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Absence of Tapasin Alters Immunodominance against a Lymphocytic Choriomeningitis Virus Polytope

Denise S. M. Boulanger, Roberta Oliveira, Lisa Ayers, Stephen H. Prior, Edward James, Anthony P. Williams, and Tim Elliott

Tapasin edits the peptide repertoire presented to CD8+ T cells by favoring loading of slow off-rate peptides on MHC I molecules. To investigate the role of tapasin on T cell immunodominance we used poxvirus viral vectors expressing a polytope of lymphocytic choriomeningitis virus epitopes with different off-rates. In tapasin-deficient mice, responses to subdominant fast off-rate peptides were clearly favored. This alteration of the CD8+ T cell hierarchy was a consequence of tapasin editing and not a consequence of the alteration of the T cell repertoire in tapasin-deficient mice, because bone marrow chimeric mice (wild-type recipients reconstituted with tapasin knockout bone marrow) showed the same hierarchy as the tapasin knockout mice. Tapasin editing is therefore a contributing factor to the phenomenon of immunodominance. Although tapasin knockout cells have low MHC I surface expression, Ag presentation was efficient and resulted in strong T cell responses involving T cells with increased functional avidity. Therefore, in this model, tapasin-deficient mice do not have a reduced but rather have an altered immune response. The Journal of Immunology, 2010, 184: 73-83.
bone marrow chimeric (BMC) mice (C57BL/6 mice transplanted with Tpn KO BM) that would contain a normal T cell repertoire selected on Tpn" cells but Tpn-deficient APCs.

Materials and Methods

Cells

Primary chicken embryo fibroblasts were prepared using 10-d-old embryonated eggs from Rhode Island or Brown Leghorn chicken and grown in MEM containing 10% FCS (Globepharm, Surrey, UK). RMA-S, TAP2-deficient mouse T cell line, was cultured in RPMI 1640 (Lonza, Verviers, Belgium) supplemented with 10% FCS and 2 mM glutamine (Lonza), Kb-transfected L cells (K89) and Kb-transfected L cells (D8l), kindly provided by N. Shastri, California, were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1% HEPE, 1 mM pyruvate, and 50 μg/ml β-mercaptoethanol.

Pepitides

Pepitides CSANNSHHYLVGVYAGGQGYL, FQPQGGGGQI, KAVYNFATCGI, CYS41 (Cys41 was substituted into Met to avoid dimerization (10)), ISHFCNHL, and YTVKYPNL (Peptide Protein Research, Fareham, U.K.) and YAHINALEY, GLLSDHKSN, KAVYNFLATCGI, AAEFFNSL, TSYKGSVESP, and VSLDVINTM (GL Biochem, Shangai, China) were synthesized by fluorescent methoxy carbonyl chemistry and were >95% pure by HPLC and mass spectrometry.

Tetramers

Tetramers were manufactured essentially as described (11), with minor modifications. Mice MHC class I and mouse β2-microglobulin (β2-m) expression vectors were kindly provided by Prof. Dirk Busch (Munich, Germany), cloned into pET-3a (Novagen, Madison, WI) and transformed into BL21(DE3) CodonPlus RIPL (Stratagene, La Jolla, CA) cells. β2-m was refolded by slow dilution to a final concentration of 2 μM in the presence of 10 μM peptide. The heavy chain was then refolded in the presence of β2-m and peptide to a final concentration of 1 μM. The refolded complex was then concentrated and applied to a HiLoad 26/60 Superdex 200 column (GE Healthcare, Buckinghamshire, England). The refolded complex was biotinylated by incubation at 16˚C with 50 μg/ml streptavidin (Invitrogen, Paisley, U.K.) at 4˚C.

RMA-S cells were incubated overnight at 26˚C to maximize MHC I surface expression. After being washed, 5 × 10^6 cells were pulsed at different time intervals with peptides at a final concentration of 20 μM for 1 h at 26˚C. After washing with medium, the MHC I surface was blocked by the addition of BFA, and peptide-loaded RMA-S cells were incubated at 37˚C to allow decay of unstable molecules. Cells were stained with Y3 or B22 primary monoclonal Abs (anti-H-2Kb and anti-H-2Db, respectively) and goat anti-mouse IgG-PE (Abcam, Cambridge, U.K.) secondary Abs to detect peptide-loaded MHC I molecules. Samples were analyzed by flow cytometry on a Beckton Dickinson (Oxford, U.K.) FACSCalibur flow cytometer, and data were analyzed with the Cell Quest software.

