Proteasomes shape the repertoire of T cells participating in antigen-specific immune responses

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Differences in the cleavage specificities of constitutive proteasomes and immunoproteasomes significantly affect the generation of MHC class I ligands and therefore the activation of CD8-positive T cells. Based on these findings, we investigated whether proteasomal specificity also influences CD8-positive T cells during thymic selection by peptides derived from self proteins. We find that one of the self peptides responsible for positive selection of ovalbumin-specific OT-1 T cells, which is derived from the f-actin capping protein (Cpα1), is efficiently generated only by immunoproteasomes. Furthermore, OT-1 mice backcrossed onto low molecular mass protein 7 (LMP7)-deficient mice show a 50% reduction of OT-1 cells. This deficiency is also observed after transfer of BM from OT-1 mice in LMP7-deficient mice and can be corrected by the injection of the Cpα1 peptide. Interestingly, WT and LMP7-deficient mice mount comparable immune responses to the ovalbumin-derived epitope SIINFEKL. However, their cytotoxic T lymphocytes (CTL) differ in the use of T cell receptor Vβ genes. CTL derived from WT mice use Vβ8 or Vβ5 (the latter is also used by OT-1 cells), whereas SIINFEKL-specific CTL from LMP7-deficient mice are exclusively Vβ8-positive. Taken together, our experiments provide strong evidence that proteasomal specificity shapes the repertoire of T cells participating in antigen-specific immune responses.

selection | T cell repertoire

Cytotoxic T lymphocytes (CTL) recognize a complex of MHC class I molecules and peptides derived mainly from intracellular proteins. The generation of these peptides crucially depends on the activity of proteasomes, which represent the main proteolytic activity present in the cytoplasm and are responsible for the generation of the C termini of most peptides presented by MHC class I molecules (1–5). The 20S proteasome represents the proteolytic core complex, which is composed of seven different α- and seven different β-subunits organized in a complex of four stacked rings with $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry (6–8). The specificity of this protease is influenced by the incorporation of three IFN-inducible β-subunits (β1i, β2i, and β5i), which replace the three proteolytically active constitutive subunits (β1, β2, and β5) during proteasome assembly, resulting in the formation of so-called immunoproteasomes (9, 10). This type of proteasome generates reduced numbers of peptides with acid C termini and increased numbers of peptides with hydrophobic C termini (11, 12), which is in favor of a more efficient transporter associated with antigen-presentation transport and binding to MHC class I molecules (13). In addition, a change in proteolytic specificity is apparent from the numerous examples reporting that either constitutive proteasomes or immunoproteasomes are required for the efficient generation of certain MHC class I ligands and consequently for the activation of CTL (14–19). As a result, proteasomal specificities provide a major contribution to the hierarchies of epitopes recognized by pathogen-specific CTL and therefore to immunodominance (20).

However, T cells make their first contact with MHC–peptide complexes not during activation in the periphery but during their development in the thymus. Here, peptides derived from self proteins are presented by MHC class I molecules and are involved in positive and negative selection processes leading to the development of CD8-single positive T cells, which then enter the pool of peripheral T cells (21). The nature of peptides able to induce positive selection of CD8-positive T cells was enigmatic until 1997. At that time, Hogquist *et al.* (22) were able to identify the first naturally occurring peptide involved in thymic positive selection, followed by other peptides discovered 5 years later (23). Because proteasomes will also be involved in the generation of peptides recognized during CD8-positive T cell development, their specificity can be expected to influence T cell selection and therefore the repertoire of T cells in the periphery. The first evidence for this hypothesis was obtained in a study by Chen *et al.* (20), where it was shown that CTL of LMP2$^{-/-}$ mice display a defect in the recognition of a peptide derived from influenza nucleoprotein due to alterations in the CD8-positive T cell repertoire. In support of this, Nil *et al.* (24) reported recently that P14 T cells, which express a transgenic (tg) TCR specific for an H2-D^b-restricted lymphocytic choriomeningitis virus epitope, show an altered selection in the thymus of LMP2$^{-/-}$ mice as compared with WT.

To address the issue of proteasomal influence on the development of the CD8-positive T cell repertoire in more detail at the level of self-peptide generation, we analyzed the development of OT-1 T cells specific for an ovalbumin-derived peptide presented by H2-K^b molecules in WT and LMP7$^{-/-}$ mice (25). The latter, similar to LMP2$^{-/-}$ mice, display a deficiency in the formation of immunoproteasomes and show a defect in the production of immunoproteasome-dependent CTL epitopes. Because self peptides responsible for positive selection of OT-1 T cells are known, we determined whether immunoproteasomes are required for their generation and whether a proteasomal defect would influence the development of OT-1 T cells and in addition the repertoire of ovalbumin-specific CTL in WT mice.

