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Research Article

The immunoproteasome subunit LMP7 is required in the murine thymus for filling up a hole in the T cell repertoire

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Cells of hematopoietic origin express high levels of the immunoproteasome, a cytokine-inducible variant of the proteasome which has been implicated in regulating inflammatory responses and antigen presentation. In the thymus, medullary thymic epithelial cells (mTECs) and cortical thymic epithelial cells (cTECs) do express different proteasome subunits exerting chymotrypsin-like activities suggesting distinct functions in thymic T cell selection. Employing the lymphocytic choriomeningitis virus (LCMV) infection model, we could show that the immunoproteasome subunit LMP7 was absolutely required for the generation of LCMV $GP_{118-125}$-specific T cells although the class I mediated presentation of $GP_{118-125}$ was not dependent on LMP7. Using bone marrow chimeras and adoptive transfer of LMP7-deficient $CD8^+$ T cells into RAG1-deficient mice we show that LMP7-deficient mice lacked $GP_{118-125}$-specific T cell precursors and that LMP7 was required in radioresistant cells – most likely thymic epithelial cells - to enable their selection. Since LMP7 is strongly expressed in negatively selecting mTECs but barely in positively selecting cTECs our data suggest that LMP7 was required to avoid excessive negative selection of $GP_{118-125}$-specific T cell precursors. Taken together, this study demonstrates that the immunoproteasome is a crucial factor for filling up holes within the cytotoxic T cell repertoire.

Keywords: Antigen processing · Immunoproteasome · LCMV · Proteasome · Thymic selection

Introduction

The proteasome is the main protease responsible for generating ligands for MHC class I molecules for the presentation to cytotoxic T lymphocytes (CTLs) [1, 2]. The proteolytic core complex of the proteasome system is the 20S proteasome, which possesses a barrel shaped structure consisting of four rings with seven subunits each. The inner two rings are made of β-subunits and bear the catalytically active subunits β1c, β2c, and β5c [3]. In hematopoietic cells and in cells stimulated with interferon (IFN)-γ or tumor necrosis factor (TNF)-α these proteolytically active subunits are replaced by β1i (low molecular mass polypeptide (LMP)2), β2i (multicatalytic endopeptidase complex-like (MECL)-1), and β5i (LMP7) forming an inducible variant of the 20S proteasome designated the immunoproteasome. The immunoproteasome is functionally involved in the generation of MHC-I ligands [4–12], in

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T cell expansion [13, 14], in the protection from immunopathological damage in the brain [15, 16], and in autoimmune diseases [17–23]. Immunoproteasome-deficient mice have been used to investigate the impact of the inducible proteasome subunits in various studies (reviewed in [24]). Apart from defects in class I antigen presentation, LMP7-deficient mice show alterations in the clearance of *Listeria monocytogenes* [25], in *Toxoplasma gondii* infection [26], in murine cytomegalovirus (MCMV) infection [27, 28], in *Trypanosoma cruzi* infection [29], and in experimental colitis [18, 30, 31]. Cells from LMP7$^{-/-}$ mice [32] express reduced levels of surface MHC-I molecules [17, 32], emphasizing the crucial role of LMP7 in MHC-I restricted antigen processing. It has recently been reported that differences in MHC-I presentation between standard and immunoproteasomes were due to quantitative rather than qualitative differences in the spectrum of proteasome-generated antigenic peptides [33].

The repertoire of T cells is primarily formed in the thymus through positive and negative selection of developing thymocytes. After T cell receptor (TCR) rearrangement, developing thymocytes undergo the process of positive and negative selection, which is mediated by TCR-MHC-self peptide interaction. It is generally accepted that the proteasome is the main protease involved in generating class-I ligands for negative and positive selection. Distinct proteolytically active proteasome subunits have been described in the thymus [24]. Apart from the constitutive proteasome subunits β1c, β2c, and β5c the expression of the immunosubunits β1i, β2i, and β5i have been identified in the thymus [34]. Additionally, β1i, β2i, and β5t are expressed in cortical thymic epithelial cells (cTECs) building the so-called thymoproteasome [35].

In this study, we describe a strongly reduced glycoprotein (GP)$_{118\text{-}125}$-specific CTL response in LMP7-deficient mice infected with lymphocytic choriomeningitis virus (LCMV). We could show that this effect was not due to inefficient processing of this epitope, but due to a lack of GP$_{118\text{-}125}$-specific precursors in LMP7-deficient mice. Since LMP7 is abundant in negatively selecting medullary thymic epithelial cells (mTECs) but not [34] or hardly [36, 37] detectable in positively selecting cTECs, our data suggests that LMP7 is required to prevent negative selection of GP$_{118\text{-}125}$ specific T cell precursors by so far unknown self antigens. Hence, we show that LMP7 is an important factor in determining the cytotoxic T cell repertoire and for filling up holes in the spectrum of T cell receptor specificities.

