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Immunoproteasome Subunit Deficiencies Impact Differentially on Two Immunodominant Influenza Virus-Specific CD8+ T Cell Responses

Ken C. Pang,* Megan T. Sanders,* John J. Monaco,† Peter C. Doherty,‡ Stephen J. Turner,‡ and Weisan Chen2*

Primary CD8+ T cell (T_{CD8+}) responses to viruses are directed toward multiple Ags and shaped by both the level of Ag presentation and the underlying Ag-specific T_{CD8+} repertoire. The relative importance of these factors in deciding the hierarchy of T_{CD8+} responses and how they are influenced by the immunoproteasome are not well understood. Using an influenza infection model in mice deficient in various immunoproteasome subunits, we observe that Ag presentation and T_{CD8+} repertoire are altered in an epitope-specific and immunoproteasome subunit-dependent manner. More importantly, we find that the level of Ag presentation and the extent of the underlying repertoire can work either alone or in concert to determine definitively the magnitude of the individual T_{CD8+} responses and hence the overall T_{CD8+} hierarchy. Together, these results provide a clearer understanding of how immunodominance hierarchies are established. The Journal of Immunology, 2006, 177: 7680–7688.

The CD8+ T cells (T_{CD8+}) are critical effector cells of the adaptive immune system and mediate protection against viruses via recognition of viral peptides complexed to MHC class I molecules on the surface of APCs. During infection, a phenomenon known as immunodominance ensures that the host T_{CD8+} response is narrowly directed against a tiny fraction of the many possible viral peptide epitopes, and, within this fraction, hierarchically divided between immunodominant epitopes that reproducibly elicit the strongest reaction and “subdominant” epitopes that induce relatively weaker responses (1).

Understanding the mechanisms that determine immunodominance is an important goal of cellular immunity and should ultimately aid in the design of future T_{CD8+}-based vaccines against both pathogens and tumors. In recent years, our knowledge of the factors that influence immunodominance, including differences in not only the levels of peptide-MHC-I complexes presented on APC but also T_{CD8+} repertoire and avidity (2–4) has increased but remains incomplete. Earlier studies have also examined the effects of MHC-I binding ability on the rank of a particular peptide in the immunodominance hierarchy (3, 5, 6) and found that some weak binders elicit more robust T_{CD8+} responses than strong binders and vice versa, indicating that there was minimal correlation between immunodominance status and binding affinity. Moreover, it has been reported that an MHC binding affinity threshold of KD lower than ~50 nM determines the capacity of a peptide to elicit a CTL response in both humans (7) and mice (8). It would therefore appear that some factors such as peptide binding act in a permissive, all-or-nothing manner with respect to the establishment of an immunodominance hierarchy; i.e., beyond a minimum threshold further alterations fail to influence the magnitude of the T cell response, whereas below that same threshold no response is elicited. What then is actually responsible for shaping immunodominance hierarchies? Clearly, in the establishment of a graded T_{CD8+} hierarchy, there must be some means by which responses are influenced in an incremental or quantitative manner and not all factors can exert a binary, yes-or-no effect.

The 26S proteasome degrades intracellular proteins and polypeptides and plays a crucial role in the production and processing of peptides for MHC-I Ag presentation (9). At its catalytically active core lies the 20S proteasome, a multisubunit complex that exists in two forms: the standard 20S proteasome found in most cells; and the immunoproteasome. The presence of three unique subunits, LMP2 (β1i), LMP7 (β5i), and MECL1 (β2i), that can be induced by IFN-γ distinguishes the immunoproteasome from the standard 20S proteasome (10). The immunoproteasome is constitutively expressed in mature dendritic cells (11), the principal APC involved in priming T_{CD8+} responses. The immunoproteasome also favors the production of peptides with C-terminal hydrophobic residues (12–15), which often act as anchor residues for MHC-I binding. This suggests that immunoproteasomes play an important role in the production of peptides toward which T_{CD8+} immune responses are mainly directed in vivo, a finding supported by previous studies (16, 17). It has been shown, however, the sequences of some antigenic peptides from both viral and self Ags are cleaved internally by the immunoproteasome and that their production is instead favored by the standard proteasome (18–20). Immunoproteasome-deficient mice have also been found to mount largely normal T_{CD8+} responses to lymphocytic choriomeningitis virus infection

* T Cell Laboratory, Melbourne Centre for Clinical Sciences, Ludwig Institute for Cancer Research, Austin Health, Heidelberg, Australia; †Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH 45267; and ‡Department of Microbiology and Immunology, University of Melbourne, Parkville, Australia.

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2 Address correspondence and reprint requests to Dr. Weisan Chen, T Cell Laboratory, Melbourne Centre for Clinical Sciences, Ludwig Institute for Cancer Research, Austin Health, Heidelberg, Victoria 3084, Australia. E-mail address: weisan.chen@ludwig.edu.au

3 Abbreviations used in this paper: T_{CD8+}, CD8+ T cell; NP, nucleoprotein; NP366, NP366–374; PA, acidic polymerase; PA224, PA224–233; PB1F2, PB1F2_242–250; BMDC, bone marrow-derived dendritic cell; ICS, intracellular cytokine staining; NA, neuraminidase; BFA, brefeldin A; PR8, A/Puerto Rico/8/34 (HINI) influenza virus; NA, neuraminidase.

