Immunoproteasomes Shape Immunodominance Hierarchies of Antiviral CD8 T Cells at the Levels of T Cell Repertoire and Presentation of Viral Antigens

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Abstract

Vertebrates express three cytokine-inducible proteasome subunits that are incorporated in the place of their constitutively synthesized counterparts. There is increasing evidence that the set of peptides generated by proteasomes containing these subunits (immunoproteasomes) differs from that produced by standard proteasomes. In this study, we use mice lacking one of the immunoproteasome subunits (LMP2) to show that immunoproteasomes play an important role in establishing the immunodominance hierarchy of CD8 T cells (TCD8+) responding to seven defined determinants in influenza virus. In LMP2−/− mice, responses to the two most dominant determinants drop precipitously, whereas responses to two subdominant determinants are greatly enhanced. Adoptive transfer experiments with naive normal and transgenic TCD8+ reveal that the reduced immunogenicity of one determinant (PA224–233) can be attributed to decreased generation by antigen presenting cells (APCs), whereas the other determinant (NP366–374) is less immunogenic due to alterations in the TCD8+ repertoire, and not, as reported previously, to the decreased capacity of LMP2−/− APCs to generate the determinant. The enhanced response to one of the subdominant determinants (PB1F262–70) correlates with increased generation by LMP2−/− virus–infected cells. These findings indicate that in addition to their effects on the presentation of foreign antigens, immunoproteasomes influence TCD8+ responses by modifying the repertoire of responding TCD8+.

Key words: antigen processing • immunodominance • T cells • proteasome • repertoire

Introduction

Proteasomes are multicatalytic enzymes complexes that are responsible for the turnover of most cellular proteins and also for the generation of the bulk of antigenic peptides transported by transporter associated with antigen presentation (TAP) and presented by MHC class I molecules (1). 26S proteasomes consists of catalytic 20S proteasomes and either the 19S or 11S regulatory complexes. The 20S proteasome is a four-ring structure with seven different subunits in each ring, arrayed as α7β7α7β7α7β7 (2). The regulatory complexes provide the specificity of polypeptide recognition. They also open the gated channel formed by the outer ring of 20S proteasomes to control substrate access to the catalytic chamber (3). Substrates of 26S proteasomes are largely targeted to 19S regulatory subunits by the addition of polyubiquitin chains (4). Three of the β subunits (β1, β2, β5) of 20S proteasomes are known to possess protease activity. Exposing cells to IFN-γ induces the synthesis of alternatives for these subunits (respectively, LMP2, MECL1, LMP7) that together are incorporated into an alternative form of proteasomes known as immunoproteasomes (5).

Differences in the cleavage patterns of standard and immunoproteasomes have been demonstrated using purified 20S proteasomes and synthetic peptide substrates. Although there were several conflicts in the original reports, it now appears that in general, 20S immunoproteasomes are more adept at producing peptides with hydrophobic and positively charged COOH-terminal residues. These are precisely the types of residues preferred by class I molecules.
Materials and Methods

Cell Culture. The DC cell line DC2.4 (H-2b; provided by Dr. K. Rock, University of Massachusetts Medical School, Worcester, MA; reference 15) and the thymoma cell line EL-4 were maintained in RPMI 1640 containing 10% fetal calf serum, 5 × 10⁻³ M 2-mercaptoethanol, antibiotics, and 2 mM glutamine (RP-10). To generate Con A or LPS blasts, 2 × 10⁷ spleen cells were cultured with RP-10 medium in 6-well plates in the presence of 5 μg/ml of Con A or LPS for 48 h. T<sub>CD8⁺</sub> were stimulated with 1/20–1/25 the number of IV-infected or peptide-pulsed APCs, irradiated with 200 Gy. Stimulated live T cells were harvested through Ficoll-Hypaque gradient and enriched for CD8⁺ cells by depleting B220⁺ and CD4⁺ cells using mAb-coated M-450 Dynal beads (Dynal).