Results

Strongly reduced GP$_{118\text{-}125}$-specific CTL response in LCMV-infected LMP7-deficient mice

To investigate the CD8$^+$ T cell response in LMP7-deficient mice in viral infection, C57BL/6 wild-type and LMP7$^{-/-}$ mice were infected with LCMV. On day 8 post infection, the CTL response directed against GP$_{33–41}$/D^b/K^b, NP$_{396–404}$/D^b, GP$_{118–125}$/K^b, and NP$_{205–212}$/K^b was determined by ICS for IFN-γ (Fig. 1A). No alteration in GP$_{33\text{-}41}$-, NP$_{396\text{-}404}$-, and NP$_{205\text{-}212}$-specific CD8$^+$ T cell responses between wild-type and LMP7-deficient mice could be observed. In contrast, the response to GP$_{118\text{-}125}$ was reduced to background levels in LMP7-deficient mice (Fig. 1A and B). In order to investigate whether the reduced GP$_{118\text{-}125}$ CTL response in LMP7$^{-/-}$ mice is dependent on the virus type, we used a recombinant vaccinia virus expressing the LCMV glycoprotein (VVG2). Similar to LCMV, the GP$_{118\text{-}125}$-specific CTL response in VVG2-infected LMP7$^{-/-}$ mice was strongly reduced compared to wild-type mice (Fig. 1C). Interestingly, as previously reported [8], the GP$_{276\text{-}286}$ CTL response was increased in LMP7-deficient mice.

It has been reported that LMP2 and MECL-1 incorporation into immunoproteasomes is strongly reduced in mice lacking LMP7 [38]. Hence, an altered incorporation of LMP2 and MECL-1 into immunoproteasomes of LMP7-deficient mice might contribute to the reduced GP$_{118\text{-}125}$ CTL response as seen in Fig. 1. We have previously observed that the GP$_{118\text{-}125}$ response is not affected in MECL-1-deficient mice [13]. Analysis of LMP2-deficient mice showed a similar GP$_{118\text{-}125}$ response in wild-type and LMP2 deficient mice (Fig. 2A). Immunosubunit incorporation into 20S proteasomes can be influenced via the regulatory particle PA28αβ in PA28β$^{-/-}$ mice [39], although these results could not be confirmed in mice lacking both PA28α and PA28β [40]. Moreover, PA28αβ was recently shown to preferentially associate with the immunoproteasome in vitro and in cells [41]. To investigate the contribution of PA28αβ to the GP$_{118\text{-}125}$ response, PA28α$^{-/-}$/β$^{-/-}$ mice were infected with LCMV-WE and the CTL response was analysed on d8 post infection (Fig. 2B). No alteration of the GP$_{118\text{-}125}$ response could be observed in these mice, indicating that LMP7 alone is responsible for the reduced GP$_{118\text{-}125}$ response observed in LMP7-deficient mice (Fig. 1).

GP$_{118\text{-}125}$ presentation is not altered by LMP7-deficient cells

In IFN-γ-stimulated cells, LMP2, MECL-1, and LMP7 are incorporated into newly synthesized proteasomes. We have previously shown that IFN-γ stimulation of cells led to a reduced presentation of the LCMV-derived T cell epitope GP$_{276\text{-}286}$ and to an increased GP$_{33\text{-}41}$ presentation [8]. To analyse whether IFN-γ stimulation of cells leads to an altered GP$_{118\text{-}125}$ presentation, MC57 (H-2^b) cells were stimulated with IFN-γ for 2 days and infected with LCMV for 24 h. These cells were used as stimulators for mono-specific CTL lines specific for GP$_{33\text{-}41}$, NP$_{396\text{-}404}$, and GP$_{118\text{-}125}$. IFN-γ stimulation of MC57 cells markedly increased GP$_{33\text{-}41}$, NP$_{396\text{-}404}$, and GP$_{118\text{-}125}$ presentation as assessed by IFN-γ production by the peptide-specific CTLs (ICS) (Fig. 3). Since the stimulation of cells with IFN-γ induces, apart from immunoproteasome subunits, many other proteins from the MHC class I presentation pathway [1] - thus leading to an improved MHC-I presentation - we investigated GP$_{118\text{-}125}$ presentation directly in LMP7-deficient cells. Thioglycolate-elicited peritoneal macrophages derived from wild-type or LMP7-deficient mice were infected in vitro for 20 h with LCMV-WE and analysed for GP$_{118\text{-}125}$ presentation by ICS of GP$_{118\text{-}125}$-specific CTLs (Fig. 4A). Compared to uninfected

