Brief Definitive Report

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

By Weisan Chen, Christopher C. Norbury, Yunjung Cho, Jonathan W. Yewdell, and Jack R. Bennink

From the Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

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 (T_{CD8+}) 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 T_{CD8+} reveal that the reduced immunogenicity of one determinant ($PA_{224–233}$) can be attributed to decreased generation by antigen presenting cells (APCs), whereas the other determinant ($NP_{366–374}$) is less immunogenic due to alterations in the T_{CD8+} 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 ($PB1F2_{62–70}$) 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 T_{CD8+} responses by modifying the repertoire of responding T_{CD8+}.

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 $\alpha_7\beta_7\beta_7\alpha_7$ (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

W. Chen's present address is Cancer Vaccine Unit, Ludwig Institute for Cancer Research, Austin & Repatriation Medical Centre, Studley Rd., Heidelberg VIC 3084, Australia.

Address correspondence to Dr. J.W. Yewdell, Rm. 211, Bldg. 4, 4 Center Dr., MSC 0440, National Institutes of Health, Bethesda, MD 20892-0440. Phone: 301-402-4602; Fax: 301-402-7362; E-mail: jyewdell@nih.gov

(for a review, see reference 6). More recently, these findings have been extended to the generation of defined peptides by APCs (7–11).

The most direct method for studying the influence of immunoproteasomes on immune responses in vivo is to compare wild-type mice to mice with targeted knockout of genes encoding the immunoproteasome subunits. Although mice lacking either LMP2 or LMP7 have been available for several years, characterization of their T_{CD8+} responses has been performed at a fairly rudimentary level, both in the methods used for enumeration of T_{CD8+} responses and the number of determinants surveyed. The effects of the targeted disruptions on the immunodominance hierarchy of T_{CD8+} responding to a viral infection remain to be characterized.

Immunodominance is the term given to the universal phenomenon that only a small percentage of the multitude of peptides present in a given antigen elicit T_{CD8+} responses. Even among the chosen few, the numbers of responding T_{CD8+} can vary enormously. The mechanisms underlying immunodominance have recently come under increased scrutiny because of its obvious importance for vaccine design and T_{CD8+}-determinant prediction (for a review, see reference 12). The rekindled interest is also due to technological advances in T cell quantitation, such as the introduction of intracellular cytokine staining (ICS; reference 13) and MHC-peptide tetramers (14). This has enabled direct counting of multiple specific CTL directly ex vivo without the intervening in vitro culture that can skew the true frequencies of responding cells. With the improved technology, it is clear that greater numbers of determinants are recognized by T_{CD8+} than originally believed. Thus, understanding the rules of determinant hierarchy becomes an even more interesting (and difficult) problem. In this study, we have used $LMP2^{-/-}$ mice to examine the effects of immunoproteasomes on the immunodominance hierarchy in the T_{CD8+} response to influenza virus (IV) infection.

Materials and Methods

Cell Culture. The DC cell line DC2.4 (H-2^b; 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^{-5} M 2-mercaptoethanol, antibiotics, and 2 mM glutamine (RP-10). To generate Con A or LPS blasts, 2×10^7 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_{CD8+} were stimulated and maintained in RP-10 medium supplied with 10 U/ml recombinant human IL-2 (see below). Peritoneal exudate cells were harvested from mice 3 d after intraperitoneal injection with 1 ml thioglycollate. Dendritic cells were prepared as described (16).

mAbs and Other Reagents. All mAbs used for flow cytometry were purchased from BD PharMingen. All were fluorescein labeled except anti-CD8α which was Cy-Chrome labeled. PE-labeled D^b-$NP_{366–374}$-tetramers were provided by the MHC Tetramer Core Facility of National Institute of Allergy and Infectious Diseases, National Institutes of Health. Anti-CD4 mAb culture supernatants from hybridoma GK1.5 (TIB 205; American Type Culture Collection) and unlabeled anti-B220 Ab RA3-6B2 (BD PharMingen) were used to coat M450 Dynal beads (Dynal) for depleting $CD4^+$ cells and $B220^+$ expressing NK cells when establishing T_{CD8+} lines.