Immunization and intracellular cytokine (IFN-γ) staining

Mice were primed and boosted at a 2-wk interval by i.p. injection of 10^7 PFU of recombinant or wt virus. Splenocytes were harvested 7 d after the boost. Red blood cells were eliminated by centrifugation through a Ficol cushion. Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit with GolgiPlugs (BD Biosciences, San Jose, CA). Splenocytes (2 × 10^6 splenocytes from immunized mice or 10^6 splenocytes from immunized mice + 10^6 splenocytes from naive mice) were incubated with peptides at the concentration indicated in the figure legends. After 2 h of incubation at 37˚C, BFA (GolgiPlug) was added. After 2 h at 37˚C, the plates were transferred at 4˚C and stored overnight before staining. Dead cells were stained with ethidium monoazide bromide (Molecular Probes, Eugene, OR) simultaneously to block with Fcγ block (2.4G2; BD Pharmingen, San Diego, CA). Extra-cellular staining was performed for 30 min on ice with an anti-CD8a-APC (BD Pharmingen) Ab. Cells were permeabilized with Cytofix/Cytoperm and stained with an anti-IFN-γ-PE-Cy7 Ab (BD Pharmingen) for 30 min on ice. Cells were fixed in 1% paraformaldehyde solution and analyzed using a Beckton Dickinson FACScan and the Diva software. In some experiments, surface staining included an anti-TCRβ-PE Ab (eBio-sciences, Hatfield, U.K.).

Tetramer staining and tetramer decay

Splenocytes were prepared as above. After the blocking step, the cells were stained with a predetermined saturating concentration of tetramer for 45 min on ice. Anti-CD8-APC Abs were added and incubated for a further 15 min. After three washings in FACS buffer (PBS, 2% FCS, 0.02% NaN₃), the cells were either fixed in 1% PFA or incubated at room temperature for different periods of time in the presence of 100 μg/ml Y3 mAb. After a final wash, the cells were fixed, and samples were analyzed as described above. Total fluorescence was determined by multiplying the mean fluorescence intensity (MFI) of the gated tetramer-positive population by the number of events within the gate (13).

Hybridoma generation

TSKY- and KAV-specific hybridoma were generated according to Sanderson and Shastri (14). Splenocytes harvested from C57BL/6 mice, immunized as described above, were restimulated with each peptide in vitro for 4 d in the presence of 20 U/ml IL-2 before being fused with BWZ.36/CDS8 fusion partners. The SGVE-specific hybridoma was kindly provided by Dr. Marcus Groettrup, Konstanz, Germany.

Epitope presentation assay

Splenocytes harvested from C57BL/6 or Tpn KO mice were stimulated with 10 μg/ml Escherichia coli LPS (Sigma-Aldrich, St. Louis, MO) for 48 h. Approximately 10^7 or 10^8 cells per well of a 96 well plate were then infected for 0, 2, 4, or 6 h with MVA-vt or MVA-ME1 at a multiplicity of infection (moi) of 3 PFU per cell. After being washed to eliminate any residual virus, appropriate T cell hybridoma (10^5 cells per well) were added and incubated at 37˚C O/N. After freeze–thawing, IL-2 production was measured by ELISA.
Reverse phase HPLC fractionation of peptides

LPS-blasts (10^8 cells) were infected with recombinant MVA at an moi of 3 PFU per cell for 6 h. Surface peptides were eluted by incubation for 1 min in 0.13 M citrate phosphate buffer (pH 3.1) supplemented with 1% BSA. After centrifugation, the supernatants were filtered through 10-kDa cut-off Microcon Ultracell YM-10 filters (Millipore, Bedford, MA). Filtrates were fractionated by HPLC on a C18 column (Vydac, Kinesis, St Neots, Cambs, U.K.) using a 0.1% trifluoroacetic acid 15–50% acetonitrile/water gradient. Fractions were collected in 96-well plates and dried by vacuum centrifugation.