Results

***In Vitro* Generation of the f-Actin Capping Protein (Cpα1) Peptide Responsible for Positive Selection of the OT-1 TCR Is Strictly Immunoproteasome-Dependent.** We first determined the need for immunoproteasomal activity in the generation of the peptide Cp$\alpha1_{92–99}$, one of the peptides that contribute to positive selection of OT-1 T cells (22). The use of proteasomal prediction programs (13) suggested that the generation of the Cp$\alpha1_{92–99}$ C

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Abbreviations: TCR, T cell receptor; CTL, cytotoxic T lymphocyte; tg, transgenic; BM, bone marrow; BMT, BM transfer; Cpα1, f-actin capping protein.

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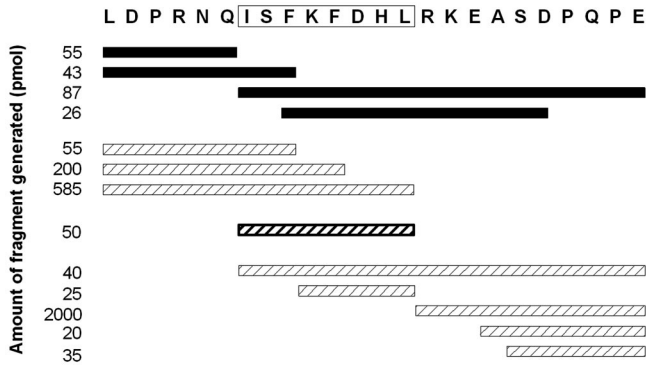

Fig. 1. The peptide responsible for positive selection of the OT-1 TCR can be efficiently generated only by immunoproteasomes. Ten nanomolar (μg) of LDPRNQISFKFDHLRKEASDPQPE (Cpα1$_{86-109}$) was incubated for 6 h with 2 μg of purified constitutive proteasome (filled bars) or immunoproteasome (hatched bars), respectively. Replacement of the β5 subunit by β5i in the immunoproteasome preparation was 90%, as determined by Western blot analysis and MS (Fig. 5, which is published as supporting information on the PNAS web site). Fragments were separated via reversed-phase HPLC and fractions analyzed by Edman degradation and MALDI MS. Amounts of fragments generated by constitutive proteasomes (filled) or immunoproteasomes (hatched) >20 picomol are shown. The Cpα1$_{92-99}$ peptide (ISFKFDHL) is marked in bold.

terminus will be much more efficiently performed by immunoproteasomes (data not shown). This prediction was confirmed by the *in vitro* digestion of a N- and C-terminally extended Cpα1 peptide (Cpα1$_{86-109}$) using purified constitutive proteasomes and immunoproteasomes followed by MS analysis and Edman sequencing for quantification of the fragments generated. Indeed, immunoproteasomes generated the C terminus of Cpα1$_{92-99}$ ≈1,000-fold more efficiently than constitutive proteasomes (data not shown) and were able to directly produce the Cpα1$_{92-99}$ epitope (Fig. 1).

Mice Lacking the *LMP7* Gene Display a Prominent Reduction of OT-1 T Cells in the Thymus and the Periphery. Inspired by the clear outcome of the *in vitro* peptide digest, we crossed OT-1 mice onto the LMP7$^{-/-}$ background and analyzed the numbers of CD8-positive T cells expressing a Vα2/Vβ5 TCR, which is used by OT-1 T cells. We observed that OT-1/LMP7$^{-/-}$ mice have ≈30% reduced numbers of CD8-single positive T cells in the thymus (Fig. 2*A*). Comparing CD8-positive T cells among the Vα2- or Vβ5-expressing T cells, we observed a reduction of ≈50% in OT-1/LMP7$^{-/-}$ versus OT-1/LMP7 WT mice. A similar situation was seen in the periphery. OT-1/LMP7$^{-/-}$ mice have 40% reduced numbers of CD8/Vα2-positive T cells in the blood, whereas the number of CD4-positive T cells is identical in both mouse strains (Fig. 2*B*). That CD4 T cell development is not altered in the LMP7$^{-/-}$ background supports the hypothesis that the observed difference in OT-1 T cell selection might be due to a suboptimal processing and presentation of peptides like the one derived from Cp1α by MHC class I on the selecting thymus epithelial cells due to the lack of functional immunoproteasomes. To exclude the possibility that potential differences in the genetic background of OT-1/LMP7$^{-/-}$ and OT-1/LMP7 WT mice contribute to the observed differences in the selection of CD8/Vα2/Vβ5-positive T cells, we transferred bone marrow (BM) of OT-1 mice into LMP7$^{-/-}$ and WT mice and analyzed the development of OT-1 T cells. We basically obtained the same picture. As shown in Fig. 3*A*, ≈50% more tg OT-1 T cells can be found in the blood of WT mice compared with LMP7$^{-/-}$ mice 4 weeks after BM transfer (BMT). This was not observed when we transferred control BM from St42 mice (26),

