80% although these inducible subunits were barely detectable in the livers of uninfected mice. This finding implies that the mouse can tolerate such an extensive exchange of active site proteasome subunits in the liver for several days arguing against their functional insufficiency for proteolysis. A second implication of this result is that the *in vivo* subunit exchange in the livers of LCMV infected mice goes beyond a level of 50%. Since a 50% exchange would provide the optimal diversity this finding is a point against the 'optimal diversity' hypothesis. A second argument may be deduced from our previous analysis of immature and mature dendritic cells (DCs) which are crucially involved in shaping the cellular immune response [36]. Before an antigen encounter in the periphery, the immature DCs display already a 50% exchange of active site proteasome subunits but after stimulation with lipopolysaccharide, the DCs mature and switch their proteasome neosynthesis to the exclusive production of immunoproteasomes and migrate to the lymph nodes where they stimulate T cells. If a maximal diversity was the prime goal of inducing LMP2, LMP7, and MECL-1, an exchange greater than 50% on the steady state level would not make sense.

7. Concluding with a hypothesis

When the mild phenotype of LMP2- and LMP7-deficient mice was reported in 1994 the interest in the function of LMP2 and LMP7 declined. It became clear, that the bulk of MHC class I ligands can be made in absence of LMP2 and LMP7. However, the recent analyses of antigen presentation of distinguished epitopes showed that the impact of the inducible proteasome subunits can be pivotal for the generation of a given epitope. It appears that the induction of immunoproteasomes can promote [25, 26, 37, 38] but it can also abrogate the presentation of an epitope as was recently shown for a number of epitopes from tumor antigens which had been previously identified in tumor cells which often do not express LMP2 and LMP7 [39]. The small number of studied epitopes suggest that the change in the spectrum of presented peptides in the presence or absence of immunoproteasomes may be greater than expected and we are further investigating this issue.

We would like to hypothesize that a prime function of inducing LMP2, LMP7, and MECL-1 is the selective

stimulation of T cells which are specific for epitopes produced in inflammatory sites and the prevention of autoimmunity. It has to be kept in mind that the basal expression of LMP2, LMP7, and MECL-1 is very low in most tissues and it is only prominent in the lung and lymphoid tissues like the thymus, the spleen, and lymph nodes [40]. However, the initial priming of cytotoxic T cells occurs in lymph nodes or the spleen by mature DCs which express high levels of immunoproteasomes. Consequently, the spectrum of peptides for which T cells are sensitized is dominated by the products specifically made by immunoproteasomes. Cytotoxic T cells may not be properly activated against those epitopes which are preferentially made by constitutive proteasomes because they are presented on class I molecules by the non professional antigen presenting cells of non-lymphoid organs and presentation in the absence of appropriate costimulation and cytokines is known to induce T cell anergy. This would imply on the other hand that T cells directed against epitopes which are only made by immunoproteasomes would not be confronted with the full tolerizing force of the peripheral tissues and may remain activated for a longer time. If cytotoxic T cells which are specific for peptides that are processed by immunoproteasomes from the products of housekeeping genes are activated in an inflammatory site, they may not see the same peptides in uninfamed tissues which lack immunoproteasomes and by this means an autoimmune attack of uninfamed tissues could be prevented.

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