T cell activation assay

DbL or K89 were added to the dried fractionated samples and cocultured with the appropriate T cell hybridoma. The lacZ activity induced upon hybridoma activation was measured by the conversion of chlorophenolred-β-d-galactopyranside (CPRG) (Roche, Mannheim, Germany) to chlorophenol red by its absorbance at 595 nm with 655 nm as the reference wavelength (14).

Results

Immune response profile to competing vaccine epitopes in the presence of Tpn

To investigate the role of Tpn on CD8+ T cell immunodominance in an in vivo setting where multiple epitopes are expressed simultaneously, we used poxvirus vectors expressing a polytope containing five LCMV epitopes of different off-rates, described previously (15–17) as either immunodominant (gp33-43, KAV, Db- and Kb-restricted (18), and np396-404, FQPO, Db-restricted) or subdominant (gp276-286, SGVE, Db-restricted; np205-212, YTUK, Kb-restricted; and gp92-101, CSAN, Db-restricted). The relative kinetic stability (off-rate) of all of the peptides used in this study was determined in a BFA decay experiment (Fig. 1). KAV9 (KAVYNFATM) and FQPO peptide had a much slower off-rate than SGVE, CSAN, and KAV11 (KAVYNFATCGI) presented on H-2Db. The slowest off-rate LCMV peptide presented on H-2Kb was also KAV9 followed by KAV11, ISHN, and then YTUK.

The hierarchy of CD8+ T cell response against the different LCMV epitopes was determined by ex vivo intracellular cytokine (IFN-γ) staining of splenocytes harvested from immunized C57BL/6 mice. A single injection with either the fowlpox or the vaccinia MVA recombinant viruses (FWPV-ME1 or MVA-ME1, respectively) did not generate a detectable response. C57BL/6 mice were therefore primed with the fowlpox recombinant virus and boosted with the MVA recombinant virus. T cells specific for

![FIGURE 1. Decay of peptide-loaded MHC I molecules on RMA-S cells. Db-restricted peptides (top panel) and Kb-restricted peptides (bottom panel) were loaded on RMA-S cells. MHC I decay was assessed by Db or Kb surface staining after different incubation times at 37°C. Results are plotted as percentages of the maximum MFI obtained at time 0 for each peptide. Dashed lines represent the controls. TSYK Kb-restricted peptide was used as negative control for Db stabilization, and DMSO was used as a negative control (top panel). SIINFEKL (SIIN) was used as a positive control for high-affinity Kb-restricted peptide, and DMSO was used as a negative control (bottom panel). TSYK and YAHI are MVA-specific epitopes used in this study to monitor the T cell response against the MVA vector (see text).](http://www.jimmunol.org/)

![FIGURE 2. T cell response against an LCMV polytope. A, Hierarchy of T cell response against an LCMV polytope in C57BL/6 mice immunized with 10^7 PFU of FWPV-ME1 and 10^7 PFU of MVA-ME1 at 2-wk intervals. Percentage of CD8+ live lymphocytes expressing IFN-γ determined by intracellular cytokine staining after ex vivo stimulation with the LCMV-specific peptides indicated on the x-axis. ISHN and DMSO were used as negative controls. Results from 19 mice tested in 10 individual experiments after stimulation with 4 or 5 × 10^6 M peptide are shown. B, Hierarchy of response in Tpn KO mice. Results from nine mice from six individual experiments are shown. C, Comparison of the T cell response in C57BL/6, Tpn KO, and BMC mice after ex vivo stimulation using 5 × 10^6 M concentrations of the respective peptides. One representative experiment out of five is shown.](http://www.jimmunol.org/)
three of the LCMV epitopes (KAV, CSAN, and SGVE) were detected (Fig. 2A). An immunodominant response against the KAV epitope was consistently observed. Most mice also developed a detectable response against the CSAN epitope. A response against the SGVE epitope was infrequently observed. Surprisingly, the Db-restricted immunodominant FQPQ epitope was recognized poorly in this system, probably due to an inefficient proteasomal cleavage from the polytyl peptide compared with the LCMV nucleoprotein. No response against the Kb-restricted subdominant YTVK epitope was ever observed. The overall response profile against the three recognized epitopes (higher T cell response against KAV than against CSAN and SGVE) (Fig. 2A) therefore matched the kinetic stability hierarchy determined by the BFA decay experiment (Fig. 1), consistent with our previous findings (9).