Intracellular Cytokine and Tetramer Staining. CTL activities were tested either with ICS using peptides corresponding to the known immunodominant and subdominant determinants in H-2b-restricted response to influenza or assayed with D<sup>9</sup>-NP<sub>366–374</sub> tetramers. ICS was performed as described (17). In brief, splenic and peritoneal cells were incubated with synthetic peptides at 0.5–1 μM for 2 h at 37°C in Iscove’s modified DMEM supplemented with 10% FBS. After addition of brefeldin A (BFA) to a concentration of 10 μg/ml, cells were incubated for an additional 4 h at 37°C. Cells were then incubated on ice with Cy-labeled anti-CD8α and PE-labeled anti-CD45.1 for 1 h, washed, and fixed with 1% paraformaldehyde. Cells were then incubated in the presence of 0.2% saponin (Calbiochem) with fluorescein-labeled anti–IFN-γ. Tetramer staining was performed at room temperature for 1 h in the presence of Cy-Chrome-labeled anti-CD8 (BD PharMingen). For ICS or tetramer staining, 100,000–300,000 cells were analyzed on a FACScan™ (Becton Dickinson). Total Ag-specific cell numbers were calculated using Ag-specific percentage of total CD8⁺ cells multiplied by the total T<sub>CD8⁺</sub> percentage of total spleen or peritoneal cells acquired on the FACS®.

Infection of Cells for Antigen Presentation Kinetic Assay. Cells were washed with Autopow (Life Technologies) supplemented with 0.1% BSA, 25 mM Heps, and adjusted to pH 6.6, and resuspended in the same buffer containing 60 HAU IV per 10⁶ cells and incubated for 1 h at 37°C in 96-well plates before adding T<sub>CD8⁺</sub> in 200 μl of RP-10. BFA was then added at different time points to a final concentration of 10 μg/ml. 2 h after the last addition of BFA, cells were harvested and T cell activation was determined by ICS staining as described above.

Peptides and Binding Assays. All peptides were synthesized, HPLC purified, and analyzed by mass spectrometry by or under the supervision of the Biologic Resource Branch, National Institutes of Allergy and Infectious Diseases, National Institutes of Health. All peptides were >95% purity. Peptides were dissolved in DMSO at 1 mM as stock solutions and stored at −30°C.

Naive T<sub>CD8⁺</sub> Precursor Transfer. Splenocytes were T cell enriched with anti-CD90 (Thy1.2)-coated microbeads (Miltenyi Biotec). Generally, ~85% purity was achieved as assessed by flow cytometry. ~1.5 × 10⁶ purified cells were transferred into a naive host animal intravenously. After transfer, animals were immediately primed intraperitoneally with IV. 7 d later, the responses...
of both host and donor cell population were followed with CD45.1 Ab specific for B6.SJL cells or CD45.2 Ab specific for normal B6 cells.

Transgenic TCR T<sub>CD8</sub><sup>+</sup> Transfer. B6 or LMP2<sup>−/−</sup> mice were irradiated with 800 rads 2 d before T cell transfer. On the day of transfer, mice (in groups of three) were infected by intraperitoneal injection with IV HK/X31 (X31), A/NT60/68 (NT60), or left uninfected. 5 h later, mice were given 5 × 10<sup>6</sup> transgenic T cells by intravenous injection. T cells were purified from homogenized spleen and lymph nodes (popliteal, inguinal, brachial, axillary, and superficial cervical) obtained from F5 mice. Purification entailed Ficoll gradient centrifugation followed by positive selection of Thy1.2 (CD90) positive as described above. 16 h after T cell transfer, spleens were removed, homogenized, and centrifuged in a Ficoll gradient to yield viable mononuclear cells. Cells were incubated in 20% normal mouse serum and mAb 2.4G2 (Fc block) supernatant for 20 min before staining with directly conjugated FITC-labeled Abs to CD69, CD25, or CD62L. Cells were analyzed by flow cytometry, gating on CD8<sup>+</sup> cells (the V<sub>B</sub> class of the F5 TCR) to ascertain the cell surface levels of these T cell activation markers.