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dominant. More generally, we found that changes in Ag presentation in mice deficient in LMP2, MECL1, or both LMP7 and MECL1 and compared the results with those in wild-type mice. A/Puerto Rico/8/34 (H1N1) (PR8) influenza infection of C57BL/6 (hereafter called B6) mice results in a well-established immunodominance hierarchy of primary TCD8 responses, with the primary immunodominant responses directed against peptides from nucleoprotein (NP366–374; NP366) and acidic polymerase (PA224–233; PA224) (8, 22, 23). We found that influenza-specific TCD8 responses can be exquisitely sensitive to alterations in Ag presentation, with immunodominant responses rendered subdominant or subdominant responses enhanced to immunodominant status based solely on the effective level of presentation in subunit-deficient animals. We also showed that the influenza-specific TCD8 repertoire was impaired in some, but not all, of the knockout animals and that by itself a defective repertoire was sufficient to render a normally immunodominant response subdominant. More generally, we found that changes in Ag presentation and TCD8 repertoire can work in concert to shape immunodominance patterns. Taken together, these results provide a clearer understanding of how immunodominance hierarchies are established.

**Materials and Methods**

**Mice**

Female B6 mice were purchased directly from WEHI Animal Services. The following mouse strains were bred at the animal facility of the Melbourne Branch of the Ludwig Institute for Cancer Research: B6.1Ly5.1 (originally from WEHI Animal Services), B6.LMP2−/− (a gift from Dr. L. Van Kaer, Department of Microbiology and Immunology, Vanderbilt University, Nashville, TN), B6.MECL1−/−, B6.LMP7−/− MECL1−/−. Animals were generally used at 8–12 wk of age. Experiments were conducted under the auspices of the Austin Health Animal Ethics Committee, and conforms to the National Health and Medical Research Council Australian code of practice for the care and use of animals for scientific purposes.

**Viruses**

Wild-type and mutant PR8 influenza A viruses were grown in 10-day embryonic chicken eggs. The mutant recombinant PR8 PA2A virus was generated using an eight plasmid reverse genetics system as described previously (24, 25) and carried a disabling single amino acid mutation in the acidic polymerase (PA224–233; PA224) (8, 22, 23). We found that influenza-specific TCD8 responses can be exquisitely sensitive to alterations in Ag presentation, with immunodominant responses rendered subdominant or subdominant responses enhanced to immunodominant status based solely on the effective level of presentation in subunit-deficient animals. We also showed that the influenza-specific TCD8 repertoire was impaired in some, but not all, of the knockout animals and that by itself a defective repertoire was sufficient to render a normally immunodominant response subdominant. More generally, we found that changes in Ag presentation and TCD8 repertoire can work in concert to shape immunodominance patterns. Taken together, these results provide a clearer understanding of how immunodominance hierarchies are established.

**Cell lines, bone marrow-derived dendritic cells (BMDC), and CD8+ T cell line culture**

The thymoma cell line EL-4 (H-2b) was cultured in RPMI 1640 containing 10% FCS, 50 μM 2-ME, and 2 mM glutamine (RP-10). BMDCs were cultured from femoral bone marrow cells as previously described (27) in RP-10 supplemented with GM-CSF containing supernatant produced from the X63-GM-CSF cell line (a gift from Dr. Brigitta Stockinger, Division of Molecular Immunology, National Institute of Medical Research, London, U.K.). For culturing TCD8 cell lines, 3 × 106 splenocytes were stimulated with 3 × 106 peptide-pulsed autologous splenocytes in six-well plates with RP-10 containing 10 U/ml IL-2. Media were changed every 2 days, and the T cells were generally used within 10–20 days.

**Abs and peptides**

For flow cytometry, FITC-labeled anti-IFN-γ, CyChrome-labeled anti-CD8α, PE-labeled anti-CD45, purified anti-B220, and anti-CD4 mAb were purchased from BD Biosciences. For Western blotting, anti-LMP2 (Abcam), anti-LMP7 (Abcam), and anti-MECL1 (BIO-MOL) rabbit polyclonal Abs were used at 1/2000 dilution in PBS with 0.05% Tween 20 (ICN Biomedicals) and 1% skim milk powder (Bonalac). Peptides were procured and characterized by the Biologic Resource Branch, National Institute of Allergy and Infectious Diseases (gifts from Drs. J. Yewdell and J. Bennink, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). They are NP366–374 (ASDNENMETM), PA224–233 (SSLENFRAYV), PB1F2–226 (PB1F2; LSRLNPILV), NS2114–121 (RTFSFQILD), PB1703–711 (SSYRRNPILVQ), PB1718–207 (PB1; MGILYNKR), and M1128–313 (MLGILYRKM).

