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Immunoproteasome-Deficient Mice Mount Largely Normal CD8⁺ T Cell Responses to Lymphocytic Choriomeningitis Virus Infection and DNA Vaccination

Alexander K. Nussbaum, Maria P. Rodriguez-Carreno, Nicola Benning, Jason Botten, and J. Lindsay Whitton

During viral infection, constitutive proteasomes are largely replaced by immunoproteasomes, which display distinct cleavage specificities, resulting in different populations of potential CD8⁺ T cell epitope peptides. Immunoproteasomes are believed to be important for the generation of many viral CD8⁺ T cell epitopes and have been implicated in shaping the immunodominance hierarchies of CD8⁺ T cell responses to influenza virus infection. However, it remains unclear whether these conclusions are generally applicable. In this study we investigated the CD8⁺ T cell responses to lymphocytic choriomeningitis virus infection and DNA immunization in wild-type mice and in mice lacking the immunoproteasome subunits LMP2 or LMP7. Although the total number of virus-specific cells was lower in LMP2 knockout mice, consistent with their having lower numbers of naive cells before infection, the kinetics of virus clearance were similar in all three mouse strains, and LMP-deficient mice mounted strong primary and secondary lymphocytic choriomeningitis virus-specific CD8⁺ T cell responses. Furthermore, the immunodominance hierarchy of the four investigated epitopes (nuclear protein 396 (NP₃₉₆) > gp33 > gp276 > NP₂₃₆) was well maintained. We observed a slight reduction in the NP₂₃₆-specific response in LMP2-deficient mice, but this had no demonstrable biological consequence. DNA vaccination of LMP2- and LMP7-deficient mice induced CD8⁺ T cell responses that were slightly lower than, although not significantly different from, those induced in wild-type mice. Taken together, our results challenge the notion that immunoproteasomes are generally needed for effective antiviral CD8⁺ T cell responses and for the shaping of immunodominance hierarchies. We conclude that the immunoproteasome may affect T cell responses to only a limited number of viral epitopes, and we propose that its main biological function may lie elsewhere.

Virus infections and conventional vaccines induce CD8⁺ T cell responses to only a few of the many potential epitopes present, and for those epitopes to which CD8⁺ T cell responses are mounted, the relative abundance of CD8⁺ T cells can vary dramatically, in a phenomenon termed immunodominance. Both the initial priming of CD8⁺ T cells and their subsequent abundance are affected by several factors (reviewed in Ref. 1); one key component is the presentation of peptide epitopes by MHC class I proteins, without which a CD8⁺ T cell response cannot occur. The great majority of these epitopes are generated within the cell by proteolytic degradation of newly synthesized proteins. This hydrolysis, which preferentially targets misfolded proteins (2), is conducted by the proteasome, a cytosolic enzyme complex that is crucial for the generation of the C termini of MHC class I-bound peptides (3, 4). There are at least two types of proteasome: the constitutive form, which is present in all cells, and the immunoproteasome, so-called because it is induced by the inflammatory cytokine IFN-γ, which is synthesized in response to many viral infections. The constitutive proteasome contains the δ (β1), MB1 (β5), and MC14 (Z and β2) proteins, but these are absent from the immunoproteasome, which is assembled de novo after the IFN-γ-driven induction of three replacement proteins, (LMP2) (β1i), LMP7 (β5i), and MECL-1 (β2i). Constitutive proteasomes and immunoproteasomes have distinct proteolytic activities (5–8), resulting in the generation of different, but overlapping, peptide pools (9, 10); for this reason and because an increase in immunoproteasome abundance has been reported during viral infection (11), immunoproteasomes are considered likely candidates for modifying the array of epitopes that are presented during virus infection for the perusal of naive CD8⁺ T cells. However, published data do not allow a clear conclusion to be drawn. The immunoproteasome has been reported to enhance the presentation of some epitopes (12–15), but to abrogate the presentation of others (16). The emerging picture is that self and tumor epitopes are poorly processed by immunoproteasomes, whereas viral epitopes are more efficiently generated by immunoproteasomes (17, 18). However, it is still a matter of debate whether immunoproteasomes have evolved specifically to process Ags from infectious organisms.