Results and Discussion

We first compared anti-IV T<sub>CD8</sub><sup>+</sup> responses of wild-type B6 to that of LMP2<sup>−/−</sup> mice. After intraperitoneal infection, peritoneal exudate cells and splenocytes were assayed from 4 to 9 d after infection for responses to six of the seven defined peptides from IV gene products (see Table I) that are known to be recognized by T<sub>CD8</sub><sup>+</sup> from IV-infected H-2<sup>b</sup> mice (18; and unpublished data). Two determinants, NP<sub>366-374</sub> and PA<sub>224-233</sub>, have been reported to dominate local and splenic responses of B6 mice after intranasal infection with influenza. As seen in Fig. 1, the same peptides dominate the local and splenic response after intraperitoneal infection. LMP2<sup>−/−</sup> mice exhibited dramatic differences from B6 mice. Responses to NP<sub>366-374</sub> and PA<sub>224-233</sub> were greatly reduced whereas responses to NS2<sub>114-122</sub> and PB1F<sub>262-70</sub> were enhanced. This effect was particularly prominent with peritoneal T<sub>CD8</sub><sup>+</sup>. Overall, the LMP2<sup>−/−</sup> response was less vigorous both in the percentage of responding virus-specific T<sub>CD8</sub><sup>+</sup>, and in the overall number of responding cells. Of interest, ∼1/2 the number of total T<sub>CD8+</sub> were recovered from infected LMP2<sup>−/−</sup> mice compared with B6 mice.

This experiment indicates that immunoproteasomes play an important role in determining the immunodominance hierarchy (summarized in Table I). To distinguish the contributions to this phenomenon of T<sub>CD8+</sub> repertoire versus antigen presentation, we examined the response of

![Figure 1. Immunodominance hierarchy to IV infection in B6 and LMP2<sup>−/−</sup> mice. Spleen and peritoneal cells were prepared at various times after IV priming and their determinant-specific responses were assessed by ICS using a panel of synthetic peptides corresponding to defined H-2<sup>b</sup>-restricted determinants. All responses were normalized by subtracting background values obtained using cells receiving no peptides. In this experiment, twice as many T<sub>CD8+</sub> were recovered from B6 mice than from LMP2<sup>−/−</sup> mice.](image-url)
LMP2<sup>−/−</sup> mice after transfer of T<sub>CD8</sub> from wild-type mice. To enable discrimination of host versus donor cells we used B6.SJL mice as donors. T<sub>CD8</sub> from these mice express a marker (CD45.1) absent in B6 mice that can be detected by mAb staining. CD90 (Thy1.2)-enriched splenocytes from B6.SJL mice were transferred into either B6 or LMP2<sup>−/−</sup> mice that were then infected with IV. Spleen and peritoneal cells were harvested 7 d later and analyzed by ICS using the panel of seven IV peptides or by staining with PE-labeled D<sup>b</sup>-NP<sub>366–374</sub> tetramers.

The left panel of Fig. 2 A shows the typical pattern of tetramer staining of splenocytes in obtained from IV-infected B6 mice; in the mouse examined, 7% of T<sub>CD8</sub> are tetramer positive. The middle panel shows that of the tetramer-positive T<sub>CD8</sub> in this spleen, only 2% are derived from donor cells. The right panel shows the summarized composite results of tetramer-positive cells for host- (white

**Table I. Properties of H-2<sup>b</sup>-restricted IV-derived Determinants**

| Determinant | Sequence      | ID rank | Restriction element |
|-------------|---------------|---------|---------------------|
| PA<sub>224–233</sub> | SSLENFRAYV  | 5       | D<sup>b</sup>       |
| NP<sub>366–374</sub> | ASNENMETM  | 3       | D<sup>b</sup>       |
| PB1F2<sub>202–207</sub> | LSLRPILV  | 1       | D<sup>b</sup>       |
| NS2<sub>114–121</sub> | RTESFQLI    | 2       | K<sup>b</sup>       |
| M1<sub>128–135</sub> | MGLIVNRM    | 5       | K<sup>b</sup>       |
| PB1<sub>703–711</sub> | SSYRRPGVI   | 3       | K<sup>b</sup>       |
| PB2<sub>198–206</sub> | ISPCLMVAYM  | 5       | K<sup>b</sup>       |

The ID rank is based on averaging the frequency of splenic and peritoneal T<sub>CD8</sub> following intraperitoneal infection with PR8. Determinants with similar immunogenicity are assigned the same rank.