**Intracellular cytokine staining (ICS)**

Splenic and peritoneal cells from primed animals were suspended in 200 μl of RP-10 at 1.5–2 × 106 cells per well in round-bottom 96-well plates. Peptides were added to cells at a final concentration of 1 μM unless otherwise stated. The cells were incubated with peptides for 2 h at 37°C and then for 4 h with brefeldin A (BFA; Sigma-Aldrich) at 10 μg/ml. Cells were stained with CyChrome-labeled anti-CD8α mAb PE-labeled anti-B220, and FITC-labeled anti-CD4 mAb for 30 min, washed, and fixed with 1% paraformaldehyde in PBS at room temperature for 20 min. Fixed cells were then further stained with fluorescein-anti-IFN-γ in PBS containing 0.2% saponin (Sigma-Aldrich). Stained cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Ag presentation assays**

The method for Ag presentation kinetics assessment has previously been described (3, 28). Briefly, aliquots of 105 BMDCs, EL-4 cells, or DC2.4 cells were infected with either wild-type or mutant PR8 for 60 min at 37°C (designated as t = 0 h). At various time points, 106 peptide-specific TCD8+ were added in conjunction with 10 μg/ml BFA to freeze Ag presentation. Four hours after TCD8/BFA addition, the cells were harvested and stored at 4°C until all samples were ready for ICS. For Ag presentation assays ex vivo, peritoneal cells were harvested 8–12 h after i.p. influenza infection, and 5 × 106 cells were cocultured with 5 × 106 peptide-specific TCD8+ for 4 h, then ICS was performed.

**Tetramer and TCR Vβ staining**

PA244-specific TCD8+ were identified with tetrameric H-2Dβ/PA244 (ImmunoID). Splenocytes were stained with PE-conjugated tetramer for 30 min at room temperature, washed, and then incubated with CyChrome-labeled anti-CD8α mAb and selected FITC-labeled TCR Vβ mAbs (BD Pharmingen) at 4°C for 20 min.

**Western blotting**

Splenocytes and BMDC samples were lysed in 1% Triton-X (Sigma-Aldrich), and SDS-PAGE analyses were performed. The proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore). After transfer, the membranes were incubated with the primary anti-immunoproteasome subunit Ab (see above) at 4°C overnight. The membranes were washed in PBS, peroxidase-labeled sheep anti-rabbit immunoglobulins (Silenus Labs) were added at a 1/2500 dilution in PBS with 0.05% Tween 20. After further washing, the proteins were visualized radiographically using an electrochemiluminescence (ECL Plus) substrate (Amersham Biosciences).

**Results**

The codominant PA244 response becomes subdominant after influenza infection in immunoproteasome subunit knockout mice

Fig. 1 demonstrates that the absence of different immunoproteasome subunits has different effects on the TCD8+ responses in both the peritoneum and spleen as measured by ICS 7 days after i.p. influenza infection. Consistent with our previous findings, loss of LMP2 resulted in relegation of the normally immunodominant NP366 and PA224 TCD8+ responses to subdominant status. In contrast, mice deficient in either MECL1, or both LMP7 and MECL1, retained the immunodominant NP366 response of wild-type animals, but had a diminished proportion of PA224-specific TCD8+ compared with B6 mice. This effect was more pronounced in the double knockout animals. The alteration in the immunodominance
hierarchy in the MECL1\(^{-/-}\) and LMP7\(^{-/-}\)/MECL1\(^{-/-}\) mice was observed consistently, and did not change when the primary response was measured over an extended duration of time (data not shown). Thus, the codominant PA224 response became subdominant in all three immunoproteasome subunit knockout mice, albeit to varying extents. Previously, we have observed that the total number of T\(_{CD8}\) in LMP2\(^{-/-}\) mice was reduced to ∼50% that of B6 mice (16). Reduced T\(_{CD8}\) numbers have also been recently described in MECL1\(^{-/-}\) and LMP7\(^{-/-}\)/MECL1\(^{-/-}\) animals (29, 30). Our own results were consistent with these findings (data not shown).

Ag presentation of PA224 is diminished in immunoproteasome subunit knockout mice

Given the role of immunoproteasomes in Ag processing, one likely explanation for the observed changes in immunodominance hierarchy in mice lacking immunoproteasome subunits was a difference in Ag presentation. To explore this possibility, we grew BMDCs from each of the strains, infected them with PR8 in vitro, and compared their ability to present different epitopes to short-term T\(_{CD8}\) lines. By adding BFA to BMDCs at various times after infection to block export of nascent MHC-I-peptide complexes to the cell surface and then monitoring the activation of peptide-specific T\(_{CD8}\) effectors by ICS, we were able to assess Ag presentation in a dynamic manner.