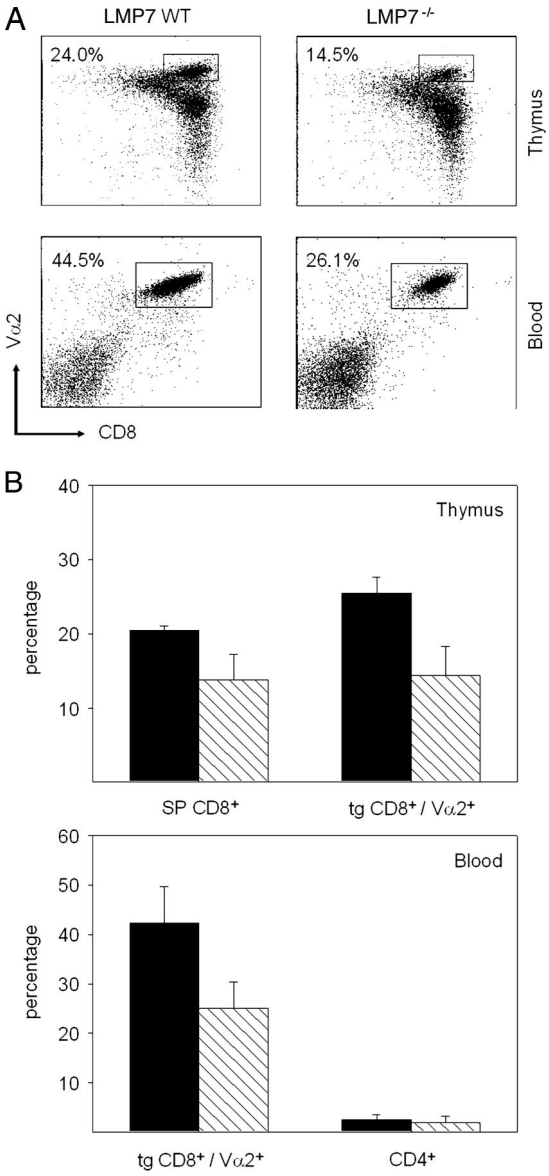

Fig. 2. OT-1 mice crossed onto the LMP7$^{-/-}$ background show a 40% reduction of tg CD8$^+$/Vα2$^+$ T cells. (*A*) Thymocytes (*Upper*) or blood lymphocytes (*Lower*) were analyzed with regard to proportion of tg CD8$^+$/Vα2$^+$ T cells. Thymus data are representative of two experiments with four mice each; blood data are representative of 20 mice per mouse strain. (*B*) Graphical depiction of single positive (sp) CD8$^+$ and tg CD8$^+$/Vα2$^+$ thymocytes and tg CD8$^+$/Vα2$^+$ as well as CD4$^+$ T cells in the periphery. Filled bars represent WT and hatched bars LMP7$^{-/-}$ mice. Error bars represent standard deviation of triplicates from one of two independent experiments.

which give rise to TCR-tg T cells specific for an adenoviral peptide (Fig. 3*A*). These experiments exclude potential genetic differences being responsible for the observed differences between OT-1/LMP7$^{-/-}$ and OT-1/LMP7 WT mice and demonstrate that LMP7$^{-/-}$ mice have no general defect in the development of CD8-positive T cells. This is also supported by the fact that LMP7$^{-/-}$ and WT mice show no differences in the numbers of CD8-positive T cells in the thymus and the periphery (data not shown). We also did not observe differences in the homeostatic proliferation of OT-1 T cells adoptively transferred into irradiated LMP7$^{-/-}$ and WT mice, which suggests there is no difference between LMP7$^{-/-}$ and WT mice in maintaining OT-1 T cells in the periphery (data not shown).