Immune response profile in the absence of Tpn

To determine whether the immunodominance of CD8+ T cell responses seen following recombinant virus immunization was under the control of Tpn, we immunized Tpn KO mice with the same prime–boost strategy. In Tpn KO mice, the response against SGVE and CSAN increased, whereas the response against the KAV epitope decreased compared with the response observed in C57BL/6 mice. The hierarchy of CD8+ T cell response became SGVE > CSAN > KAV (Fig. 2B, 2C). In some mice, the response to CSAN was as high or sometimes slightly higher than the response to SGVE, but the response to KAV was always much lower. Thus, the role of Tpn in favoring presentation and therefore T cell response of slow off-rate peptides was also confirmed in this vaccine setting and was potent enough to induce a shift in immunodominance hierarchy.

Immune response against MVA-specific epitopes

After prime–boost immunization we noted that the percentage of CD8+ T cells with low CD8 surface expression (activated lymphocytes) was always higher in Tpn KO mice than that in C57BL/6 mice, showing that the Tpn KO mice are highly responsive. Because the percentage of CD8+ T cells responding to the LCMV epitopes was low compared with the percentage of activated CD8+ T cells, we suspected that this might be a consequence of responses to competing epitopes from the viral vector and therefore tested the CD8+ T cell response against five vaccinia-specific epitopes: YAHINALEY (A47L 157–165, Db-restricted), GLLSDHKS (J6R 543–551, Kb-restricted) (19), TSYKFESV (B8R 20–27, Kb-restricted), AAFEFINSL (A47L 138–146, Kb-restricted), and VLSDYINTM (A19L 47–55, Kb-restricted) (20). After two immunizations with 10⁷ PFU of MVA-wt, C57BL/6 and Tpn KO mice reacted strongly and consistently against the TSYK epitope and, in some experiments, against YAHI (Fig. 3A). The TSYK epitope is present in the vaccinia B8R protein, which has no homologue in FWPV. To determine the importance of TSYK in the immune response generated after a heterologous immunization protocol with the recombinant viruses, groups of two C57BL/6 mice were immunized either with two injections of MVA-ME1 or with two injections of both vectors in either order (MVA-ME1 first or FWPV-ME1 first). Both mice injected twice with MVA-ME1 responded strongly against TSYK (22% of IFN-γ–producing CD8+ splenocytes) but very weakly against the LCMV epitopes (maximum 0.9% against KAV9). Both mice primed with MVA-ME1 and boosted with FWPV-ME1 showed a lower response against TSYK (4% or 5%) but did not respond better against the LCMV epitopes (0.8% maximum). Both mice primed with FWPV-ME1 and boosted with MVA-ME1 reacted better against TSYK (6–9%) and also responded to the LCMV epitopes (3–4% against KAV9). TSYK was therefore immunodominant over the LCMV epitopes even in heterologous immunizations (Fig. 3B) and represents one of the major components of the immune response in both C57BL/6 and Tpn KO mice (compare the level of response against TSYK in Fig. 3C and the response against the LCMV epitopes in the same mice in Fig. 2C).

The higher response of Tpn KO mice against TSYK (Fig. 3C) as well as against CSAN and SGVE (Fig. 2C) was not an artifact due to the low percentage of CD8+ T cells found in naive Tpn KO mice, because immunized mice also had a higher total number of IFN-γ–producing CD8+ T cells per spleen (Fig. 3D). This is remarkable, because naive Tpn KO mice have half the percentage of CD8+ T cells compared with C57BL/6 mice. Therefore, at most a similar or more likely a lower number of naive T cell precursors in Tpn KO mice compared with C57BL/6 mice must have been primed, activated, and proliferated more efficiently until homeostasis constraints were reached.

Generation of BMC mice and comparison of their immune response to C57BL/6 and Tpn KO mice

The difference in the hierarchy of response against the LCMV epitopes seen in Tpn KO mice could be due either to a difference in the hierarchy of epitope presentation or to a difference in the T cell repertoire altered by impaired thymic selection. To assess the respective influence of the editing function of Tpn and the altered T cell repertoire on the T cell response of Tpn KO mice, we generated Tpn KO chimeric mice by transplanting BM cells harvested from Tpn KO mice into irradiated C57BL/6 recipient mice. All hematopoietic cells, including dendritic cells, in the BMC mice should derive from the Tpn KO BM transplant and should not express Tpn. The percentage of CD8+ splenocytes in the BMC mice was normal, and Tpn could not be detected in either splenocytes or BM dendritic cells by RT-PCR (Supplemental Table I, Supplemental Fig. 1A). MHC I levels on BMC hematopoietic cells were the same as those on Tpn KO cells and were 10–20% of the surface expression seen on C57BL/6 cells (Supplemental Fig. 1B), confirming the donor-origin of circulating lymphocytes and restoration of normal positive selection in BMC mice.