Fig. 2A demonstrates that presentation of both NP366 and PB1F2 is very similar for BMDCs propagated from both B6 animals and mice lacking immunoproteasome subunits. For both these epitopes, presentation by BMDCs from all four strains of mice resulted in activation of the majority of peptide-specific T cells. The response started to plateau ∼2–4 h postinfection, with PB1F2 presentation slightly slower than that of NP366. Interestingly, PA224 presentation was quite different between BMDCs propagated from the different strains of mice. The most effective presentation was by B6 BMDCs, although even here only a minority of PA224-specific T\(_{CD8}\) was successfully activated. Presentation of PA224 by BMDCs derived from LMP2\(^{-/-}\) and MECL1\(^{-/-}\) mice was similar and at maximal levels resulted in only one-half the T cell activation stimulated by B6 BMDCs. Lowest of all was presentation by BMDCs derived from LMP7\(^{-/-}\)/MECL1\(^{-/-}\) cells, which was only slightly higher than background levels.

Although dendritic cells are believed to be the critical APC involved in priming T\(_{CD8}\) responses and cultured BMDCs have been shown to function as APCs in vivo (31–34), it was unclear whether the results of our BMDC studies in vitro accurately reflected presentation of the individual epitopes following influenza infection in the mice. To address this, we primed B6 and immunoproteasome subunit-deficient mice with PR8 influenza i.p. and recovered peritoneal cells 8–12 h later. We reasoned that resident or recently migrated APCs within the peritoneum would be capable of presenting influenza epitopes ex vivo. We then used these peritoneal cells to stimulate NP366- or PA224-specific T\(_{CD8}\) lines and measured the activation of the T\(_{CD8}\) by ICS to assess Ag presentation from each of the strains. It was possible that the harvested peritoneal cells might contain T\(_{CD8}\), which could confound assessment of the Ag-specific T\(_{CD8}\) lines. To exclude this complication, the T\(_{CD8}\) used to assess Ag presentation were grown from congenic B6.Ly5.1 animals, which express a CD45.1 cell surface marker that can be detected with mAbs by flow cytometry, to enable discrimination between cultured T\(_{CD8}\) and T\(_{CD8}\) from the peritoneal cells.

As shown in Fig. 2B, NP366 presentation by the peritoneal cells ex vivo was similar for B6 and immunoproteasome subunit-deficient mice, consistent with our earlier findings using BMDCs infected in vitro. Meanwhile, PA224 presentation again showed clear differences between the strains. Peritoneal cells from B6 mice stimulated a reasonable proportion of PA224-specific T cells (17.6 and 8.6% of total CD8\(^{+}\) cells for each mouse). In contrast, peritoneal cells from LMP2\(^{-/-}\) and MECL1\(^{-/-}\) mice activated only ∼1% of total T\(_{CD8}\), whereas PA224-specific T cell recognition of LMP7\(^{-/-}\)/MECL1\(^{-/-}\) cells was <0.5% and close to background levels. Thus, these results provided further evidence that the presentation of PA224 was diminished after influenza infection in immunoproteasome subunit-deficient mice.

The PA224 T\(_{CD8}\) repertoire is intact in MECL1\(^{-/-}\) but impaired in LMP2\(^{-/-}\) and LMP7\(^{-/-}\)/MECL1\(^{-/-}\) mice

Up to this point, we had evidence that the fall of the PA224 T\(_{CD8}\) response to subdominant status in the immunoproteasome subunit-deficient mice was a result of reduced Ag presentation of the PA224 epitope. The possibility remained, however, that differences in the T\(_{CD8}\) repertoire between wild-type and knockout
BMDCs were added at a 1:1 ratio to short-term T CD8 peptide), and the avidities of the NP366- and PA224-specific T CD8 and PA224 peptide were 58.4 and 84.9%, respectively (as judged by IFN- secretion suggesting that the PA224 response in MECL1 not only indicating that the relevant repertoires are alike but also the level of Ag presentation by peritoneal APC in each well. Results from individual mice (two per group) are shown as fluorescence (FL) dot plots of CD8 BMDCs in each well. Results are plotted as a percentage of maximal response, as determined by stimulating T CD8 peptide concentrations. B, B6, LMP2−/−, MECL1−/−, and LMP7−/−/MECL1−/− animals were infected with PR8 influenza via i.p. injection. From 8 to 12 h later, peritoneal cells were harvested ex vivo and added at a 10:1 ratio to short-term TCD8 lines from Ly5.1 mice specific for either NP366 or PA224 in the presence of BFA. Individual wells were harvested 4 h after BFA addition, and activation of CD45.1+ TCD8 cells was assessed via ICS to gauge the level of Ag presentation by peritoneal APC in each well. Results from individual mice (two per group) are shown as fluorescence (FL) dot plots of CD8 and IFN-γ expression and have been gated to display CD45.1+CD8+ cells. For the experiments shown, the proportions of total TCD8− specific for NP366 and PA224 peptide were 58.4 and 84.9%, respectively (as judged by IFN-γ production upon stimulation with EL-4 cells and saturating levels of cognate peptide), and the avidities of the NP366- and PA224-specific TCD8+ were very similar.

mice might also be contributing to the observed changes in immunodominance hierarchy. Indeed, we have previously shown that the reduced immunogenicity of NP366 in LMP2−/− mice is due to alterations in the T cell repertoire (16), and we were interested to determine whether this was also true for PA224 in any of the immunoproteasome subunit-deficient mice.