Mice, Viruses, and CTL Stimulation. C57BL/J6 (B6) mice and B6.SJL-ptprc (B6.SJL) mice, which carry a CD45.1 marker, were purchased from Taconic. B6.$LMP2^{-/-}$ mice were provided by L. Van Kaer (Vanderbilt University, Nashville, TN) and were maintained under barrier conditions by Taconic. For priming, 8–10-wk-old female mice were infected with 600 hemagglutination units (HAU) of IV A/Puerto Rico/8/34 by intraperitoneal injection. IVs were propagated in the allantoic cavity of 10-d embryonated chicken eggs. Splenic and peritoneal cells were generally prepared 7 d after priming unless otherwise indicated. For generation of T_{CD8+} lines, animals were generally used >30 d after priming. T_{CD8+} stimulation was always carried out in RP-10 with 10 U/ml recombinant human IL-2. In brief, 3×10^7 splenocytes 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-2^b–restricted response to influenza or assayed with D^b-$NP_{366–374}$-tetramers. ICS was performed as described (17). In brief, splenic or 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-CD8a 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_{CD8+} 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 Hepes, and adjusted to pH 6.6, and resuspended in the same buffer containing 60 HAU IV per 10^6 cells and incubated for 1 h at 37°C in 96-well plates before adding T_{CD8+} 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_{CD8+} 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. $\sim 1.5 \times 10^7$ 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_{CD8+} Transfer. B6 or LMP2$^{-/-}$ 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 5×10^6 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$^+$Vβ 11$^+$ cells (the Vβ 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_{CD8+} responses of wild-type B6 to that of LMP2$^{-/-}$ 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_{CD8+} from IV-infected H-2^b mice (18; and unpublished data). Two determinants, $NP_{366–374}$ and $PA_{224–233}$, 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$^{-/-}$ mice exhibited dramatic differences from B6 mice. Responses to $NP_{366–374}$ and $PA_{224–233}$ were greatly reduced whereas responses to $NS2_{114–122}$ and $PB1F2_{62–70}$ were enhanced. This effect was particularly prominent with peritoneal T_{CD8+}. Overall, the LMP2$^{-/-}$ response was less vigorous both in the percentage of responding virus-specific T_{CD8+}, and in the overall number of responding cells. Of interest, ~1/2 the number of total T_{CD8+} were recovered from infected LMP2$^{-/-}$ 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_{CD8+} repertoire versus antigen presentation, we examined the response of