The left panel of Fig. 2 A shows the typical pattern of tetramer staining of splenocytes in obtained from IV-infected B6 mice; in the mouse examined, 7% of T<sub>CD8</sub> are tetramer positive. The middle panel shows that of the tetramer-positive T<sub>CD8</sub> in this spleen, only 2% are derived from donor cells. The right panel shows the summarized composite results of tetramer-positive cells for host- (white

**Figure 2.** IV-specific responses of naive host and donor T cells. CD90-enriched spleen cells from naive B6.SJL were transferred into either normal B6 or LMP2<sup>−/−</sup> mice followed by IV infection. After 7 d, both host and donor cells were assayed for ICS and CD45.1 expression. Donor- and host-derived NP<sub>366–374</sub>-specific T<sub>CD8</sub> were also tested for NP<sub>366–374</sub>-tetramer staining and CD45.1 expression. Panel A shows typical NP<sub>366–374</sub>-tetramer staining of spleen cells (left). The background-positive staining on naive B6 spleen cells was <0.5%. Tetramer-positive cells were gated and displayed as CD45.1 positive (donor) and negative (host) (middle). The percentage of each group was then plotted as the fraction of tetramer positive cells (right). B and C show, respectively, the determinant-specific hierarchy of host and donor cells. Both are shown as the total antigen-specific cell number, either per spleen or per peritoneum after subtracting background values obtained from the no peptide control for each mouse. Data represent the average values from two individual mice.
bars) and donor-derived (filled bars) $T_{CD8^+}$ in B6 and LMP2$^{−/−}$ mice. Remarkably, donor cells comprise 50% of the tetramer-positive response in LMP2$^{−/−}$ mice: more than 10-fold higher than in B6 mice. These findings were confirmed by ICS (Fig. 2, B and C), which demonstrated a much greater host $T_{CD8^+}$ response to NP366-374 in B6 mice, and conversely a much greater (~sixfold) donor NP366-374-specific $T_{CD8^+}$ response in spleens of LMP2$^{−/−}$ mice. Note the 20-fold difference in scale used to display the results of host and donor cells. This difference in abundance between host and donor cells can be attributed to both the number of $T_{CD8^+}$ used for transfer (~1/10 the amount of $T_{CD8^+}$ present in mice), and also an anticipated loss in cells due to imperfect trafficking to spleen and peritoneum. The marked expansion of transferred naive NP366-374-specific $T_{CD8^+}$ in LMP2$^{−/−}$ mice indicates that deficiencies in the $T_{CD8^+}$ repertoire are a major factor in the limited response of LMP2$^{−/−}$ mice to NP366-374.

Examination of the results for host cell responses to the six other determinants tested in this experiment reveals that the typical immunodominance hierarchies of B6 and LMP2$^{−/−}$ host cells were basically unaltered by the cell transfer (compare Fig. 2 B with Fig. 1). In B6 mice, NP366-374 and PA224-233-specific CTL remain dominant in both peritoneal and spleen cell responses. In LMP2$^{−/−}$ mice, $T_{CD8^+}$ specific for PB1F262-70 and NS214-122 dominate the response. Notably, the total number of responding host cells in LMP2$^{−/−}$ mice is much less than in B6 mice, particularly in the spleen.