After heterologous prime–boost immunization, BMC mice developed a strong response against SGVE and CSAN and a reduced response against KAV as in Tpn KO mice (Fig. 2C). BMC mice also developed a strong response against TSYK after either homologous prime–boost immunizations with MVA-wt or heterologous prime–boost immunizations with both recombinant viruses (Fig. 3C). Because BMC mice share the same APCs as Tpn KO mice but a T cell repertoire educated on high MHC I like C57BL/6 mice, the BMC T cell profile showed that the hierarchy of T cell response was essentially a consequence of Tpn editing and not a difference in the T cell repertoire.

Quantitation of relative amounts of peptides presented on the cell surface

Because TSYK has a similar off-rate as KAV9 (Fig. 1) and on the basis of the published data on Tpn function, we would have expected the response to TSYK to be lower in Tpn KO mice than that in C57BL/6. However, the response against TSYK in Tpn KO mice was consistently higher. It is not clear why TSYK is not presented poorly like KAV in Tpn KO mice. This could be due to a difference in abundance, processing efficiency, duration of exposure (21), or pathway of presentation.

To answer this question and to confirm Tpn editing of the peptide repertoire in this model, we attempted to quantitate the relative amount of each peptide presented on the surfaces of infected cells. C57BL/6 and Tpn KO LPS-blasts infected for 2, 4,
or 6 h were cocultured with T cell hybridomas specific for TSYK (3E1), KAV presented by H-2Kb (D9 or G10), KAV presented by H-2Db (5E2), or SGVE. We used LPS-blasts to maximize MHC I expression. Although LPS treatment increased MHC I expression on both C57BL/6 and Tpn KO LPS-blasts, the latter still expressed less surface MHC I than C57BL/6 LPS-blasts (Fig. 4A). Fig. 4B shows that KAV was presented efficiently by H-2Kb on C57BL/6 LPS-blasts but was not detected on Tpn KO LPS-blasts (Fig. 4B). Interestingly, KAV was presented only very weakly by H-2Db on C57BL/6 LPS-blasts (note the difference of sensitivity of the 5E2 H-2Db-restricted hybridoma compared with the D9 H-2Kb-restricted hybridoma in Fig. 4C) and not at all on Tpn KO blasts. Because SGVE was neither detected on C57BL/6 nor on Tpn KO LPS-blasts in this assay (data not shown), we therefore infected a larger number of LPS-blasts, acid-eluted peptides from MHC I surface molecules, and fractionated them by HPLC. Using this method, we could show that approximately two times more SGVE was recovered from Tpn KO than C57BL/6 LPS-blasts ($6 \times 10^{-10}$ M compared with $3 \times 10^{-10}$ M, respectively) (Fig. 5B). When the relative level of H-2Db MHC I expression on C57BL/6 compared with Tpn KO LPS-blasts was taken into consideration (Fig. 4A), this translated into approximately six times more on Tpn KO on a per-cell basis. KAV was detected only in one extract from C57BL/6 LPS-blasts (data not shown), but the amount was too low to be quantified (maximum lower than $10^{-10}$ M). These data therefore confirm that the subdominant epitope SGVE, which has a faster off-rate from H-2Db than the immunodominant epitope KAV, is presented better in Tpn KO than in wt cells, whereas the reverse is true for KAV. These semiquantitative data therefore correlate well with the response hierarchies observed following recombinant virus infection.

Fig. 4B also shows that C57BL/6 LPS-blasts presented slightly more TSYK than Tpn KO LPS-blasts. In acid elution experiments, ~50 times more TSYK was recovered from C57BL/6 than from Tpn KO LPS-blasts (maximum concentrations of $2 \times 10^{-8}$ M and $4 \times 10^{-10}$ M, respectively, average from 4 experiments, one of them shown in Fig. 5A). This increase could not be accounted for by the fivefold increase of H-2Db MHC I expression on C57BL/6 compared with Tpn KO LPS-blasts. We conclude therefore that like KAV the high-affinity peptide TSYK is also subject to Tpn-mediated editing but that its high abundance is sufficient to breach a threshold for priming even in the absence of tapasin.