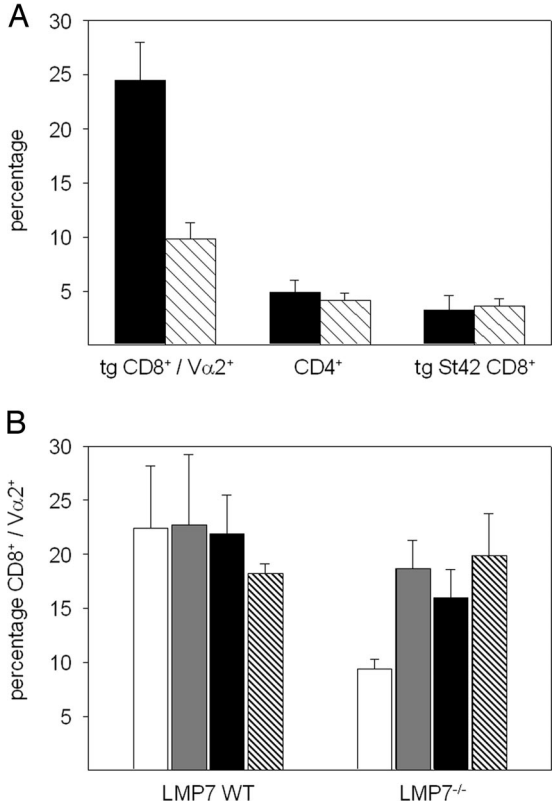

Fig. 3. Selection of OT-1 T cells after OT-1 BMT into LMP7$^{-/-}$ recipients can be restored by injection of Cpα1. (*A*) T cell-depleted OT-1 bone-marrow cells (1.5×10^7) were injected into lethally (8.5 Gy) irradiated WT (filled bars) or LMP7$^{-/-}$ hosts (hatched bars), and tg CD8$^+$ numbers as well as CD4$^+$ lymphocytes were monitored in the blood 4 weeks after BMT. BM cells (7.5×10^6) of CD8$^+$ TCR tg St42 mice were injected for control reconstitution. (*B*) Four weeks after OT-1 BMT, Cpα1 peptide (ISFKFDHL) was i.v.-injected three times on 3 consecutive days into OT-1 BM-reconstituted WT or LMP7$^{-/-}$ mice, and tg CD8$^+$ numbers in the blood were monitored before (open) and on days 14 (gray), 65 (filled), and 180 (hatched) after the last peptide injection. Error bars represent standard deviation of triplicates from one of three independent experiments.

Deficiency in Selection of OT-1 T Cells After OT-1 BMT into LMP7$^{-/-}$ Recipients Can Be Corrected by Injection of the Cpα1 Peptide. To support the hypothesis that a defect in the immunoproteasome-mediated generation of positive-selecting peptides like Cp$\alpha1_{92-99}$ is responsible for the impaired development of OT-1 T cells, we i.v.-injected this peptide into LMP7$^{-/-}$ and WT mice after they had received OT-1 BM. The i.v. injection of peptide variants of viral CTL epitopes had previously been reported to mediate the positive selection and emigration of antigen-specific thymocytes *in vivo* (27). Before and after three consecutive injections of the Cp$\alpha1_{92-99}$ peptide, we analyzed the numbers of CD8/Vα2-positive T cells in the blood of OT-1 BM-transferred LMP7$^{-/-}$ and WT mice at days 0, 14, 65, and 180. As shown in Fig. 3*B*, peptide injection doubles the number of CD8/Vα2-positive T cells in LMP7$^{-/-}$ mice. This effect is visible at day 14 and remains constant until day 180. Peptide injection had no effect in WT mice. This experiment strongly supports that indeed a immunoproteasomal defect in the processing and presentation of self peptides involved in positive selection is responsible for the impaired development of OT-1 T cells in LMP7$^{-/-}$ mice.

TCR Vβ Usage of SIINFEKL-Specific CD8+ T Cells Differs in LMP7 WT and LMP7$^{-/-}$ Mice. Next, we wondered whether we would be able to detect a proteasomal influence not only on the development of tg T cells but also on a naive T cell repertoire. A TCR Vβ analysis of CD8-positive T cells from naive LMP7$^{-/-}$ and WT mice did not reveal any differences (Fig. 4*A*). We immunized LMP7$^{-/-}$ and WT mice with tg *Listeria monocytogenes*, which contains the ovalbumin gene and has been described to induce a strong SIINFEKL-specific CTL response. Both LMP7$^{-/-}$ and WT mice were able to control the *L. monocytogenes* infection, judged by assessing viable bacteria on day 3 of primary infection in the liver ($4.03 \times 10^6 \pm 1.38 \times 10^6$ in LMP7$^{-/-}$ mice and $4.17 \times 10^6 \pm 6.24 \times 10^5$ in WT mice). Thirty days after primary infection, animals were rechallenged with a 100-fold higher dose of tg *L. monocytogenes* and recall responses of CD8-positive T cells directed against SIINFEKL as well as responses of CD4-positive T cells directed against the immunodominant MHC-II epitope $LLO_{190-201}$ investigated. Intracellular cytokine staining revealed that comparable amounts of both IFN-γ- and TNF-α-producing CD8$^+$ and CD4$^+$ T cell populations were detectable upon *in vitro* restimulation of both LMP7$^{-/-}$ and WT splenocytes (Fig. 4*B*). Thus, both strains of mice are able to mount a similar immune response in terms of bacteria control and cytokine production against recombinant *L. monocytogenes*. Because our initial experiments demonstrated that LMP7$^{-/-}$ mice have a deficiency in the selection of Vα2/Vβ5-positive OT-1 T cells specific for the H2-K^b ligand SIINFEKL, we analyzed the Vβ usage of CD8-positive T cells specific for this MHC/peptide combination after infection with ovalbumin-tg *L. monocytogenes*. Total numbers of SIINFEKL-H2-K^b tetramer-positive CD8-positive T cells were comparable in LMP7$^{-/-}$ and WT mice (data not shown). With regard to the usage of Vβ chains, we observed the following: CD8-positive SIINFEKL-specific T cells in WT mice basically reflect the Vβ-chain usage of the naive CD8-positive T cell repertoire. Vβ5.1 and Vβ8.1 are both used to form the SIINFEKL-specific TCR. However, LMP7$^{-/-}$ mice showed a strong preference for the use of the TCR Vβ 8.1/8.2 chains within the SIINFEKL-specific CD8-positive T cells. On the other hand, the proportion of CD8-positive SIINFEKL-specific T cells expressing Vβ 5.1, which is also used by the tg OT-1 T cells, was substantially reduced in LMP7$^{-/-}$ mice (Fig. 4*C*). This effect is specific, because all other Vβ chains were used at similar levels in LMP7$^{-/-}$ and WT mice, as exemplified by Vβ10 usage. The specificity of this observation is further supported by the analysis of the CD8-positive T cells that are not specific for SIINFEKL. These T cells exhibit a Vβ usage comparable between LMP7$^{-/-}$ and WT mice.