In B6 mice, the response of donor cells basically mirrored the host cell response (Fig. 2 C). There were several interesting differences in the response of donor cells in LMP2$^{−/−}$ mice compared with B6 mice. First, there was the noticeable increase in the response of splenic NP366-374-specific cells observed with tetramers. This was somewhat less apparent in the peritoneal cavity, perhaps due to increased immunodominance from PB1F262-70-specific $T_{CD8^+}$. Second, with one important exception, more donor cells specific for each determinant were recovered from LMP2$^{−/−}$ mice than B6 mice, indicating that antigen presentation is not limiting for these determinants. This effect was most marked with PB1F262-70-specific $T_{CD8^+}$. Third, for the exceptional determinant, PA224-233, less specific $T_{CD8^+}$ were recovered from both peritoneal and splenic populations in LMP2$^{−/−}$ mice than from B6 mice, pointing to a defect in antigen presentation in these mice.

To confirm that presentation of NP366-374 was similar in wild-type and LMP2$^{−/−}$ mice, we adoptively transferred purified $T_{CD8^+}$ from F5 TCR transgenic mice (19). Mice were then infected intraperitoneally with NT60 IV, which expresses NP with the cognate D$^b$-restricted NP366-374 peptide (ASNENMDAM) recognized by F5 $T_{CD8^+}$. As a control, mice were infected with X31, which expresses NP with the non–cross-reactive ASNENMETM peptide. Peritoneal exudate cells were harvested 16 h after infection and the activation state of F5 $T_{CD8^+}$ was assessed by enhanced expression of CD25 and diminished expression of CD62 (Fig. 3). Importantly, F5 cells were activated at similar levels after transfer into LMP2$^{−/−}$ and wild-type mice. The antigen specificity of this activation is shown by the similarity between F5 cells recovered from X31 infected mice and uninfected mice.

We next performed an adoptive transfer experiment using LMP2$^{−/−}$ donor splenocytes and B6.SJL recipients (Fig. 4). The response of host $T_{CD8^+}$, similar to unmanipulated mice. Despite these robust responses, we failed to detect activation of the transferred cells. This finding cannot be attributed to differences in the handling or purification of the transferred $T_{CD8^+}$, as we observed similar results in three separate experiments. Rather, it is consistent with a general defect in the ability of LMP2$^{−/−}$ mice to mount an IV-specific response that is exacerbated in the presence of $T_{CD8^+}$ from normal mice.

Finally, we examined the capacity of LMP2$^{−/−}$ and wild-type APCs to present endogenous IV antigens to antigen-specific $T_{CD8^+}$ lines. To avoid uncertainties associated with the clonal variation of established APC cell lines, we

![Figure 3](image-url) Activation of adoptively transferred transgenic $T_{CD8^+}$. Purified $T_{CD8^+}$ from F5 TCR transgenic mice were transferred into B6 or LMP2$^{−/−}$ mice infected with NT60. X31 (control), or left uninfected as indicated. Cells were assessed by flow cytometry for activation based on enhanced binding of a CD25-specific mAb or decreased binding of a CD62-specific mAb.

![Figure 4](image-url) IV-specific responses of transferred naive LMP2$^{−/−}$ $T$ cells. CD90-enriched spleen cells from naive LMP2$^{−/−}$ mice were transferred into either naive B6.SJL or B6 mice followed by IV pMNNG. 7 d later, both the host and donor cells were assayed for ICS and CD45.2 expression. The determinant-specific hierarchy among host (left) and donor (right) splenic $T_{CD8^+}$ is shown. Data were processed as described in the Fig. 2 legend. Note the 10-fold difference in the Ag-specific $T_{CD8^+}$ scale.
used cell populations obtained directly from mice. APCs were incubated with BFA at various times after infection and antigen presentation was quantitated by the capacity of the cells to trigger determinant-specific T<sub>CD8+</sub> lines as measured by ICS. In additional experiments (not shown), we found that this assay parallels presentation kinetics as measured by 51Cr release, which itself is proportional to the number of peptide-class I complexes recovered from cells (20), and we believe that the data in Fig. 5 demonstrate that there is no significant difference between the capacities of wild-type and LMP<sup>2</sup>−/− cells to generate NP<sub>366-374</sub>-specific T<sub>CD8+</sub> cells. Peritoneal exudate cells derived from LMP<sup>2</sup>−/− mice exhibit diminished responses to NP<sub>366-374</sub>. This was attributed to a defect in antigen presentation based on the decreased abilities of LPS-activated splenocytes or peritoneal exudate cells derived from LMP<sup>2</sup>−/− mice to activate a NP<sub>366-374</sub>-specific T cell hybridoma. By contrast, we failed to observe a similar defect in presentation by either LPS- or Con A-activated splenocytes, peritoneal exudate cells, or dendritic cells. We previously demonstrated that the kinetics of presentation as determined using BFA reflects the number of peptide class I complexes recovered from cells (20), and we believe that the data in Fig. 5 demonstrate that there is no significant difference between the capacities of wild-type and LMP<sup>2</sup>−/− mice to generate NP<sub>366-374</sub> from the PR8 NP.