Mice lacking LMP2 or LMP7 were generated about a decade ago (19, 20) and show the anticipated changes in peptide activities (20, 21). In addition, LMP2 knockout (LMP2KO) mice differed from wild-type (WT) mice in 1) a reduced percentage of CD8⁺ T cells in blood and spleen, 2) 5- to 6-fold reduced numbers of virus-specific cells, 3) a reduced number of virus-specific cells in secondary lymphocytic choriomeningitis virus infection and DNA immunization in wild-type mice and in mice lacking the immunoproteasome subunits LMP2 or LMP7. Although the total number of virus-specific cells was lower in LMP2 knockout mice, consistent with their having lower numbers of naive cells before infection, the kinetics of virus clearance were similar in all three mouse strains, and LMP-deficient mice mounted strong primary and secondary lymphocytic choriomeningitis virus-specific CD8⁺ T cell responses. Furthermore, the immunodominance hierarchy of the four investigated epitopes (nuclear protein 396 (NP₃₉₆) > gp33 > gp276 > NP₂₃₆) was well maintained. We observed a slight reduction in the NP₂₃₆-specific response in LMP2-deficient mice, but this had no demonstrable biological consequence. DNA vaccination of LMP2- and LMP7-deficient mice induced CD8⁺ T cell responses that were slightly lower than, although not significantly different from, those induced in wild-type mice. Taken together, our results challenge the notion that immunoproteasomes are generally needed for effective antiviral CD8⁺ T cell responses and for the shaping of immunodominance hierarchies. We conclude that the immunoproteasome may affect T cell responses to only a limited number of viral epitopes, and we propose that its main biological function may lie elsewhere.


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3 Abbreviations used in this paper: ICCS, intracellular cytokine staining; KO, knockout; LCMV, lymphocytic choriomeningitis virus; NP, nuclear protein; WT, wild type.
of CTL precursors specific for an influenza virus epitope, and 3) reduced presentation of this viral epitope by infected splenocytes (20). The latter finding was not confirmed in a more recent study (22). In LMP7KO mice, normal numbers of B and T cells were reported, but the surface expression of both H-2D\(^{\beta}\) and H-2K\(^{\alpha}\) MHC class I alleles was reduced by 25–45% on all major lymphoid subpopulations (19). Two recent studies of virus infection in LMPKO mice suggested that immunoproteasomes do, indeed, shape in vivo CD8\(^{+}\) T cell responses. The immunodominance hierarchy of CD8\(^{+}\) T cell responses to influenza virus was altered in mice lacking LMP2 (22), and this was attributed to changes in either proteasomal Ag generation or the T cell repertoire. In addition, immunoproteasomes were found to limit the CD8\(^{+}\) T cell response to the gp33 epitope of lymphocytic choriomeningitis virus (LCMV), but this was not observed during normal LCMV infection; it was detected only when the LCMV protein was expressed from a recombinant vaccinia virus (23).

The aim of the present study was to establish whether there is a general influence of immunoproteasomes on the strength and specificity of CD8\(^{+}\) T cell responses virus infection. To this end, we investigated the CD8\(^{+}\) T cell responses against four epitopes from two different LCMV proteins, nuclear protein (NP) and glycoprotein (gp), in the context of LCMV infection. We found that the absence of LMP2 or LMP7 had no substantial effect on overall CD8\(^{+}\) T cell responses to primary or secondary LCMV infection and did not alter the kinetics of virus clearance. Furthermore, the immunodominance hierarchies remained virtually unchanged in LMP-deficient mice; only a slight reduction in the already weak response to the NP\(_{395}\) epitope was observed, and this was limited to LMP2KO mice. We also established that mice lacking LMP2 or LMP7 can mount normal CD8\(^{+}\) T cell responses to DNA vaccines. Our data suggest that immunoproteasomes are not generally required for effective antiviral CD8\(^{+}\) T cell responses. These conclusions differ from those recently reported (23) and lead us to propose that immunoproteasomes may have evolved to generate novel epitopes from the host, rather than microbial, proteome.

Materials and Methods

Mice and CD8\(^{+}\) T cell epitopes

LMP2KO mice were originally generated by van Kaer et al. (20); LMP7KO mice were originally made by Fehling et al. (19). LMP2KO mice on the C57BL/6 background were a gift from Dr. F. V. Chisari (The Scripps Research Institute, La Jolla, CA); Dr. M. Gaczynska (University of Texas, San Antonio, TX); and Dr. L. van Kaer (Vanderbilt University, Nashville, TN). LMP7KO mice on the C57BL/6 background were a gift from Dr. F. V. Chisari (The Scripps Research Institute) and Dr. H. Fehling (University of Ulm, Ulm, Germany). C57BL/6 (H-2\(^{b}\)) mice were obtained from Dr. F. V. Chisari (The Scripps Research Institute) and Dr. L. van Kaer (Vanderbilt University, Nashville, TN). LMP7- and LMP2-deficient mice were originally generated by van Kaer et al. (20); mice from The Jackson Laboratory. The genotypes of both LMPKO strains were maintained in DMEM supplemented with 10% FBS, 20 mM L-glutamine, penicillin G (50 U/L), and streptomycin sulfate (50 \(\mu\)g/L). Liver or spleen samples were weighed, thawed, and homogenized in 0.5 ml of OptiMEM (Invitrogen Life Technologies). The homogenates were then serially diluted in OptiMEM and used to infect Vero cells. After 1-h absorption at 37°C, the supernatant was removed, and the cells were overlaid with DMEM FBS-0.25% Seakem ME agarose (Cambrex). After 5 days of incubation at 37°C, the monolayers were fixed in 25% formaldehyde-PBS and stained with 0.05% crystal violet in PBS containing 20% ethanol.