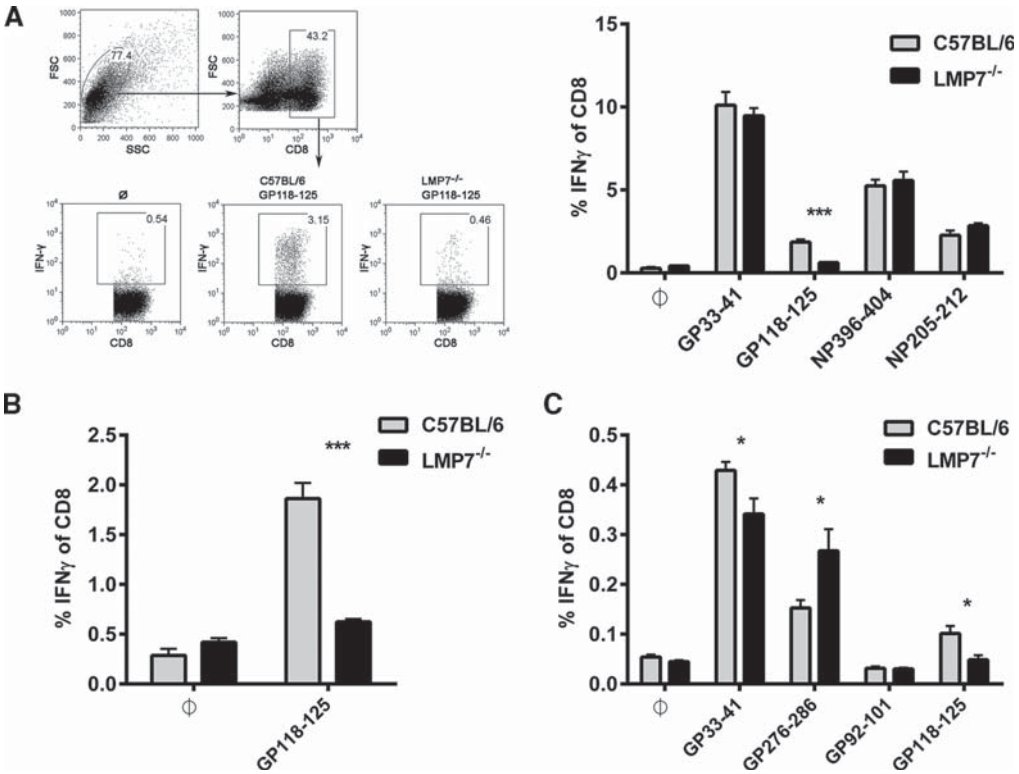

Figure 1. Reduced $GP_{118\text{-}125}$ response in LMP7-deficient mice. C57BL/6 wild-type or $LMP7^{-/-}$ mice were infected with LCMV-WE (A, B) or recombinant vaccinia virus expressing the LCMV glycoprotein (VVG2) (C). 8 days post infection, spleen cells were harvested, stimulated in vitro with the indicated LCMV peptides for 5 h, and analysed by flow cytometry after staining for CD8 and intracellular IFN-γ. Panel B is extracted from panel A and displayed with another y-axis scale. The gating strategy is shown exemplarily in dot plots for the $GP_{118\text{-}125}$ epitope on the left side of panel A. Shown are the mean percentages ± SEM ($n = 4$) of IFN-γ-positive cells of $CD8^+$ cells (y-axis). $^*p<0.05$; $^{***}p<0.001$; unpaired Student's *t*-test. Unstimulated cells (Ø) were used as negative controls. Data are from a single experiment representative of at least five (A, B) or two (C) experiments with 4 mice per experiment, yielding similar results.

macrophages, both LCMV-infected wild-type as well as LMP7-deficent macrophages presented $GP_{118\text{-}125}$ at similar levels. Next, we investigated antigen presentation ex vivo by dendritic cells. Wild-type and LMP7-deficient mice were infected with LCMV-WE. On day 4 post infection, $CD11c^+$ cells were magnetically isolated from the spleen and analysed for $GP_{118\text{-}125}$ presentation by ICS of $GP_{118\text{-}125}$-specific CTLs (Fig. 4B). Compared to naïve uninfected dendritic cells, wild-type and LMP7-deficient cells stimulated $GP_{118\text{-}125}$-specific CTLs to a similar extent. Hence, the reduced $GP_{118\text{-}125}$ presentation observed in LCMV-infected $LMP7^{-/-}$ mice