Discussion

To assess the influence of immunoproteasomes on the TCR repertoire, we started by *in vitro* digesting the precursor of one of the peptides shown to be responsible for positive selection of the OT-1 TCR with constitutive proteasomes or immunoproteasomes purified from human LCL721 cell lines, respectively. The analysis of the digests revealed that only immunoproteasomes were capable of generating the correct C terminus of the peptide and additionally were able to directly produce the respective Cpα1 peptide. To test whether our *in vitro* findings would have functional consequences *in vivo*, we crossed OT-1 mice onto the LMP7$^{-/-}$ background or transferred OT-1 BM into irradiated LMP7$^{-/-}$ and WT recipients, respectively, and analyzed the development of the tg CD8$^+$ lymphocyte population in the thymus as well as the periphery. We observed a 50% reduction in TCR tg CD8$^+$ T cells in OT-1 mice harboring the LMP7 defect in the periphery as well as the site of selection of T cells, the thymus. The specificity of this effect is supported by the observation that the reconstitution of LMP7$^{-/-}$ and WT mice with BM from St42 mice results in equally efficient selection of St42 T cells specific for an adenoviral peptide. OT-1 selection might not be completely abolished, because not only the Cp$\alpha1_{92-99}$ peptide but also additional peptides, as reported

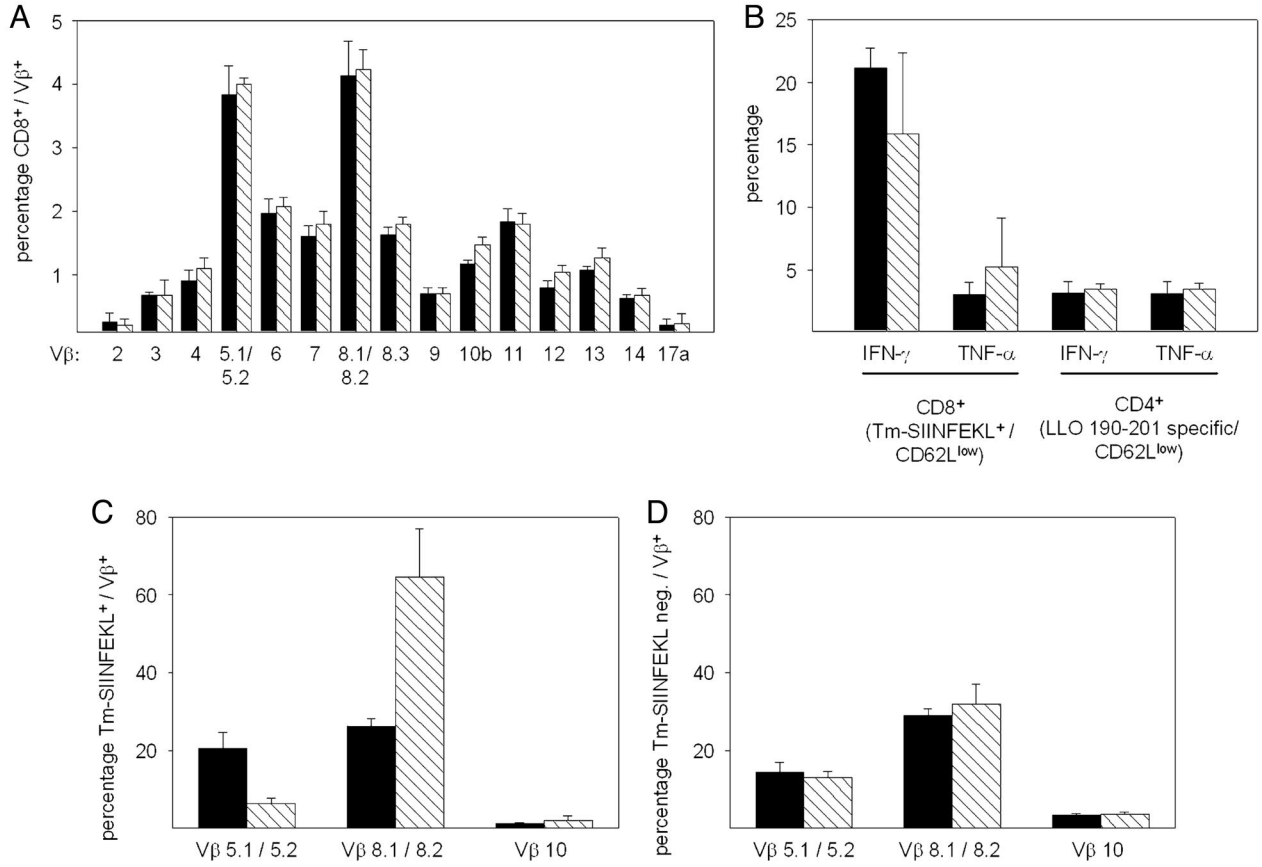

Fig. 4. Different TCR Vβ usage of SIINFEKL-specific CD8$^+$ T cells in LMP7$^{-/-}$ and LMP7 WT mice. (*A*) TCR Vβ usage in splenocytes from naive WT (filled bars) and LMP7$^{-/-}$ mice (hatched bars). (*B*) LMP7$^{-/-}$ or control LMP7$^{+/+}$ mice were recall-infected with 2×10^5 bacteria (Lm OVA) on day 35 after primary infection with 2×10^3 bacteria 5 days after recall infection. Harvested splenocytes were restimulated with peptide (SIINFEKL, $LLO_{190-201}$, 1 μg/ml final) for 5 h in the presence of the Golgi plug. Intracellular TNF-α and IFN-γ were measured for SIINFEKL-specific CD62L^{low} CD8$^+$ or $LLO_{190-201}$-specific CD62L^{low} CD4$^+$ lymphocytes. (*C*) LMP7$^{-/-}$ or control LMP7$^{+/+}$ mice were recall-infected with 2×10^5 bacteria (Lm OVA); 5 days after recall infection, SIINFEKL-specific CD62L^{low}CD8$^+$ or (*D*) SIINFEKL-negative CD8$^+$ lymphocytes from the spleen were analyzed with a panel of Vβ-TCR FITC-conjugated antibodies. Error bars represent standard deviation of quadruplicates from one of two independent experiments.