Infection of mice with LCMV

Primary LCMV infections were conducted by i.p. injecting mice with 2 \(\times\) 10\(^5\) PFU of LCMV (Armstrong strain). In a viral dosage experiment, 2 \(\times\) 10\(^5\) and 1 \(\times\) 10\(^5\) PFU also were used. For secondary LCMV infection of LCMV-immune mice, 10\(^5\) PFU were inoculated i.o.

DNA vaccinations and challenge

DNA vaccinations were conducted as previously described (25). In brief, plasmid DNAs encoding the full-length LCMV NP and gp were mixed and dissolved in 1 N saline at 1 mg/ml, and mice were immunized with 50 \(\mu\)l (50 \(\mu\)g) into the right and left anterior tibial muscles using a 28-gauge needle. Control mice received the same dose of empty plasmid vector. Mice were vaccinated on days 0, 21, and 35 and were bled on days 14, 28, and 42 to permit enumeration of virus-specific CD8\(^{+}\) T cells in the PBMC. Three weeks after the final DNA immunization, the mice were infected with 2 \(\times\) 10\(^5\) PFU of LCMV, and animals were killed 4 days after infection. At this early time point after infection, previously nonimmune mice (e.g., those vaccinated with empty plasmid) will show little or no detectable CD8\(^{+}\) T cell response; in contrast, mice that had virus-specific CD8\(^{+}\) memory T cells at the time of infection (i.e., memory cells induced by the DNA vaccine encoding LCMV NP and gp) will have readily detectable numbers of virus-specific cells.

Flow cytometry and intracellular cytokine staining (ICCS)

Ex vivo ICCS was performed as previously reported (24). In brief, up to 2 \(\times\) 10\(^5\) lymphocytes from blood (tail bleeding) or spleens from LCMV-infected or naive mice were cultured in round-bottom, 96-well plates at 37°C in 5% CO\(_2\) for 5–7 h in the presence or the absence of peptide in RPMI 1640 (containing 5–10% FBS, 50 \(\mu\)M 2-ME, t-glutamine, and antibiotics). The peptides used represent dominant and subdominant T cell epitopes from LCMV; NP\(_{396–404}\); gp33–41, gp276–286 (all restricted by H-2\(^{D}\)), and NP\(_{205–212}\) (H-2\(^{K}\)), at a final concentration of 0.5–1 \(\mu\)g/ml. Brefeldin A (Sigma-Aldrich) was added at a final concentration of 5 \(\mu\)g/ml. For ICCS, cells were washed and permeabilized with the Cytofix-Cytoperm kit (BD Pharmingen), according to the manufacturer’s directions. Samples were resuspended in PBS containing 2% formaldehyde, acquired on a FACScan or FACScalibur flow cytometer (50,000–100,000 events acquired/sample) and analyzed with CellQuest software (BD Biosciences).

Antibodies

Abs were purchased from CalTag Laboratories (anti-CD8-Tricolor, anti-IFN-\(\gamma\)-FITC, and anti-CD62L-PE), BD Pharmingen (anti-CD44-PE, anti-H-2D\(^{\beta}\)-PE (clone KB95), and anti-H-2K\(^{\alpha}\)-PE (clone AF6-88.5)), and eBioscience (anti-MHC class I-PE (recognizes H-2\(^{D}\) and non-H-2\(^{D}\) determinants) and anti-MHC class II-PE (recognizes I-A\(^{b}\) and non-H-2\(^{b}\) determinants).
a reduced antiviral CD8+ T cell response to influenza virus infection (20, 22). LMP7KO mice, in contrast, have been shown to have normal cell numbers, in normal relative proportions, but reduced surface expression of MHC I molecules (19). We considered it important to reassess these factors in the LMP-deficient mice on the B6 background, which were used in the present study. To this end, the relative proportions and total numbers of five different immune cell types were evaluated in the spleens of these mice. Contrary to our expectations, we found that the percentages of the various cell types, including CD8+ T cells, in naïve LMP2KO mice were not statistically different from those in wild-type animals (Fig. 1A). However, the total number of each of the five splenic cell types measured was markedly reduced in LMP2KO mice (Fig. 1B); consequently, naïve LMP2KO mice have ~50% fewer CD8+ T cells (and other cell types) than normal animals. In contrast, both the proportions and total numbers of these five cell types in LMP7KO mice were indistinguishable from those observed in WT animals.