Quantitative aspects of the CD8+ T cell response

The intracellular cytokine staining assays described above were performed after stimulation with a standard concentration of 4 or $5 \times 10^{-8}$ M of peptide. However, as shown in Fig. 1, the peptides used in this study have different affinities. Therefore we tested responses at saturating concentrations of peptide (Fig. 6A, 6B).

The intensity of the response depended on the concentration of peptide used in the ex vivo stimulation. The response to KAV and ME1 (F7M7) (all immunizations with $10^7$ PFU of virus). Both mice primed with FWPV-ME1 reacted to a high extent against TSYK and gave the strongest response against the LCMV epitopes. This immunization strategy was therefore used throughout this study. C, Comparison of the IFN-γ response of C57BL/6 mice immunized with two injections of MVA-ME1 recombinant virus (M7m7) or primed with MVA-ME1 and boosted with FWPV-ME1 (M7F7) or primed with FWPV-ME1 and boosted with MVA-ME1 (F7M7) (all immunizations with $10^7$ PFU of virus). Both mice primed with FWPV-ME1 reacted to a high extent against TSYK and gave the strongest response against the LCMV epitopes. This immunization strategy was therefore used throughout this study. C, Comparison of the IFN-γ response of C57BL/6, Tpn KO, and BMC mice primed with FWPV-ME1 and boosted with MVA-ME1 and stimulated ex vivo with $10^{-9}$ M TSYK. The T cell response was expressed as the percentage of CD8+ live lymphocytes expressing IFN-γ. D, Same as C expressed as numbers of CD8+ live lymphocytes expressing IFN-γ per spleen.
TSYK was already maximal at $10^{-6}$ M, but the response to CSAN and SGVE could be increased at higher concentrations, reaching a maximum at $10^{-4}$ M. Interestingly, Tpn KO CD8$^+$ T cells seemed to be activated by lower concentrations of peptides (Fig. 6A, 6B). Because Tpn KO T cells have been selected on a low MHC I background, we hypothesized that T cells with a higher avidity would have been selected in Tpn KO mice compared with C57BL/6 mice. We therefore compared the T cell responses of

**FIGURE 4.** Epitope presentation on LPS-blasts. A, C57BL/6$^{+/+}$ and Tpn KO$^{-/-}$ LPS-blasts were stained for H-2Db (left panel) and H-2Kb (right panel) surface molecules. Samples were analyzed by flow cytometry using a Beckton Dickinson FACSCalibur flow cytometer, and data were analyzed with FlowJo 7.5. Filled histograms show the unstained controls. B, Approximately $10^5$ LPS-blasts were infected with MVA-ME or MVA-wt at an moi of 3 PFU per cell for 2 h (light gray histograms), 4 h (dark gray), or 6 h (black histograms). Uninfected controls are shown as white histograms. LPS-blasts were cocultured with the following hybridoma cells: 3E1 hybridoma specific for TSYK (left panels), D9 hybridoma specific for KAV presented by H-2Kb (middle panels), 5E2 hybridoma specific for KAV presented by H-2Db molecules (right panels). Hybridoma activation could not be tested by CPRG staining, because the MVA-ME recombinant virus expresses β-galactosidase. Therefore, overnight IL-2 production was measured by ELISA. (C) KAV peptide titrations. Approximately $10^5$ DbL or K89 cells were incubated with two step dilutions of peptide and $10^5$ hybridoma cells (D9 on K89 or 5E2 on DbL). After overnight incubation hybridoma activation was revealed with CPRG. (D) TSYK peptide titration. As in C, K89 cells were incubated with peptide and with the 3E1 hybridoma.
C57BL/6, Tpn KO, and BMC mice by intracellular cytokine staining using decreasing concentrations of peptides for the ex vivo stimulation, referred to as the functional avidity. The concentrations necessary to obtain 50% of the maximum IFN-γ response varied greatly between peptides (Fig. 6C–E) and were lower in Tpn KO than those in C57BL/6 for TSYK (30-fold), CSAN (4-fold), and SGVE (5-fold). This implied that TSYK-, CSAN-, and SGVE-specific T cells have a higher avidity in Tpn KO mice compared with those in C57BL/6 mice. To confirm that the lower peptide requirement for activating Tpn KO T cells was not due to the different quality of APCs, responders from immunized mice were mixed with naive C57BL/6 and Tpn KO splenocytes in the presence of different concentrations of peptide. Although Tpn KO splenocytes improved the efficiency of the stimulation compared with C57BL/6 (Fig. 6F), a difference in functional avidity was still clearly observed between C57BL/6 and Tpn KO responders. Similarly, the functional avidity of the BMC T cells was higher than that in C57BL/6 but lower than that in Tpn KO mice although they share the same Tpn−/− APCs (Fig. 6C). Therefore the higher Tpn KO T cell response and functional avidity was only partly due to the enhanced capacity of Tpn−/− APCs to load with extracellular peptides.