We therefore performed adoptive transfer experiments in which donor T cells from either wild-type or immunoproteasome subunit-deficient mice (isolated via Thy1.2 magnetic bead separation) were injected i.v. into congenic B6.Ly5.1 host animals. After i.p. influenza infection, the primary responses of host and donor T cells were directly compared by ICS, and the underlying functional repertoires were assessed. Fig. 3 compares the peritoneal and splenic T CD8+ responses, respectively. As expected, there was no difference between donor B6 and host B6.Ly5.1 responses to influenza, with both showing typical immunodominance of PA224 and NP366 T cells. In contrast, LMP2−/− donor cells specific for both NP366 and PA224 responded very poorly compared with B6.Ly5.1 host T CD8+, which not only confirmed our previous finding that the NP366 T cell repertoire was impaired in these mice (16) but also highlighted that the PA224 repertoire was adversely affected. Interestingly, MECL1−/− donor cells displayed an immunodominance hierarchy very similar to that of wild-type host cells (with NP366 and PA224 sharing immunodominant status), not only indicating that the relevant repertoires are alike but also suggesting that the PA224 response in MECL1−/− mice could rise to immunodominant status if Ag presentation was improved.

After adoptive transfer of LMP7−/−/MECL1−/− donor cells into B6.Ly5.1 mice, the host PA224 T CD8+ response in both the spleen and peritoneum became subdominant (~50% that of the NP366 response that remained unaffected). This result was consistent in repeated experiments. The reason for this was unclear but may have been related to the observation that LMP7−/−/MECL1−/− donor T cells survived suboptimally in B6.Ly5.1 hosts (Fig. 3C, showing donor TCD8+), which prompts the possibility that the host immune response had been skewed toward rejection of donor cells because of either reduced surface MHC-I expression (29, 35) or minor histocompatibility Ag differences that had arisen due to variation in the immunoproteasome composition. Similarly, a lower number of donor T CD8+ was recovered after LMP2−/− T cells were transferred into B6.Ly5.1 mice (Fig. 3C). It is also possible that both the LMP2−/− and LMP7−/−/MECL1−/− donor T cells mounted a simultaneous antihost response. With these caveats in mind, it was nonetheless possible to observe that LMP7−/−/MECL1−/− donor T CD8+ responses to PA224 were generally lower than those of the B6.Ly5.1 host cells, indicating that the PA224 repertoire in LMP7−/−/MECL1−/− mice might have been impaired.

We also performed adoptive transfer experiments in the opposite direction, using B6.Ly5.1 animals as donors and either B6 or immunoproteasome subunit-deficient mice as hosts. The results obtained were consistent with the findings presented above (data not shown), although in the case of the transfer into LMP7−/−/MECL1−/− host, the PA224 repertoire could not be adequately
assessed given that presentation of this epitope is severely diminished in these animals (Fig. 2), and consequently the donor wild-type PA224 T cells may not have been adequately stimulated.

**PA224 is better presented when inserted into the NA stalk**

Next, we wanted to explore the effects of improving PA224 presentation on the immunodominance hierarchy after influenza infection. To do so, we used mutant PR8 influenza viruses engineered using an established eight-plasmid reverse genetics system (24, 26). One of these viruses (PR8 PA Na) expressed a PA224 epitope inserted into the NA stalk at aa 42 and also carried a disabling asparagine to glutamine substitution at position 5 in the native PA224 epitope (4).

Using the same methods as earlier, we compared the presentation of PA224 in vitro following either PR8 wild-type or PA Na infection (Fig. 4A). PR8 PA Na infection of B6 BMDCs improved PA224 presentation compared with infection with wild-type virus. A similar pattern was seen for each of the immunoproteasome subunit-deficient BMDCs. When comparing presentation between BMDCs, it was evident that after infection with PR8 PA Na virus, LMP2−/−, MECL1−/−, and LMP7−/−/MECL1−/− BMDCs were capable of presenting PA224 at levels that were 1) greater than that observed for B6 BMDCs infected with wild-type virus and 2) similar to the enhanced levels seen for B6 BMDCs infected with PR8 PA Na virus. We also examined whether ex vivo peritoneal cells from the different mice were able to present PA224 more effectively after PR8 PA Na infection, and these data were consistent with our findings in vitro (data not shown).