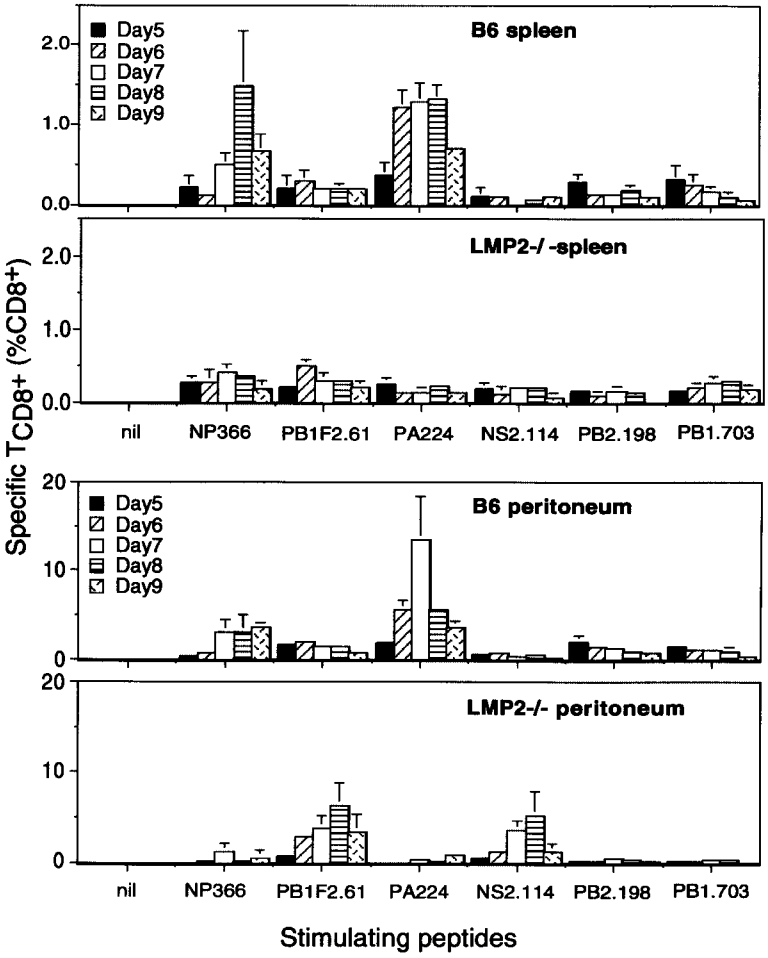

Figure 1. Immunodominance hierarchy to IV infection in B6 and LMP2$^{-/-}$ mice. Spleen and peritoneal cells were prepared at various times after IV priming and their determinant-specific responses were assessed by ICS using a panel of synthetic peptides corresponding to defined H-2^b–restricted determinants. All responses were normalized by subtracting background values obtained using cells receiving no peptides. In this experiment, twice as many T_{CD8+} were recovered from B6 mice than from LMP2$^{-/-}$ mice.

Table I. *Properties of H-2^b–restricted IV-derived Determinants*

Determinant	Sequence	ID rank LMP2$^{-/-}$	ID rank B6	Restriction element
$PA_{224–233}$	SSLENFRAYV	5	1	D^b
$NP_{366–374}$	ASNENMETM	3	2	D^b
$PB1F2_{62–70}$	LSLRNPILV	1	3	D^b
$NS2_{114–121}$	RTFSFQLI	2	6	K^b
$M1_{128–135}$	MGLIYNRM	5	7	K^b
$PB1_{703–711}$	SSYRRPVGI	3	3	K^b
$PB2_{198–206}$	ISPLMVAYM	5	3	K^b

The ID rank is based on averaging the frequency of splenic and peritoneal T_{CD8+} following intraperitoneal infection with PR8. Determinants with similar immunogenicity are assigned the same rank.

LMP2$^{-/-}$ mice after transfer of T_{CD8+} from wild-type mice. To enable discrimination of host versus donor cells we used B6.SJL mice as donors. T_{CD8+} 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$^{-/-}$ 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^b-$NP_{366–374}$ 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_{CD8+} are tetramer positive. The middle panel shows that of the tetramer-positive T_{CD8+} 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