**Normal kinetics of LCMV clearance in LMP2KO and LMP7KO mice**

Our results, showing markedly reduced numbers of CD8+ T cells in naïve LMP2KO mice, suggested that they might be limited in their ability to eradicate LCMV, the clearance of which is known to be almost entirely dependent on CD8+ T cells. Groups of naïve LMP2KO and LMP7KO mice and WT B6 mice were infected with 2 × 10^5 PFU of LCMV, and three mice from each group were killed daily between days 4 and 8 after infection. Virus titers in the spleen were determined, and as shown in Fig. 2, both LMP-deficient strains cleared the virus with kinetics very similar to those observed in WT mice. By 8 days after infection, virus was undetectable. Similar conclusions were drawn from virus titrations of livers of the same animals (not shown), suggesting that CD8+ T cells in LMP-deficient mice can exert their effects in peripheral tissues. Therefore, even in the LMP2KO mice, which had fewer naïve CD8+ T cells, LCMV is cleared with the same kinetics and efficacy as in WT mice. We conclude that neither LMP2 nor LMP7 is needed for efficient LCMV clearance. These results confirm the recent findings of another group (23).

**Efficient CD8+ T cell responses and normal immunodominance pattern after LCMV infection of LMP2KO or LMP7KO mice**

The demonstration that LMP-deficient mice could control LCMV infection did not allow us to conclude that CD8+ T cell responses in these mice were unimpaired. Indeed, earlier results have suggested that LMP deficiency leads to markedly weaker CD8+ T cell responses to virus infection (LMP2KO) (20, 22) and to self Ag (LMP7KO) (19). To test CD8+ T cell responses in the LCMV model, we infected WT and LMP-deficient mice with LCMV and evaluated the responses to four epitopes from two LCMV proteins, gp and NP. Each protein contains one dominant epitope (gp33 and NP396) and one subdominant (gp276 and NP205). Eight days after infection, at the peak of the CD8+ T cell response, total CD8+ T cell responses and their specificity for each of the four epitopes were enumerated by ICCS. The numbers of CD8+ T cells (epitope-specific and total) are shown in Fig. 3A, and the strength of each epitope-specific primary response is presented as a percentage of all CD8+ T cells (Fig. 3B). The immunodominance hierarchy induced by primary LCMV infection (NP396 > gp33 > gp276 > NP205) was very well maintained in LMP-deficient mice, indicating that neither LMP2 nor LMP7 is crucial for shaping immunodominance hierarchies in LCMV infection. Furthermore, the absolute numbers of epitope-specific CD8+ T cells at 8 days after infection were very similar in WT and LMP7KO mice, as were the relative percentages of the four epitope-specific populations. In contrast, cell numbers in all four epitope-specific populations were reduced in LMP2KO mice. There was an ~3-fold drop for the two dominant populations (NP396 and gp33), but a greater reduction (8- to 10-fold) in the subdominant populations (gp276 and NP205). The NP205-specific response was particularly weak and was more clearly seen when the relative abundance of NP205-specific cells was compared with each of the other three epitope-specific populations (Fig. 3B). In summary, LMP-deficient mice can mount strong NP- and gp-specific primary CD8+ T cell responses to...
LCMV infection, and the reduced subdominant responses in LMP2KO mice appears to have little biological effect, because these mice controlled LCMV infection normally (Fig. 2).