by Santori *et al.* (23), support the positive selection of OT-1 T cells, and their generation does not require immunoproteasomal activity. Nonetheless, injection of the Cpα1 peptide completely restores the percentage of tg OT-1 CD8$^+$ T cells in LMP7$^{-/-}$ mice to WT levels, possibly by entering the thymus via the bloodstream after i.v. injection. Because Fridkis-Hareli *et al.* (27) reported an altered positive selection after i.v. injection of peptide variants, one might speculate that, after Cpα1 injection, OT-1 T cells can be selected at a higher frequency, leave the thymus, and undergo normal homeostatic proliferation, explaining the elevated percentage of T cells even 180 days after Cpα1 injection. The finding that adoptive transfer of OT-1 T cells into sublethally irradiated LMP7$^{-/-}$ or WT hosts results in identical expansion (data not shown) further points toward a defect during thymic selection of the T cells rather than to a more general failure of T cell expansion or maintenance in the periphery. Because self peptides involved in positive selection are also suggested to play a role in homeostasis (28), immunoproteasome-independent self peptides other than $Cp\alpha1_{92-99}$ might contribute to the normal behavior of mature OT-1 cells in the periphery of LMP7$^{-/-}$ mice.

Yewdell and coworkers (20) reported a reduced CTL response against the H2-D^b-restricted influenza nucleoprotein epitope 366–374 in LMP2$^{-/-}$ mice despite the fact that cells from WT and LMP2$^{-/-}$ mice can produce the epitope at identical levels. The authors speculate this defect is due to the influence of immunoproteasomes on the repertoire of peptides involved in the selection of CD8-positive T cells. Our data now provide direct evidence that this takes place. Furthermore, our data are also reconcilable with a report by Nussbaum *et al.* (29), in which immunoproteasome-deficient mice basically show equal responses to lymphocytic choriomeningitis virus infection as infected WT mice. The authors conclude that immunoproteasomes may affect T cell responses to only a limited number of viral epitopes, and that the main biological function may lie elsewhere.