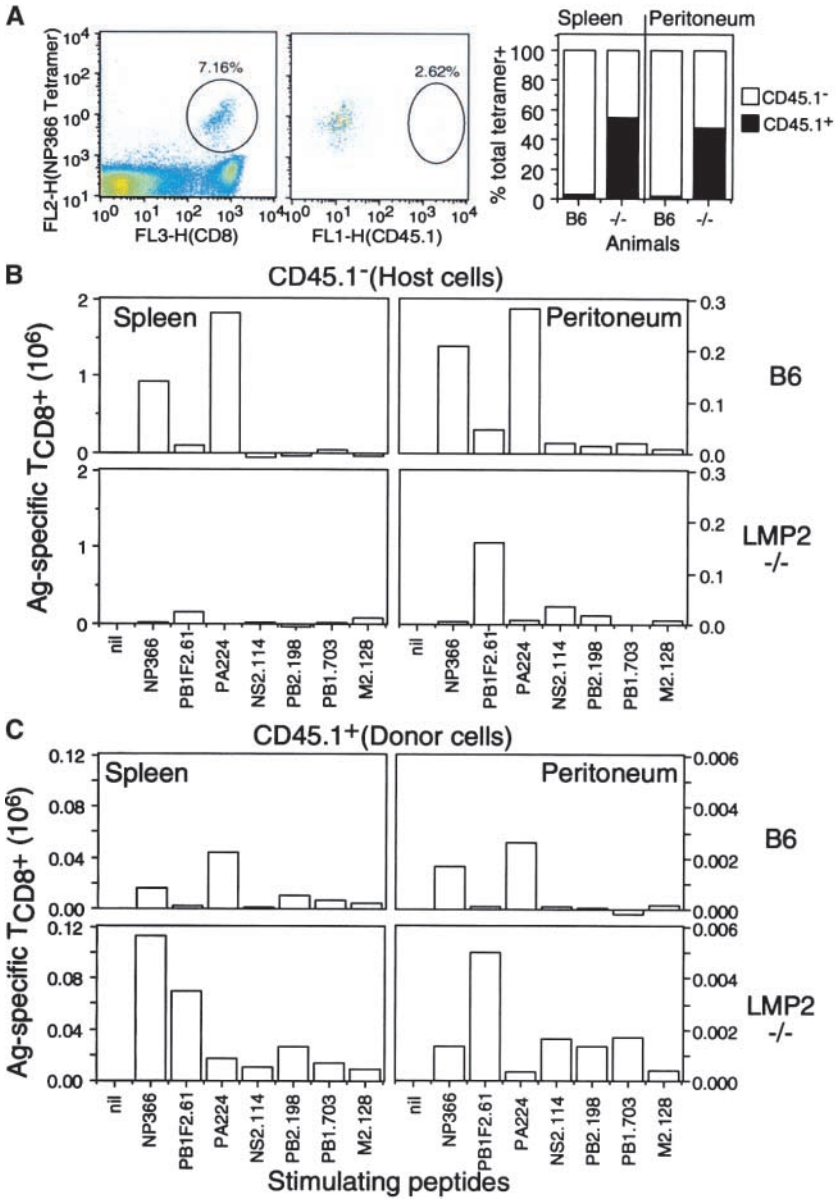

Figure 2. IV-specific responses of naive host and donor T cells. CD90-enriched spleen cells from naive B6.SJL were transferred into either normal B6 or LMP2$^{-/-}$ mice followed by IV infection. After 7 d, both host and donor cells were assayed for ICS and CD45.1 expression. Donor- and host-derived $NP_{366–374}$-specific T_{CD8+} were also tested for $NP_{366–374}$-tetramer staining and CD45.1 expression. Panel A shows typical $NP_{366–374}$-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 $NP_{366–374}$ in B6 mice, and conversely a much greater (~sixfold) donor $NP_{366–374}$–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 $NP_{366–374}$–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 $NP_{366–374}$.

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, $NP_{366–374}$ and $PA_{224–233}$-specific CTL remain dominant in both peritoneal and spleen cell responses. In $LMP2^{-/-}$ mice, T_{CD8+} specific for $PB1F2_{62–70}$ and $NS2_{114–122}$ 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 $NP_{366–374}$-specific cells observed with tetramers. This was somewhat less apparent in the peritoneal cavity, perhaps due to increased immunodomination from $PB1F2_{62–70}$-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 $PB1F2_{62–70}$-specific T_{CD8+}. Third, for the exceptional determinant, $PA_{224–233}$, 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 $NP_{366–374}$ 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 $NP_{366–374}$ 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+} was 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