**Secondary CD8⁺ T cell response to LCMV infection is similar in WT and LMPKO mice**

The relative abundances of different epitope-specific populations can change after secondary virus infection (26, 27); several explanations have been proposed, including differential Ag presentation, cross-presentation, and immunodomination (23, 28, 29). In this study we asked whether immunoproteasome deficiency might contribute to the phenomenon. LCMV-immunized mice (WT, LMP2KO, and LMP7KO; used at least 6 wk after primary LCMV infection; WT and LMP7KO mice were reduced by only ~4-fold in LMP2KO mice, which is in parallel with the reduced primary response in the LMP2KO strain; thus, memory T cell expansion as a whole appears to proceed normally in LMP-deficient mice. Compared with primary LCMV infection (Fig. 3B), the immunodominance pattern in reinfected WT mice was skewed in favor of gp276-specific CD8⁺ T cells (Fig. 3D); the response to gp276 was at least as strong as the response to gp33. This change in the immunodominance hierarchy also was seen in LMP7KO mice and, albeit to a lesser extent, in LMP2KO mice. The apparent decrease in the NP₃₉₆-specific memory response in LMP2KO mice is not statistically significant compared with the responses in WT or LMP7KO mice, in which this response was rather variable. Taken together, the data indicate that neither LMP7 nor LMP2 is responsible for controlling the expansion of CD8⁺ memory T cells or for setting the CD8⁺ T cell immunodominance hierarchy during primary or secondary LCMV infection. These findings are in contrast with a recent report that concluded that the CD8⁺ T cell response to the gp276 epitope was selectively suppressed by the immunoproteasome (23).

**Effect of reduced infectious dose of LCMV on the immunodominance pattern of CD8⁺ T cell responses**

It was recently suggested that the large Ag load during LCMV infection might obscure subtle differences in immunodominance between WT and LMPKO mice (23). Accordingly, if the immunoproteasome subunits LMP2 or LMP7 were especially important under conditions of limiting Ag load, one might expect to detect differences between WT and LMP-KO mice after infection with low doses of LCMV. This issue was addressed by infecting mice with three different doses of LCMV, which we characterized as high (2 × 10⁵ PFU), medium (2 × 10⁴ PFU), and low (1 × 10² PFU). Eight days after infection, the CD8⁺ T cell response and LCMV titers were assessed. The immunodominance hierarchy to primary infection was well maintained at all doses (Fig. 4). Both WT and LMPKO mice showed strong CD8⁺ T cell responses to all doses of LCMV, although the total number of CD8⁺ cells in LMP2KO mice was generally lower, consistent with their lower starting levels. Within each mouse strain, a 2000-fold reduction in virus dose (high vs low doses) had only a minor effect on the number of responding cells; WT and LMP7KO mice showed an ~2-fold reduction in the numbers of cells responding to the low
dose, and LMP2KO mice showed a reduction of ~3-fold. This relatively moderate decrease in response, despite a dramatic change in Ag challenge, is consistent with the in vitro observation that, once triggered, the antiviral CD8+ T cell response proceeds in a preprogrammed manner (30–32). In all mice, virus was undetectable in the spleen, and liver (not shown), indicating that the observed CD8+ T cell responses, even if slightly reduced, were sufficient to effect virus clearance by 8 days after infection. Statistical comparisons of the epitope-specific responses mounted by each of the mouse strains to each of the virus doses indicated that only the responses to the NP205 epitope in LMP2KO mice were significantly different; this is similar to the observation reported in Fig. 3. In summary, infection with reduced doses of LCMV still leads to efficient CD8+ T cell responses in LMP-deficient mice and to retention of the primary immunodominance hierarchy. Even though the LCMV-specific response is reduced in LMP2-deficient mice, virus is still efficiently cleared. This argues against a general role of LMP2 or LMP7 for the efficient generation of CD8+ T cell epitopes in low dose virus infections. Moreover, we did not detect significant virus dose-dependent changes in the immunodominance pattern of CD8+ T cell responses.

**LMP2KO and LMP7KO mice mount CD8+ T cell responses to DNA vaccines**

Even a low dose LCMV infection could lead to a relatively high Ag load; therefore, to better test whether immunoproteasomes exert stronger effects at low Ag load, we investigated the CD8+ T cell response in LMP2KO and LMP7KO mice inoculated with DNA vaccines, which have been shown to effectively prime mice and to protect against subsequent LCMV infection (25, 27). In contrast to virus infection, DNA vaccines trigger only mild local inflammation and represent a limited, nonreplicating source of Ag (33). Mice were immunized three times with a DNA vaccine comprised of a mixture of two plasmids, one encoding LCMV NP and the other encoding LCMV gp. Two weeks after the first DNA inoculation, and 1 wk after each boost, mice were bled, and their CD8+ T cell responses were determined. DNA vaccines are, in general, much less potent immunogens than virus infections, and responses against the subdominant epitopes were very low in all mouse strains. Thus, in this study we report CD8+ T cell responses only against the dominant (NP205) epitope. As shown in Fig. 5A, NP205-specific responses were detected in all mouse strains after a single inoculation of DNA, and the responses were increased after each of the two subsequent boosts. These data indicate that, even in the absence of authentic immunoproteasomes, mice can mount epitope-specific responses to DNA vaccines. However, the NP396-specific responses in both the LMP2KO and LMP7KO mice were lower than those in WT mice and, although the differences were not statistically significant, we cannot exclude the possibility that immunoproteasomes contribute to the robustness of DNA vaccine-induced T cell responses.