As PA<sub>224-233</sub> occupies the α-position in the B6 T<sub>CD8+</sub> immunodominance hierarchy, its demotion in LMP<sup>2</sup>−/− mice can influence the response to all of the other determinants due to decreased immunodomination (17). Given the opportunity to assume the α-position, the plunge of NP<sub>366-374</sub> down the immunodominance hierarchy is striking, and demonstrates the profound effect of LMP2 on the anti-NP<sub>366-374</sub> repertoire. The loss of immunodomination exerted by these two determinants probably contributes to the ascendance of PB1F2<sub>62-70</sub>- and NS2<sub>114-122</sub>-specific T<sub>CD8+</sub> cells. The enhanced presentation of PB1F2<sub>62-70</sub> in LMP<sup>2</sup>−/− mice probably due at least in part to enhanced generation by standard proteasomes relative to immunoproteasomes.

Van Kaer et al. (21) previously reported that LMP<sup>2</sup>−/− mice exhibit diminished responses to NP<sub>366-374</sub>. This was attributed to a defect in antigen presentation based on the decreased abilities of LPS-activated splenocytes or peritoneal exudate cells derived from LMP<sup>2</sup>−/− mice to activate a NP<sub>366-374</sub>-specific T cell hybridoma. By contrast, we failed to observe a similar defect in presentation by either LPS- or Con A-activated splenocytes, peritoneal exudate cells, or dendritic cells. We previously demonstrated that the kinetics of presentation as determined using BFA reflects the number of peptide class I complexes recovered from cells (20), and we believe that the data in Fig. 5 demonstrate that there is no significant difference between the capacities of wild-type and LMP<sup>2</sup>−/− mice to generate NP<sub>366-374</sub> from the PR8 NP.

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Our findings demonstrate that the loss of LMP2 has a significant effect on the T<sub>CD8+</sub> repertoire. This is not shocking given the observation that LMP2−/− mice exhibit a specific deficit in the numbers of T<sub>CD8+</sub>, relative to B6 mice. As LMP2 is expressed in the thymus (22), its absence can affect the repertoire by influencing positive and/or negative selection, as well as whatever effects it may exert in the periphery. As LMP7 and MECL1 can assemble functional proteasomes in the absence LMP2, albeit inefficiently (23, 24), the characteristics of LMP2−/− mice provide a minimal estimate of the role of immunoproteasomes on antigen presentation and the T<sub>CD8+</sub> repertoire. That deficiencies in antigen processing can influence the T<sub>CD8+</sub> repertoire was previously established in studies of TAP−/− mice (25, 26). Moreover, the effect of LMP2 on repertoire development parallels similar findings in MHC class II−mediated selection of the T<sub>CD4+</sub> repertoire, where targeted disruptions in genes encoding a protease (cathepsin L) that contributes to antigen processing or a molecule (H-2M) that aids peptide loading of class II molecules have been shown to modify the repertoire (27–29).
It is interesting that the overall number of TCDS8+ responding to IV was decreased in LMP2−/− mice. Potentially, this could be due to our failure to provide the proper determinants to LMP2−/− TCDS8+ during restimulation for the ICS assay. This possibility is unlikely, however, as a large difference was observed in head to head comparisons of ex vivo TCDS8+ activated for ICS staining by IV-infected splenocytes from autologous mice as APCs (data not shown). Rather, it appears that the absence of LMP2 decreases the ability of TCDS8+ to respond to foreign antigens, even to determinants that appear to be made equally by standard proteasomes and immunoproteasomes. Consistent with this finding, TCDS8+ from LMP2−/− mice failed to expand when transferred to B6 mice. Even if the lion’s share of this effect is due to increased relative immunodomination by host TCDS8+ (as it probably is), it still points to a diminished capacity of LMP2−/− TCDS8+ to proliferate relative to normal TCDS8+.