Another explanation for the increased functional avidity in Tpn KO mice could result from the selection of T cells bearing a higher TCR affinity. TCR avidity of C57BL/6 and Tpn KO TSYK-specific T cells was therefore compared in a "tetramer decay" assay. Splenocytes from immunized C57BL/6 and Tpn KO mice were stained with a saturating concentration of fluorescent tetrameric complexes of H-2Kb:TSYK, determined by titration (Fig. 7A, 7B), washed, and incubated for increasing periods of time at room temperature in the presence of anti-H-2Kb blocking Ab (Fig. 7C, 7D). Using this method, we found that there was no difference in TCR avidity between C57BL/6 and Tpn KO mice (Fig. 7D).

Increased functional avidity could also result from an increased expression of the TCR, increased expression of adhesion and activation molecules upon activation, or a decreased activation threshold (22) to counterbalance the low MHC I expression on APCs involved in T cell thymic selection or in the adoptive immune response. In immunized mice, the MFI of the TCR surface expression on the CD8+ population was usually lower in Tpn KO mice (Fig. 8A), reflecting the higher percentage of activated cells in Tpn KO mice. However, the MFI of the IFN-γ-expressing cells was identical in both types of mice. Interestingly, the TCR expression was slightly lower on naive Tpn KO CD8+ T cells (p < 0.0001) (Fig. 8B). Therefore, the increased functional avidity of Tpn KO T cells is not due to an increase of TCR expression. In addition, we have been unable to identify a difference in activation potential between wt and Tpn KO cells using markers such as differences in Lck phosphorylation and calcium mobilization, and this is a subject of ongoing investigation.

**Discussion**

Previous work using variants of the OVA 323-331 peptide SIINFEKL characterized by different off-rates, expressed as minigenes in fibroblasts transfected with DNA plasmids, showed that in vitro the presence of Tpn favors presentation of the slow off-rate peptides (6). This hierarchy correlated with the in vivo CD8+ T cell response hierarchy obtained against the same variants administered to C57BL/6 mice individually as DNA immunogens expressing either of the variant epitopes (9). We show that in C57BL/6 mice immunized with poxvirus recombinants expressing an LCMV polytope the T cell response against the slow off-rate KAV epitope was higher than those against CSAN and SGVE, whereas in Tpn KO mice the T cell response was higher against the faster off-rate CSAN and SGVE epitopes, resulting in a different immunodominance hierarchy. The difference in the T cell response hierarchy in Tpn KO mice could either be due to the presence of Tpn-deficient APCs and lack of Tpn editing or be due to a different T cell repertoire affected by an abnormal thymic T cell selection. The former interpretation is favored by our demonstration that BMC mice showed a T cell response hierarchy similar to that of Tpn KO mice. Furthermore, peptide elution from C57BL/6 and Tpn KO LPS-blasts showed that Tpn KO cells present

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**FIGURE 5.** HPLC fractionation of acid-eluted peptides from LPS-blasts. A, B, Acid-eluted peptides from 107 LPS-blasts infected with MVA-ME at an moi of 3 PFU/ml were fractionated by HPLC (top panels). To confirm the identity of the eluted peptides, 10 pmol of synthetic peptides were run through the HPLC under identical conditions (bottom panels). Samples were assayed with the 3E1 hybridoma (A) or the SGVE hybridoma (B) and K89 (A) or Dbl (B) as APCs. C, Titration curves of 3E1 and SGVE hybridoma reactivity after incubation with the appropriate APCs and serial dilutions of the respective peptides. After overnight incubation, hybridoma activation was revealed with CPRG.
approximately six times more SGVE peptide than C57BL/6 cells. Tpn editing of the peptide repertoire is therefore an important mechanism underpinning T cell immunodominance. Processing, transport, and affinity for MHC have all been identified as important factors influencing immunodominance. All of these factors converge at the point of peptide loading and repertoire editing.