We have previously shown that DNA vaccination can substantially alter the immunodominance hierarchy to subsequent virus infection, that is, can cause ‘original T cell antigenic sin’ (27). To determine the role (if any) of the immunoproteasome in this phenomenon, 4 wk after the third DNA vaccination, the mice were infected with LCMV; 4 days later, CD8+ T cell responses were measured. The relative abundances of the four epitope-specific CD8+ T cell populations after primary LCMV infection of these DNA-immunized mice are shown in Fig. 5B and differ somewhat from those observed after secondary virus infection of LCMV-immunized mice (Fig. 3D). The increased secondary response to gp276 (Fig. 3D) does not occur after infection of DNA-vaccinated WT mice, and the responses to NP205 are almost undetectable. The gp276-specific response after LCMV infection of DNA-vaccinated mice is slightly higher in LMPKO mice than in WT mice, consistent with a higher frequency of DNA vaccine-induced, gp276-specific memory cells in the absence of the immunoproteasome; however, this difference was not detectable before infection. In summary, naive CD8+ T cells in LMP2KO and LMP7KO mice are efficiently primed by DNA vaccines encoding full-length LCMV proteins. Therefore, both LMP2 and LMP7 are dispensable for the generation of LCMV epitopes from DNA vaccines, suggesting that these immunoproteasome subunits are not crucial for the processing of the tested epitopes under circumstances of low Ag load.

The lower NP205-specific CD8+ T cell response in LMP2KO mice is not attributable to reduced expression of the relevant MHC class I molecule

Our findings indicate that LMP deficiency leads to little, if any, significant change in the LCMV-specific CD8+ T cell response. However, we observed somewhat reduced primary (Fig. 3, A and B, and Fig. 4) and memory (Fig. 3, C and D) responses to the NP205 epitope in LMP2KO mice, and we have attempted to determine the underlying cause of this apparent deficit. Of the four epitopes tested, three are presented by H-2Db, and only NP205 is presented by H-2Kb. It was, therefore, possible that reduced surface expression of Kb in LMP2KO mice was responsible for the observation, although surface expression of MHC class I molecules had been reported to be normal in naive LMP2KO mice (20). As shown in Fig. 6A, we confirmed that Kb expression on naive B cells, CD8+ and CD4+ T cells, and dendritic cells from LMP2KO mice was indistinguishable from that on naive cells from WT mice; furthermore, we compared Kb expression levels 8 days after primary infection and 4 days after secondary challenge and observed similar increases in both mouse strains. Kb expression was reduced by ~50% in LMP7KO mice (19) (data not shown), although this had no apparent negative effect on the primary or secondary NP205-specific response (Figs. 3 and 4); furthermore, expressions of the Dd class I allele and of MHC class II molecules were at WT levels in naive LMP2KO and LMP7KO mice and at
Discussion

In this study we report that LCMV-specific CD8⁺ T cell responses in LMP2KO and LMP7KO mice differ only marginally from those observed in WT mice. Despite lacking authentic immunoproteasomes, the two KO strains 1) eradicate the infection with normal kinetics (Fig. 2); 2) mount strong CD8⁺ T cell responses to LCMV NP and gp epitopes (Fig. 3); 3) can respond strongly over a 2000-fold range of virus doses (Fig. 4); 4) have largely normal immunodominance hierarchies in response to primary or secondary infection, indicating that differential Ag processing between constitutive and immunoproteasomes does not set the immunodominance hierarchy in LCMV infection (Fig. 3); and 5) respond to DNA vaccination (Fig. 5). Thus, as measured by these key biological parameters, immunoproteasomes are not generally needed to induce efficient CD8⁺ T cell responses to virus infection. We observed only one striking difference among the mouse strains; naïve LMP2KO mice had lower absolute numbers of all cell types in the spleen (Fig. 1). Consistent with this, after infection, these mice had lower numbers of all epitope-specific CD8⁺ T cell populations compared with WT and LMP7KO mice (Fig. 3). Although this reduction in T cell numbers had no demonstrable effect on LCMV clearance, it is possible that LMP2KO mice might show increased susceptibility to other infections. Another, more subtle, change was observed in LMP2KO mice; these animals showed a slight drop in the NP205-specific CD8⁺ T cell response (Fig. 3). This does not seem to be attributable to a reduction in K⁺ expression or to a “hole” in the T cell repertoire (Fig. 6), and it may be related to a change in epitope processing. Taken together, however, our data support the idea that immunoproteasomes affect antiviral CD8⁺ T cell priming only rarely, and that any effects are highly epitope specific (sequence specific).