To investigate whether our findings of the development of tg OT-1 T cells are also applicable to the development of a naive T cell repertoire, we monitored and compared the immune responses of LMP7$^{-/-}$ and WT mice to an infection with ovalbumin-tg *L. monocytogenes*. Although both strains were able to mount a similar immune response, substantial differences could be observed in the Vβ chains used to form the TCRs specific for the SIINFEKL epitope. LMP7$^{-/-}$ mice strongly prefer the usage of Vβ8.1/8.2 over Vβ5.1. Because Vβ5.1 is also the Vβ-chain used in the OT-1 TCR, it is very well conceivable that differences in the processing and presentation of peptides involved in positive selection by thymus epithelial cells have a profound influence on the TCR selection from a variety of double-positive precursors. In that respect, it would make sense that immunoproteasomes are part of the positive selection process, because they would increase the potential number of

TCRs specific for a certain epitope. However, our observation regarding the contribution of immunoproteasomes to the positive selection of $CD8^+$ T cells does not fit the recent finding by Nil *et al.* (24), who reported that immunoproteasomes were not detectable in cortical thymus epithelial cells (cTEC), where positive selection takes place. From our set of data, we conclude that immunoproteasomes can influence positive selection. One possible explanation for this discrepancy might be the finding that cTEC are able to express immunoproteasomes during infection (24). Because this cannot be excluded even under specialized pathogen-free conditions, we speculate this might contribute to the expression of small amounts of immunoproteasomes in cTEC, which would favor the generation of peptides involved in positive selection of OT-1 cells. Alternatively, cells other than cTEC might play an additional role in positive selection, as suggested (30, 31), and the presence of immunoproteasomes in these cells might contribute to the selection of OT-1 cells.

In conclusion, our data provide direct evidence that proteasomal specificities are indeed able to influence the selection of naive CD8-positive T cells and therefore shape the repertoire of T cells participating in antigen-specific immune responses. Thus, proteasomes not only play an important role in generating MHC ligands for T cell activation during the initiation of an immune response but also impact the thymic selection of (CD8-positive) T cells.

Materials and Methods

Mice. $LMP7^{-/-}$ mice (129/ola) were generated by Fehling *et al.* (25). OT-1 mice (32) express a tg TCR ($Va2^+/Vb5.1/5.2^+$) specific for H-$2K^b$ restricted SIINFEKL ($OVA_{257–264}$). OT-1 mice were crossed with $LMP7^{-/-}$ (129/ola) and $LMP7^{+/+}$ mice (129/ola) and bred to homozygosity. $LMP7^{-/-}$ mice on the C57BL/6 background and C57BL/6 mice at the age of 6–12 weeks were obtained from Zentrale Versuchstiereinrichtung, University of Mainz. St42 mice (26) are tg for a TCR recognizing the H-$2D^b$ restricted peptide SGPSNTPPEI from the adenovirus Ad5 E1A protein ($E1a_{234–243}$) and were generated as described. All animal procedures were conducted in accordance with institutional guidelines.

Reagents and Flow Cytometric Analyses. Antibodies were purchased from BD Biosciences (Franklin Lakes, NJ) or Caltag (South San Francisco, CA). The following mAbs were used for analyses by flow cytometry: antigen-presenting cell (APC)-Cy7 or phycoerythrin (PE)-conjugated anti-CD8α (BD Biosciences); APC-conjugated anti-CD4 (BD Biosciences); PE-conjugated anti-Vα2 (BD Biosciences); a panel of FITC-conjugated anti-Vβ antibodies (Vβ 2, 3, 4, 5.1/5.2, 6, 7, 8.1/8.2, 8.3, 9, 10b, 11, 12, 13, 14, and 17a; BD Biosciences); FITC- or APC-conjugated anti-CD62L (BD Biosciences); APC-conjugated anti-CD8α (Caltag); and for intracellular cytokine staining, FITC-conjugated anti-IFN-γ/-TNF-α (BD Biosciences). The PE-conjugated H2-K^b-SIINFEKL tetramer (Tm) was generated as described (33). Synthetic peptides were derived from chicken ovalbumin. Cpα1 and LLO and were kindly provided by Stefan Stevanovic (Institute for Cell Biology, Department of Immunology, University of Tübingen) or purchased from Affina (Berlin). Blood samples were collected after tail-vein incision into appropriate buffer, subjected to a hypotonic lysis- and wash-step, and incubated on ice with specific mAbs as indicated for each experiment. Single-cell suspensions of spleens or thymi were analyzed by flow cytometry after staining with specific antibodies at 4°C. All analyses were performed with a FACSCanto flow cytometer by using the FACSDIVA software (BD Pharmingen).