It was surprising that the response against KAV was lower than the response against CSAN and SGVE in Tpn KO mice (and BMC mice). In the absence of Tpn function, it is likely that loading is simply controlled by peptide on-rate. The peptide repertoire should therefore depend on the relative abundance of available peptides (above a certain association-rate threshold). Because KAV, CSAN, and SGVE are expressed from a polytope, we expected similar amounts of peptides to be generated and presented on the cell surface. However, differential processing has been shown to lead to presentation of very different amounts of peptides. KAV can be presented by both H-2Db and H-2Kb MHC I molecules on LCMV-infected cells. However, our data (Fig. 4B) show that KAV
expressed from the MVA recombinant vector is presented mostly on H-2Kb molecules. Due to the very low abundance, we have not been able to quantitate the amount of KAV presented on either H-2Kb or H-2Db molecules, but our data (not shown) suggest that KAV is presented at even lower amounts than SGVE. It is therefore possible that KAV is indeed processed less efficiently than CSAN and SGVE. Another explanation for the low response against KAV would be the deletion of KAV-specific T cells from the T cell repertoire of Tpn KO mice, but this is unlikely because BMC mice also showed a reduced response against KAV. Whatever the reason for the poor T cell response against KAV in the absence of Tpn, efficient presentation, at least on H-2Kb

FIGURE 7. Tetramer staining and TCR avidity. A, TSYK-Tetramer titration on splenocytes from naive (N) or F7M7 immunized C57BL/6 and Tpn KO mice. Tetramer concentration was expressed as percentage v/v. Results are expressed as the percentage of tetramer-positive cells within the CD8+ lymphocyte population. B, TSYK-Tetramer titration expressed as a percentage of the maximum response. C, TSYK-Tetramer decay from Tetramer+ CD8+ cells of one typical C57BL/6 and one Tpn KO mouse. Staining is shown after 0, 3, or 30 min of incubation at room temperature in the presence of blocking Y3 Ab. Staining without tetramer and staining of naive mice with tetramer are shown as controls. D, Tetramer decay from Tetramer+ CD8+ T cells from immunized C57BL/6 and Tpn KO mice expressed as the percentage of the maximum total fluorescence corresponding to time 0.
molecules (Fig. 4B), was restored in the presence of Tpn, contributing to the alteration of the immunodominance hierarchy.

Interestingly the response against TSYK, an MVA-specific epitope of a similar half-life as KAV on H-2 Kb molecules, was actually immunodominant in both C57BL/6 and Tpn KO mice and was higher in Tpn KO mice than that in C57BL/6 mice. Tpn editing therefore shapes the hierarchy of T cell response only to a certain extent, because some epitopes such as TSYK (CD8+ TSYK) or DMSO (CD8+ DMSO) or gated on CD8+ cells producing IFN-γ after stimulation with TSYK (IFN+ TSYK). B. Comparison of the TCR surface staining MFI of CD8+ lymphocytes from naive C57BL/6 or Tpn KO mice. Immunized controls were included in the same experiment.

Figure 8. Comparison of TCR expression in naive and immunized C57BL/6 and Tpn KO mice. A. TCR surface staining MFI of splenocytes from C57BL/6 (black) or Tpn KO (gray) immunized mice stimulated in vitro with TSYK, or DMSO as a negative control. Cells were gated on CD8+ lymphocytes after stimulation with TSYK (CD8+ TSYK) or DMSO (CD8+ DMSO) or gated on CD8+ cells producing IFN-γ after stimulation with TSYK (IFN+ TSYK). B. Comparison of the TCR surface staining MFI of CD8+ lymphocytes from naive C57BL/6 or Tpn KO mice. Immunized controls were included in the same experiment.

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