Our results can be compared with those presented in a recent intriguing study of LCMV-specific responses in LMP-deficient mice (23); some of our conclusions differ, whereas others are similar. First, it was concluded that the immunoproteasome shaped the epitope hierarchy, because it preferentially degraded the LCMV gp276 epitope. However, an apparent enhancement of the gp276-specific response was observed only in mice infected with a recombinant vaccinia virus expressing the LCMV gp; no such difference was observed during LCMV infection, as we confirm in this study. Thus, we conclude that the immunoproteasome does not regulate the position of the gp276 epitope in the hierarchy during normal LCMV infection. We did, however, observe an increase in the gp276-specific response after LCMV infection of DNA-vaccinated LMPKO mice. Although this could reflect the increased presentation of this epitope after DNA vaccination of LMPKO mice, we were unable to convincingly demonstrate an increase in DNA vaccine-induced gp276-specific T cells in LMPKO mice before infection. In addition, theoretical considerations suggest that immunoproteasomes are unlikely to exert a strong negative impact on epitope presentation. There is little evidence that immunoproteasomes completely replace their constitutive counterparts within a cell. Rather, the two forms of proteasome coexist in a single cell. Such coexistence is likely to limit the negative biological effects of immunoproteasomes, because an epitope that is destroyed or not produced by immunoproteasomes should still be generated within a cell that contains both forms of proteasome. Thus, although other negative effects of immunoproteasome expression have been reported (16), the intracellular coexistence of constitutive proteasomes means that immunoproteasomes appear more likely to increase the epitope universe than to decrease it. Second, the apparent effect using recombinant vaccinia virus led the authors to propose that the enhancement in LMPKO mice was detectable only under conditions of relatively low Ag load. We have been unable to confirm an Ag dosage effect, because we see no enhancement of the gp276-specific response in LMPKO mice even at a very low LCMV dose (Fig. 4), nor can we exclude such an effect, because LMPKO mice mounted lower (albeit not statistically different) responses to DNA vaccination (Fig. 5). Third, the authors proposed that skewing the CD8⁺ T cell response in favor of gp276-specific responses, as seen in chronic LCMV infection (34, 35), could be due to the down-regulation of immunoproteasomes in chronic infection, leading to the enhanced generation of gp276
by constitutive proteasomes. Our results suggest an alternative explanation. We show that the gp276-specific CD8+ T cell population becomes more dominant during a normal recall response (e.g., secondary LCMV infection), and that this phenomenon is present in both WT and LMP-deficient mice. Thus, dynamic changes in the immunodominance hierarchies of LCMV-specific CD8+ T cell responses can occur independently of immunoproteasomes and may be a consequence of T cell expansion and immunodominance, as has been suggested for influenza virus infection (28).

Fourth, the authors concluded that the LMP proteins could determine the dominance hierarchy during virus infection, whereas we conclude that immunoproteasomes play little, if any, role in regulating the character or intensity of the CD8+ T cell response to normal LCMV infection.

Authentic immunoproteasomes cannot be assembled in the absence of either LMP2 or LMP7, and cooperativity favors the assembly of homogeneous proteasomes, in which individual proteasomes contain either constitutive or immunosubunits, and not both (36). As a result, one might predict that the two strains of LMPKO mice would be indistinguishable in terms of Ag presentation and CD8+ T cell response. Why, then, do the two mouse strains differ phenotypically, both in their naive state and, albeit subtly, in their responses to infection? The replacement of the constitutive subunit $\beta_1\beta_6$ by LMP2 has been shown to markedly alter the related proteolytic binding pocket and thereby to abrogate cleavage after acidic amino acids (8, 10); in contrast, the replacement of $\beta_5$ by LMP7 is predicted to have little effect on its proteolytic binding pocket (37). These data suggest that LMP2 deficiency might have more marked consequences than loss of LMP7, consistent with our observation that NP$^{305\gamma}$-specific responses are slightly reduced in LMP2KO, but not in LMP7KO, mice.