BMT. For BMT experiments, recipients were immunocompromised by 8.5 Gy irradiation. BM was flushed out of the bones of naive sex-matched OT-1 mice, erythrocytes were lysed, and BM resuspended in PBS. The BM was $CD8^+$ T cell-depleted by using Dynal (Great Neck, NY) beads according to the manufacturer's protocol. Viable $CD8\alpha^+$ T cell-depleted BM cells (5×10^7) were transferred i.v. into recipients 3–4 h after lethal irradiation. Four to five weeks after reconstitution, animals were tail-vein-bled and lymphocytes FACS-analyzed with indicated mAbs. Where indicated, 25 mg of Cpα1 peptide (ISFKFDHL) was injected i.v. three times on 3 consecutive days.

***L. monocytogenes* Infection.** Recombinant *Listeria*-expressing ovalbumin (Lm Ova) were kindly provided by Hao Shen (Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia). For primary infection, mice were i.v.-infected with 2×10^3 bacteria (Lm OVA), whereas for recall infection on day 35, a 100-fold higher dose was used. To assess viable bacteria, mice were killed at day 3 (primary) or 2 (recall) after infection, and spleens and livers were harvested. Organs were processed to single-cell suspensions, mixed with Triton X-100 to break up the cells, and several dilutions plated out in triplets on brain–heart infusion ogar plates. Plates were incubated overnight at 37°C and *Listeria* colonies counted the next day.

Intracellular Cytokine Staining. Splenocytes were prepared as described (34) on day 7 after primary and day 5 after recall infection. Cells were restimulated with peptide (SIINFEKL, $LLO_{190–201}$ at a final concentration of 1 mg/ml) and incubated for 5 h at 37°C with Golgi plug present for the last 3 h (2 mg/ml). After 5 h, cells were first incubated with Fc receptor block, washed, and then stained with indicated mAbs. Cells were treated with Cytofix/Cytoperm (BD Biosciences) to permeabilize the cell walls, washed, stained with intracellular antibodies, and fixed with 1% paraformaldehyde until used for FACS analysis.

Proteasomal Processing. Purification of 20S proteasomes, *in vitro* degradation of $Cp\alpha1_{86–109}$, and separation and analysis of cleavage products were performed as described (12). Briefly, 10 nmol (μg) $Cp\alpha1_{86–109}$ were incubated for 6 h with 2 μg of constitutive proteasome or 2 μg of immunoproteasome, respectively, and the reaction stopped by freezing the mixture at −80°C.

Immunoblotting. Different amounts of purified proteasomes were separated by 12% SDS/PAGE by standard techniques and transferred to polyvinyldifluoride (DuPont) with a semidry transfer system. Human LMP7 (β_{5i}) was detected by using a rabbit polyclonal antiserum (PW8355; Biomol, Hamburg, Germany); the subunit X (β_5) was detected by using a rabbit polyclonal antiserum (PW8895; Biomol) in conjunction with goat-anti-rabbit horseradish peroxidase (Dianova, Hamburg, Germany) and chemiluminescence (Western Lightning; PerkinElmer).

Quantification of Proteasome Subunits by Using Ultraperformance-Liquid Chromatography MS. ***Sample preparation.*** Ten micrograms of proteasomes was digested with modified trypsin (Promega), as described (35).

HPLC and MS configuration. Capillary liquid chromatography of tryptic peptides was performed with a Waters NanoAcquity ultraperformance liquid chromatography block system, equipped with a Waters NanoEase BEH C_{18}, 75 μm × 10-cm reverse-phase column. Mobile phase A contained 0.1% formic acid in H_2O. Mobile phase B contained 0.1% formic acid in acetonitrile. Samples (2-μl injection) were loaded onto the column with 5% mobile phase B. Peptides were eluted from the column with a gradient of 5–40% mobile phase B over 45 min at 250 nl/min. MS analysis of tryptic peptides was performed by

using a Waters Q-Tof Premier in positive Vmode electrospray ionization. The mass spectrometer was calibrated with a [Glu-1]-fibrinopeptide solution (500 fmol/μl at 300 nl/min) delivered through the reference sprayer of the NanoLockSpray (Waters) source. For fragment identification, the instrument was run in data-directed acquisition mode, selecting the three most intense peaks for fragmentation. For relative quantification of the peptides, the samples were analyzed in liquid chromatography MS mode. Each sample was analyzed in triplicate.

Data processing and protein identification and quantification. The liquid chromatography tandem MS (LCMSMS) data were processed and searched by using PROTEINLYNX GLOBAL SERVER, Ver. 2.2. (Waters). Protein identifications were assigned by searching the SwissProt database with the precursor and fragmentation data afforded by the LCMSMS acquisition method. The mass error tolerance values were typically <5 ppm. Peptide identifications were restricted to tryptic peptides with no more than one missed cleavage and cysteine carbamidomethylation. For selected tryptic fragments of the proteasome subunits, the chromatographic peak area was determined in LCMS mode from the combined intensity of all of the isotopes associated to each fragment.

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