**Changes in the immunodominance hierarchy after PR8 PA Na infection**

Given the improved Ag presentation of PA224 with the mutant virus, we speculated whether PR8 PA Na infection might enhance the primary T CD8+ response to PA224. We therefore assessed the T CD8+ immunodominance hierarchies in both wild-type and immunoproteasome-deficient mice by ICS 7 days after PR8 PA Na infection. As shown in Fig. 4B, challenge with the mutant virus did not result in any major alteration of either the splenic or peritoneal T CD8+ response in B6 animals when compared with PR8 wild-type infection at equivalent viral doses (10^7 PFU per mouse i.p.). In both cases, NP366 and PA224 shared immunodominant status. When PR8 PA Na virus was used to infect LMP2−/− mice, however, the PA224 T CD8+ response rose to a clear position of immunodominance. Similarly, the outcome of infection of both MECL1−/− and LMP7−/−/MECL1−/− animals with mutant virus was that the PA224 response, which had previously been subdominant following infection with wild-type virus, returned to immunodominant status. Despite the fact that PA224 was now immunodominant in all four strains of mice, we observed that the proportion of responding PA224 T CD8+ cells following PR8 PA Na virus infection varied between groups. Focusing on splenic responses, which account for the majority of responding T CD8+, the highest responses were in B6 and MECL1−/− mice, with somewhat lower responses in LMP2−/− and LMP7−/−/MECL1−/− animals.

**PA224-specific T CD8+ cells show similar peptide avidity and TCR Vβ usage**

We have assumed up to this point that the PA224-specific T CD8+ cells that respond to influenza infection are qualitatively similar between wild-type and immunoproteasome subunit-deficient mice. However, as we have already observed, differences in immunoproteasome composition affect the efficiency of Ag presentation of peptides such as PA224, and this in turn might be expected to alter the nature of PA224-specific T CD8+ cells recruited not only during the acute antiviral response but also at the time of thymic selection. To explore this possibility, we compared the TCR avidity and the Vβ usage of PA224-specific T CD8+ from wild-type and immunoproteasome subunit-deficient mice. Fig. 5A shows that the TCR
avidiy of these cells across each of the different mouse strains, as measured by IFN-γ production, was equivalent, with half-maximal responses observed at \(-10^{-11}\) M for both spleen and peritoneum T

Cooperative immunoproteasome subunit expression is cell type dependent

Others have previously reported that immunoproteasome subunit assembly is a cooperative process, where MECL1 requires LMP2 for efficient incorporation and LMP7 facilitates processing of LMP2 preprotein (38–40). It is therefore possible that some of the findings that we observed in the immunoproteasome subunit-deficient mice might reflect this codependency between the subunits. For instance, if the requirement of MECL1 for LMP2 is absolute, then one would predict that the defects we observed in LMP2-/- mice might be at least partly attributable to a relative lack of MECL1 incorporation.

To explore this further, we performed Western blots for LMP2, MECL1, and LMP7 using lysates of either splenocytes or BMDCs from B6 and immunoproteasome subunit-deficient mice (Fig. 6). As expected, each subunit was readily detected in B6 lysates, whereas LMP2 and LMP7 were absent from LMP2-/- and LMP7-/-/MECL1-/- samples, respectively, and MECL1 was absent from both MECL1-/- and LMP7-/-/MECL1-/- mice. Interestingly, looking at the LMP2-/- lysates, we consistently observed that MECL1 was not detected in spleen but was present in BMDCs. This result indicated that although MECL1 may be dependent on LMP2 for efficient incorporation into the immunoproteasome, consistent with earlier reports, such cooperation might be cell type dependent. For the MECL1-/- lysates, the spleen sample appeared to contain less LMP2 protein, which is consistent with a recent report (30), but in a subsequent experiment the difference in protein levels was less obvious. Finally, the LMP7-/-/MECL1-/-
we found that the TCD8 modified mice deficient in various immunoproteasome subunits, primary influenza A virus infection in B6 mice. Using genetically ing peptide-specific T cell repertoire work either together or separated how the overall level of Ag presentation and the underly- nant status based on partial changes in the effective levels of Ag reduced to a subdominant position or promoted to immunodominant.

Graphically, this is seen as a shift of the dose-response curve to the midslope of the curve (as we hypothesize is the case for the immunodominant PA224 response in B6 mice after wild-type virus infection) is insensitive to further increases in the level of Ag presentation but remains highly sensitive to any decreases. Additionally, from some positions (such as in the middle of a plateau), one might predict that mild changes to presentation in either direction will have no functional impact on the magnitude of the T cell response and the resulting immunodominance hierarchy.