Did immunoproteasomes evolve at least in part to optimize CD8+ T cell responses to viruses? For reasons outlined previously, this idea is persuasive. However, our data suggest that some antiviral CD8+ T cell responses may be independent of immunoproteasomes, and other considerations cause us to question the concept. First, immunoproteasomes are not generated from existing constitutive complexes by direct exchange of the three catalytic subunits. Rather, they are generated de novo, being assembled within cells that have been triggered by IFN-$\gamma$ to produce LMP2, LMP7, and MECL-1. During microbial infection, this replacement of constitutive proteasomes by immunoproteasomes is a gradual process, taking up to 7 days to reach a plateau (11). This time frame is not consistent with the observation that memory CD8+ T cells can very rapidly eradicate viruses from central and peripheral tissues. Second, although the alteration in cleavage specificity in immunoproteasomes is well established, it also is quite subtle; there is extensive overlap between the peptide sets that are generated by constitutive and immunoproteasomes. Detailed studies of the cleavage preferences of constitutive proteasomes and immunoproteasomes revealed that roughly 50% of all cleavage sites and at least 25–35% of generated peptides are shared (9, 10). These numbers might actually underestimate the similarity of the potential MHC class I ligand pool generated by constitutive and immunoproteasomes for the following reason. Despite several differences in cleavage specificity, the constitutive and immunoproteasomes share one key feature; both forms show a pronounced preference to cleave proteins after large hydrophobic amino acids (9, 10), which, therefore, form the C termini of the resulting peptides, and peptides with such residues at their C termini are favored for stable binding to MHC class I molecules (38). Thus, both constitutive and immunoproteasomes are similarly equipped to generate the C termini of potential MHC class I ligands (3, 4, 39). Our data are consistent with the idea that when considering only the C termini of potential MHC class I ligands, similar processing might be the rule, and differential processing the exception, but this awaits future studies on a larger number of epitopes.

This quite marked functional overlap between constitutive and immunoproteasomes implies that when the target proteome is small, so, too, is the likelihood of there being a dramatic difference between the MHC ligand pools generated by the two forms of proteasome. This may explain the very similar CD8+ T cell responses reported in the present study; virus proteomes are limited in size. The potential differences between the ligand pools generated by constitutive and immunoproteasomes will increase along with proteome size. We propose that the primary purpose of the immunoproteasome is not to process microbial epitopes, but is, instead, to generate additional epitopes from the much larger host proteome; consistent with this idea, others have shown that a large proportion of aging female LMP2KO mice develop uterine tumors, suggesting that immunoproteasomes may play a part in tumor surveillance, presumably by generating tumor-specific epitopes from the host proteome (40). During most virus infections, host cells will be exposed to IFN-$\gamma$, and the expression of new, immunoproteasome-generated, host-derived epitopes on APCs would trigger the induction of self-specific CD8+ T cells. These cells could aid in combating acute virus infection by recognizing virus-infected cells that began to express the new, IFN-$\gamma$-dependent, self epitopes. Furthermore, after the infection had been cleared, some of these cells could enter the memory phase and might provide bystander memory to a variety of viruses (because many different virus infections would drive the appearance of the same population of self epitopes); in this way, the immunoproteasome would drive the generation of CD8+ memory T cells that were not virus specific, but that were, instead, inflammation/infection specific. This novel explanation of immunoproteasome function provides a new perspective on previous observations. For example, it is known that infection with one virus (LCMV) can confer a substantial degree of protection against infection by a second, unrelated, virus (vaccinia virus) (41); this protection was attributed to T cells with cross-reactive TCRs capable of recognizing epitopes common to both viruses, but our hypothesis provides an alternative explanation. Our hypothesis also suggests a novel mechanism by which an autoimmune disease might be exacerbated by infection by any one of several unrelated viruses; this has been reported for multiple sclerosis (42), a disease that is worsened by the administration of IFN-$\gamma$ (43). Others also have noted that the immunoproteasome may lead to the expression of novel self epitopes, but proposed that the immunoproteasome evolved to prevent autoimmunity, because the resulting autoreactive T cells would be unable to attack uninflamed tissues (44). To our knowledge, however, LMP-deficient mice do not show an increased susceptibility to autoimmune disease. Both of the above hypotheses require that the potentially autoreactive CD8+ T cells be present in the periphery, and this, in turn, would require that they not be deleted through thymic negative selection. This issue remains open, but some LMP mRNAs are expressed in the thymus (45), and a recent report indicates that immunoproteasomes are preferentially expressed in regions of the thymus that are involved in negative selection (46). In conclusion, we show that the influence of immunoproteasomes on antiviral CD8+ T cell responses is limited and epitope specific, rather than general, and we propose a novel explanation for the evolutionary forces that have driven the development of this poorly understood response to microbial infection.

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