The poor responsiveness of LMP2−/− TCDS8+ to IV demonstrates that LMP2, and by inference immunoproteasomes, enhance the ability of TCDS8+ to respond to foreign antigens. Although this is reassuring in terms of understanding the evolution of immunoproteasomes, this comfort comes at the cost of a mechanistic conundrum: how can the absence of immunoproteasomes affect responses to foreign determinants produced equally (or more) efficiently by standard proteasomes? Immunoproteasomes may play a role in TCDS8+ cell activation and proliferation. Additionally, given that the selection of germ line TCR genes in evolution occurred in the context of MHC molecules presenting a peptide repertoire heavily influenced by immunoproteasomes, it is not hard to imagine that the repertoire suffers when the selection is limited to peptides produced by standard proteasomes. With the rapid advances in sequencing technology, it should be possible to directly compare the naive TCDS8+ repertoire of normal mice and those lacking immunoproteasomes.

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References

1. Rock, K.L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A.L. Goldberg. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell. 78:761–771.
2. Voges, D., P. Zwickl, and W. Baumeister. 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. 68:1015–1068.
3. Groll, M., M. Bajorek, A. Kohler, L. Moroder, D.M. Rubin, R. Huber, M.H. Glickman, and D. Finley. 2000. A gated channel into the proteasome core particle. Nat. Struct. Biol. 7:1062–1067.
4. Hershko, A., and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425–479.
5. Tanaka, K., and M. Kasahara. 1998. The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. Immunol. Rev. 163:161–176.
6. Rock, K.L., and A.L. Goldberg. 1999. Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu. Rev. Immunol. 17:739–779.
7. Moore, S., F. Levy, O. Burlet-Schiltz, F. Brasseur, M. Probst-Keper, A.L. Petitreguin, B. Monsarrat, R. Van Velthoven, J.C. Cerottini, T. Boon, et al. 2000. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. Immunity. 12:107–117.
8. Sijs, A.J., S. Standera, R.E. Toes, T. Ruppert, N.J. Beckman, P.A. van Veelen, F.A. Osendorp, C.J. Melief, and P.M. Kloetzel. 2000. MHC class I antigen processing of an adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. J. Immunol. 164:4500–4506.
9. van Hall, T., A. Sijs, M. Camps, R. Offringa, C. Melief, P.M. Kloetzel, and F. Osendorp. 2000. Differential influence on cytototoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. J. Exp. Med. 192:483–494.
10. Sijs, A.J., T. Ruppert, B. Rehermann, M. Schmidt, U. Koszinowski, and P.M. Kloetzel. 2000. Efficient generation of a hepatitis B virus cytototoxic T lymphocyte epitope requires the structural features of immunoproteasomes. J. Exp. Med. 191:503–514.
11. Schwarz, K., B.M. van Den, S. Kostka, R. Kraft, A. Soza, G. Schmidtke, P.M. Kloetzel, and M. Groettrup. 2000. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. J. Immunol. 165:768–778.
12. Yewdell, J.W., and J.R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. Immunol. 17:51–88.
13. Jung, T., U. Schauer, C. Heusser, C. Neumann, and C. Rieger. 1993. Detection of intracellular cytokines by flow cytometry. J. Immunol. Methods. 159:197–207.
14. Altman, J.D., P.H. Moss, P.R. Goulder, D.H. Barouch, M.G. McHeyzer-Williams, J.I. Bell, A.J. McMichael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science. 274:94–96.
15. Shen, Z., G. Reznikoff, G. Dranoff, and K.L. Rock. 1997. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J. Immunol. 158:2723–2730.
16. Norbury, C.C., B.J. Chambers, A.R. Prescott, H.G. Ljunggren, and C. Watts. 1997. Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. Eur. J. Immunol. 27:280–288.
17. Chen, W., L.C. Anton, J.R. Bennink, and J.W. Yewdell. 2000. Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. Immunity. 12:83–93.
18. Flynn, K.J., G.T. Belz, J.D. Altman, R. Ahmed, D.L. Woodland, and P.C. Doherty. 1998. Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. *Immunity*. 8:683–691.