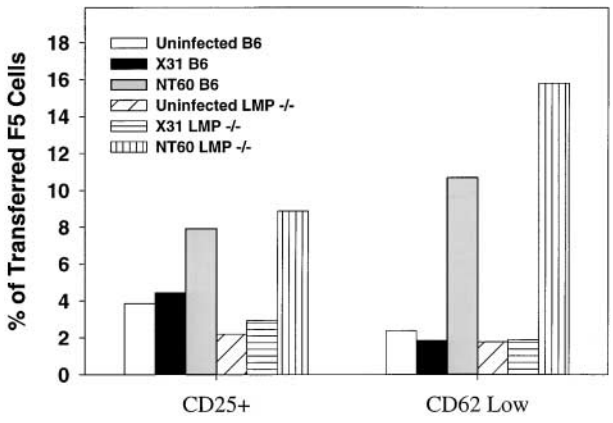

Figure 3. Activation of adoptively transferred transgenic T_{CD8+}. Purified T_{CD8+} from F5 TCR transgenic mice were transferred into B6 or $LMP2^{-/-}$ mice infected with NT60, X31 (control), or left uninfected as indicated. Cells were assessed by flow cytometry for activation based on enhanced binding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

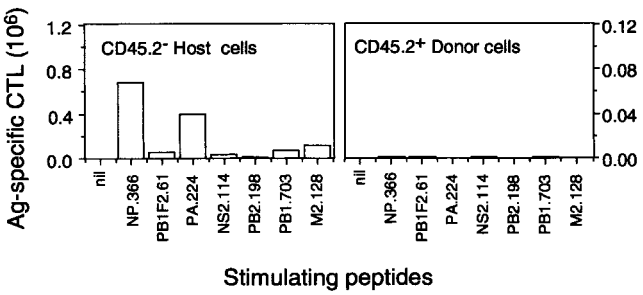

Figure 4. IV-specific responses of transferred naive $LMP2^{-/-}$ T cells. CD90-enriched spleen cells from naive $LMP2^{-/-}$ mice were transferred into either naive B6.SJL or B6 mice followed by IV priming. 7 d later, both the host and donor cells were assayed for ICS and CD45.2 expression. The determinant-specific 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.

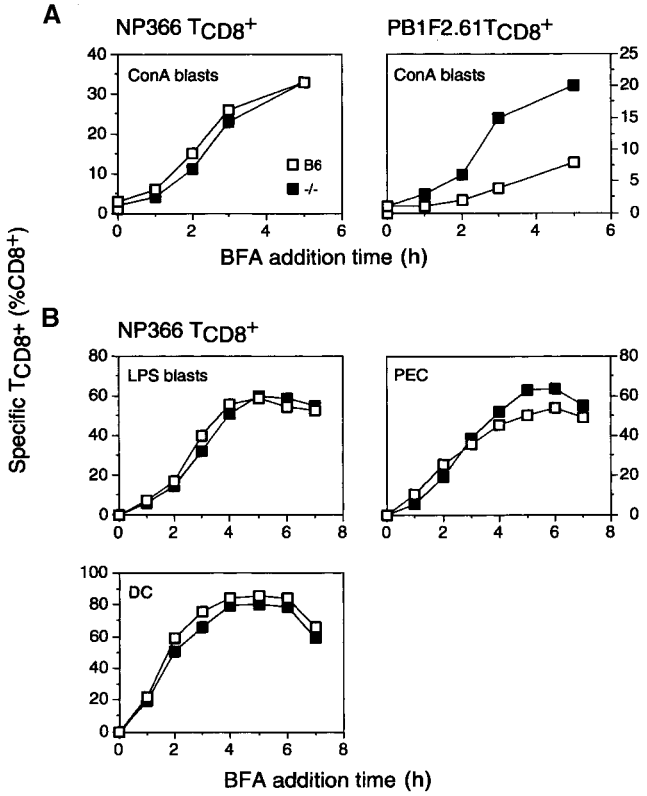

Figure 5. Kinetics of antigen presentation of B6 and LMP2$^{-/-}$ cells. The efficiency of antigen presentation was determined by the capacity of cells to activate short-term antigen-specific T_{CD8+} as determined by ICS. (A) Kinetics of antigen presentation of Con A blasts to $NP_{366–374}$-specific T_{CD8+} (left) or $PB1F2_{62–70}$-specific T_{CD8+} (right). (B) Kinetics of antigen presentation of cells as indicated to $NP_{366–374}$-specific T_{CD8+}. PEC, peritoneal cavity.