Our model also illustrates how peptide-specific T cell repertoire (diversity and/or abundance), similar to Ag presentation efficiency, can have a graded and variable impact upon immunodominance. In this schema, an impaired repertoire reduces the T cell response. Graphically, this is seen as a shift of the dose-response curve to the

Discussion
Following up on our own recent study that emphasized a role for Ag dose and TCD8 precursor frequencies (4), here we have examined how the overall level of Ag presentation and the underlying peptide-specific T cell repertoire work either together or separately to influence the immunodominance hierarchy that follows primary influenza A virus infection in B6 mice. Using genetically modified mice deficient in various immunoproteasome subunits, we found that the TCD8 responses directed toward PA224 can be reduced to a subdominant position or promoted to immunodominant status based on partial changes in the effective levels of Ag presentation. We also showed that the influenza-specific TCD8 repertoire is impaired in some but not all of the knockout animals and that by itself a defective repertoire (for NP366 in LMP2−/− mice) is sufficient to render a normally immunodominant response subdominant. More generally, we found that changes in Ag presentation and TCD8 repertoire can work in concert to shape immunodominance patterns. For instance, after infection of LMP7−/−/MECL1−/− mice with the PR8 PAA virus, enhanced presentation of PA224 in combination with an impaired repertoire resulted in a PA224 response that is immunodominant but weaker than that seen in spleens obtained from B6 mice infected with the same virus (Fig. 4B). By integrating these findings, we have developed a quantitative model based on the PA224 response that provides an understanding of how the level of Ag presentation and the T cell repertoire can function together to determine immunodominance (Fig. 7 and accompanying legend).

Understood in this way, one observes that quantitative changes in Ag presentation affect the immunodominance hierarchy in different ways, depending upon the starting position and direction of movement along the theoretical sigmoid-shaped dose-response curve. One would predict, for example, that from a position on the midslope of the curve, small changes in Ag presentation (either up or down) are likely to result in observable effects on the size of the T cell response. In contrast, a position at the tipping point on top of the curve (as we hypothesize is the case for the immunodominant PA224 response in B6 mice after wild-type virus infection) is insensitive to further increases in the level of Ag presentation but remains highly sensitive to any decreases. Additionally, from some positions (such as in the middle of a plateau), one might predict that mild changes to presentation in either direction will have no functional impact on the magnitude of the T cell response and the resulting immunodominance hierarchy.

Our model also illustrates how peptide-specific T cell repertoire (diversity and/or abundance), similar to Ag presentation efficiency, can have a graded and variable impact upon immunodominance. In this schema, an impaired repertoire reduces the T cell response. Graphically, this is seen as a shift of the dose-response curve to the

FIGURE 7. Model of the relationships among the primary PA224 TCD8 response, Ag presentation, and T cell repertoire. After infection with wild-type virus, PA224 presentation in B6 mice is sufficient to elicit a maximal, immunodominant response. In MECL1−/− mice, a partial reduction in presentation results in an incremental fall in the PA224 response to subdominant status. In LMP2−/− and LMP7−/−/MECL1−/− animals, the underlying PA224-specific TCD8 repertoires are impaired and the resulting dose-response curve is displaced to the right. Consequently, although PA224 presentation in MECL1−/− mice is similar to that in LMP2−/− animals, the PA224-specific T cell response falls back to levels at the threshold of detection. PA224 presentation in LMP7−/−/MECL1−/− mice meanwhile is lowest of all, and the resulting response remains at or below threshold. After infection with PR8 PAA virus, PA224 presentation is enhanced in all the animals tested. In B6 mice, the PA224 response does not increase any further, suggesting that saturating levels of presentation in vivo have already been achieved with wild-type virus. By comparison, in MECL1−/− animals, the T cell response to PA224 ascends the slope of the dose-response curve to achieve a maximal (Max.) response. Similarly, enhanced presentation of PA224 in LMP2−/− and LMP7−/−/MECL1−/− mice results in a shift up the curve, although the overall T cell response is still submaximal given the underlying impairment in the TCD8 repertoire.
right. What follows is that the overall impact of an impaired repertoire will depend on the original position along the Ag presentation scale (as before) as well as the degree of repertoire impairment, which manifests as the size of horizontal displacement to the right. Consider, for instance, the PA224 response in LMP2/−/− and MECL1/−/− animals. LMP2/−/− mice appear to have an impaired PA224-specific repertoire compared with their MECL1/−/− counterparts (Fig. 3), yet Ag presentation is similar between the two knockout animals after either wild-type (Fig. 2A) or mutant (Fig. 4A) virus infection. By observing the comparative magnitude of the PA224 T cell response between the LMP2/−/− and MECL1/−/− animals, it is evident that the functional TCD8 response of the underlying repertoire impairment differ depending on the level of Ag presentation achieved (as determined by the virus that is used). Thus, when wild-type virus is used and PA224 presentation is suboptimal, the repertoire difference is clearly manifested, with the PA224 TCD8 response barely detected in LMP2/−/− mice and much more vigorous (albeit still subdominant) in MECL1/−/− animals (Fig. 1). In contrast, where mutant virus is used, the PA224 response in the two knockout animals is more similar (Fig. 4B), which suggests that the underlying repertoire difference has been largely compensated by the enhanced PA224 presentation. To summarize, our model illustrates how two key factors, the level of Ag presentation and the T cell repertoire, can work either alone or together to exquisitely shape an individual T cell response; in doing so, we provide a novel schema for interpreting how immunodominance hierarchies not only arise, but also change.