19. Mamalaki, C., J. Elliott, T. Norton, N. Yannoutsos, A.R. Townsend, P. Chandler, E. Simpson, and D. Kioussis. 1993. Positive and negative selection in transgenic mice expressing a T-cell receptor specific for influenza nucleoprotein and endogenous superantigen. *Dev. Immunol.* 3:159–174.

20. Antón, L.C., J.W. Yewdell, and J.R. Bennink. 1997. MHC class I-associated peptides produced from endogenous gene products with vastly different efficiencies. *J. Immunol.* 158:2535–2542.

21. Van Kaer, L., P.G. Ashton-Rickardt, M. Eichelberger, M. Gaczynska, K. Nagashima, K.L. Rock, A.L. Goldberg, P.C. Doherty, and S. Tonegawa. 1994. Altered peptidase and viral-specific T cell response in LMP2 mutant mice. *Immunity*. 1:533–541.

22. Frentzel, S., I. Kuhn-Hartmann, M. Gernold, P. Gott, A. Seelig, and P.M. Kloetzel. 1993. The major-histocompatibility-complex-encoded beta-type proteasome subunits LMP2 and LMP7. Evidence that LMP2 and LMP7 are synthesized as proproteins and that cellular levels of both mRNA and LMP-containing 20S proteasomes are differentially regulated. *Eur. J. Biochem.* 216:119–126.

23. Griffin, T.A., D. Nandi, M. Cruz, H.J. Fehling, L.V. Kaer, J.J. Monaco, and R.A. Colbert. 1998. Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *J. Exp. Med.* 187:97–104.

24. Schmidt, M., D. Zantopf, R. Kraft, S. Kostka, R. Preissner, and P.M. Kloetzel. 1999. Sequence information within proteasomal prosequences mediates efficient integration of beta-subunits into the 20 S proteasome complex. *J. Mol. Biol.* 288:117–128.

25. Sandberg, J.K., B.J. Chambers, L. Van Kaer, K. Karre, and H.-G. Ljunggren. 1996. TAP1-deficient mice select a CD8+ T cell repertoire that displays both diversity and peptide specificity. *Eur. J. Immunol.* 26:288–293.

26. Kim, V., J.W. Yewdell, and W.R. Green. 2000. Naturally occurring TAP-dependent specific T-cell tolerance for a variant of an immunodominant retroviral cytotoxic T-lymphocyte epitope. *J. Virol.* 74:3924–3928.

27. Fung-Leung, W.P., C.D. Surh, M. Liljedahl, J. Pang, D. Leturcq, P.A. Peterson, S.R. Webb, and L. Karlsson. 1996. Antigen presentation and T cell development in H2-M-deficient mice. *Science.* 271:1278–1281.

28. Martin, W.D., G.G. Hicks, S.K. Mendiratta, H.I. Leva, H.E. Ruley, and L. Van Kaer. 1996. H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell.* 84:543–550.

29. Nakagawa, T., W. Roth, P. Wong, A. Nelson, A. Farr, J. Deussing, J.A. Villadangos, H. Ploegh, C. Peters, and A.Y. Rudensky. 1998. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science.* 280:450–453.