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_{CD8+} lines as measured by ICS. In additional experiments (not shown), we found that this assay parallels presentation kinetics as measured by ^{51}Cr release, which itself is proportional to the number of peptide-class I complexes recovered from cells (20). As seen in Fig. 5 A, Con A blasts from wild-type and LMP2$^{-/-}$ mice showed a similar kinetics of presenting $NP_{366–374}$. By contrast, LMP2$^{-/-}$ blasts presented $PB1F2_{62–70}$ much more rapidly than B6 blasts. This provides the second published example of immunoproteasomes interfering with production of a specific determinant (7). Unfortunately, we were not able to compare the presentation of $PA_{224–233}$ in this assay due to its very poor presentation in vitro even under optimal circumstances (unpublished data).

The identical presentation of $NP_{366–374}$ by wild-type and knockout cells was surprising, as it was reported that LMP2$^{-/-}$ LPS blasts and peritoneal exudate cells exhibit defective presentation of this determinant (21). We therefore repeated this experiment using LPS blasts, peritoneal exudate cells, and dendritic cells prepared from wild-type and LMP2$^{-/-}$ mice (Fig. 5 B). Once again, we failed to observe a significant difference in the presentation of this determinant.

Taken together, these findings indicate that in LMP2$^{-/-}$ mice the: (a) decreased response to $NP_{366–374}$ is based principally, if not solely, on defects in the T_{CD8+} repertoire; (b) decreased response to $PA_{224–233}$ is due principally to defects in antigen presentation; and (c) enhanced response to $PB1F2_{62–70}$ is probably due at least in part to enhanced generation by standard proteasomes relative to immunoproteasomes.

Van Kaer et al. (21) previously reported that LMP2$^{-/-}$ mice exhibit diminished responses to $NP_{366–374}$. This was attributed to a defect in antigen presentation based on the decreased abilities of LPS-activated splenocytes or peritoneal exudate cells derived from LMP2$^{-/-}$ mice to activate a $NP_{366–374}$-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 LMP2$^{-/-}$ cells to generate $NP_{366–374}$ from the PR8 NP.

As $PA_{224–233}$ occupies the α-position in the B6 T_{CD8+} immunodominance hierarchy, its demotion in LMP2$^{-/-}$ 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 $NP_{366–374}$ down the immunodominance hierarchy is striking, and demonstrates the profound effect of LMP2 on the anti-$NP_{366–374}$ repertoire. The loss of immunodomination exerted by these two determinants probably contributes to the ascendence of $PB1F2_{62–70}$- and $NS2_{114–122}$-specific T_{CD8+} in LMP2$^{-/-}$ mice. The enhanced presentation of $PB1F2_{62–70}$ in LMP2$^{-/-}$ APCs probably contributes to its rise to the α-position.

Our findings demonstrate that the loss of LMP2 has a significant effect on the T_{CD8+} repertoire. This is not shocking given the observation that LMP2$^{-/-}$ mice exhibit a specific deficit in the numbers of T_{CD8+} 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_{CD8+} repertoire. That deficiencies in antigen processing can influence the T_{CD8+} 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_{CD4+} 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 T_{CD8+} responding to IV was decreased in LMP2$^{-/-}$ mice. Potentially, this could be due to our failure to provide the proper determinants to LMP2$^{-/-}$ T_{CD8+} 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 T_{CD8+} 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 T_{CD8+} to respond to foreign antigens, even to determinants that appear to be made equally by standard proteasomes and immunoproteasomes. Consistent with this finding, T_{CD8+} 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 T_{CD8+} (as it probably is), it still points to a diminished capacity of LMP2$^{-/-}$ T_{CD8+} to proliferate relative to normal T_{CD8+}.

The poor responsiveness of LMP2$^{-/-}$ T_{CD8+} to IV demonstrates that LMP2, and by inference immunoproteasomes, enhance the ability of T_{CD8+} 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 T_{CD8+} 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 T_{CD8+} repertoire of normal mice and those lacking immunoproteasomes.

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