Our findings also add further evidence to the role of immunoproteasomes in the TCD8 immune response. We have previously shown that the TCD8 response in LMP2/−/− mice is substantially altered following influenza infection (16). Here, using wild-type virus, we confirm and extend these findings to MECL1/−/− and LMP7/−/−/MECL1/−/− animals, and we show that loss of LMP2, MECL1, or LMP7 and MECL1 has a distinct effect, which highlights the functional heterogeneity of the individual immunoproteasome subunits. Taken together, the results demonstrate that immunoproteasomes influence the TCD8 response by affecting not only Ag presentation (in this case, of the native immunodominant PA224 epitope) but also the peptide-specific TCD8 repertoire (of both PA224 and the codominant NP366 epitope). This repertoire impairment presumably arises because thymic APC express immunoproteasomes (41) and is supported by recent studies showing that TCR transgenic mice that are deficient in either LMP2 or LMP7 have a defective repertoire of transgenic TCD8 (41, 42). Alongside ours and others’ findings that immunoproteasome-deficient mice have reduced overall numbers of TCD8 (29, 30, 43), the repertoire impairments further highlight the role of the immunoproteasome during T cell development and selection. Although it is generally assumed that such a role is being mediated at the APC level, it is also possible that the immunoproteasome has a function in T cells themselves. For instance, we have shown by immunoblotting that T cells express LMP2, LMP7, and MECL1 (unpublished data), and it has recently been shown that absence of both LMP7 and MECL1 results in hyperproliferation of both CD4+ and CD8+ T cells to polyclonal and allogeneic stimuli (29). The reasons for this are not easily understood based on our current conceptions of the immunoproteasome, and we suggest that the immunoproteasome may be playing a more diverse biological role than is presently appreciated. This hypothesis is further supported by the fact that it is also expressed by nonimmune cells such as the ocular lens (44).

Our results with the mutant PR8 PA_{NA} virus deserve further comment. Most importantly, these experiments demonstrate, to our knowledge for the first time, that by enhancing Ag presentation one can overcome an impaired repertoire to achieve immunodominance, as is readily observed in the case of PA224 in the LMP2/−/− mice (compare the results of wild-type and mutant virus infection in Figs. 1 and 4B, respectively). We are also left with the question: why does insertion of the PA224 epitope within the NA stalk enhance its presentation? One suggestion is that the NA Ag is more abundant than acidic polymerase following influenza infection (4). The other likelihood is that the difference in flanking sequences (between the native PA224 and ectopic PA_{NA}) influences the processing of the epitope, as has been shown in other contexts (45). Whatever the reason, presentation of the ectopic PA_{NA} seems less dependent on the immunoproteasome than the native peptide (Fig. 4A). It is therefore tempting to hypothesize that the immunoproteasome might be particularly efficient at generating peptides from lowly abundant Ags as a way of maximizing the epitope repertoire displayed by DC, whereas the standard proteasome might be suited to generating peptides from more abundant proteins.

The work presented here has valuable implications for vaccination strategies in which TCD8 responses are targeted. The observation that the PA224 response (seen in response to wild-type influenza challenge in B6 mice) quickly falls to subdominant status following a partial reduction in Ag presentation (as observed in MECL1/−/− animals) highlights the fact that even immunodominant responses can be highly sensitive to changes in Ag presentation and thus sit precariously at the tipping point of the curve. This is in agreement with our previous assessment of the presentation of this epitope (46). Moreover, our discovery that enhanced PA224 presentation with the mutant virus can restore a subdominant response to immunodominant status strongly advocates the importance of optimizing Ag presentation with any vaccine. This study also has specific relevance for designing successful peptide and subunit-based vaccines. Consider, for instance, the finding that immunodominant epitopes can be either independent (as in the case of NP366) or dependent (as for the native PA224) on the immunoproteasome for their presentation. One obvious consequence is that the cell surface expression of such epitopes on target cells and tissues will vary depending on their proteasomal composition, affecting successful recognition by effector TCD8.

It will be intriguing to follow up our observation that enhanced Ag presentation can overcome impairments in TCD8 repertoire to generate immunodominant responses and whether similar strategies can be used to manipulate immunodominance hierarchies in other systems. Immunodominant TCD8 responses are generally regarded as being very robust, but this study raises the possibility that other immunodominant TCD8 responses may also lie at the tipping point, where they are as sensitive to partial changes in Ag presentation as PA224. And at what point does impairment in repertoire become too severe to be successfully overcome? Answering questions such as these will hopefully facilitate better vaccine design. Finally, looking further ahead, it is also important to develop better tools to accurately and directly enumerate not only naive Ag-specific T cell precursors, the frequency of which in mice has been estimated on the order of 100–200 cells (47), but also specific peptide-MHC-I complexes on the cell surface. The model we have proposed here is inferred from an indirect assessment of peptide-specific TCD8 repertoire and Ag presentation levels and, as technology improves, we would hope to develop a more detailed qualitative and quantitative understanding of how these two key factors interact to sculpt immunodominance hierarchies.

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References