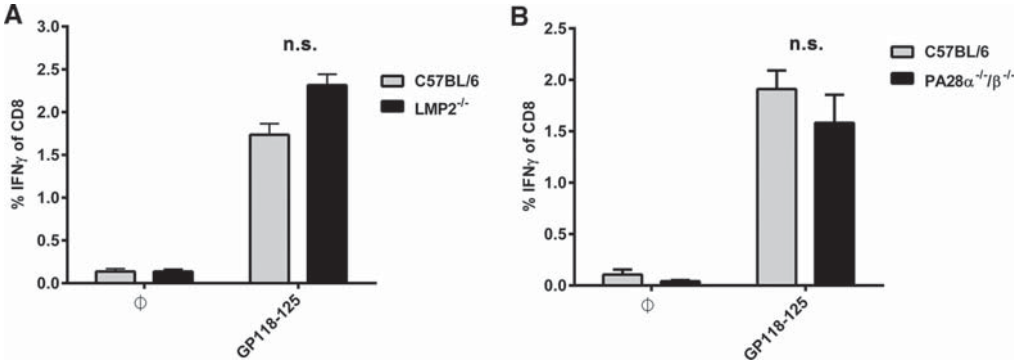

Figure 2. Unaltered $GP_{118\text{-}125}$ response in LMP2- and PA28α/β-deficient mice. C57BL/6 wild-type, $LMP2^{-/-}$ (A), and $PA28\alpha^{-/-}/\beta^{-/-}$ (B) mice were infected with LCMV-WE. 8 days post infection, spleen cells were harvested, stimulated in vitro with the $GP_{118\text{-}125}$ peptide for 5 h, and analysed by flow cytometry after staining for CD8 and intracellular IFN-γ. Shown are the mean percentages ± SEM ($n = 4$) of IFN-γ-positive cells of $CD8^+$ cells (y-axis). n.s. not significant ($p>0.05$); unpaired Student's *t*-test. Unstimulated cells (Ø) were used as negative controls. Data are from a single experiment representative of two experiments with 4 mice per experiment, yielding similar results.

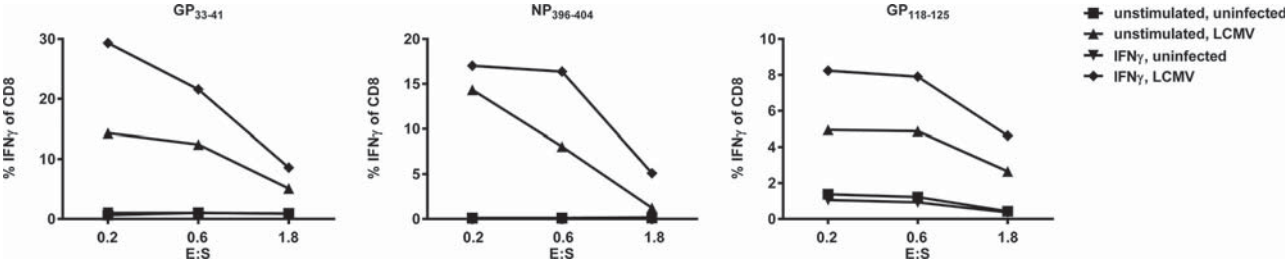

Figure 3. Comparison of the presentation of the LCMV epitopes GP_{33-41}, $NP_{396-404}$, and $GP_{118-125}$ by IFN-γ-treated and LCMV-infected MC57 cells. MC57 fibroblast stimulator cells were treated with IFN-γ or were left untreated. After 2 days, cells were infected with LCMV-WE. 24 h later, cells were used as stimulators for GP_{33-41}-, $NP_{396-404}$-, or $GP_{118-125}$-specific CTL lines. Activation of CTL-lines was analysed by staining for CD8 and intracellular IFN-γ. Shown are the percentages of IFN-γ-positive cells of $CD8^+$ cells as determined by flow cytometry. The percentage of IFN-γ^+ of $CD8^+$ cells (y-axis) is plotted versus the E:S ratio (effector (CTL lines) to stimulators (MC57 cells)). Uninfected MC57 cells were used as negative controls. All samples were measured in duplicates. The experiments have been performed twice, yielding similar results.

is not due to a different presentation of $GP_{118-125}$ on $LMP7^{-/-}$ cells.

It has been reported that the structural features rather than the proteolytic activity of an immunoproteasome subunit are required for the generation of certain epitopes [4, 10, 42]. Therefore, we tested whether the presentation of $GP_{118-125}$ is dependent on the catalytic activity of LMP7. To block LMP7-activity we used the well-described LMP7-selective inhibitor ONX 0914 (formerly designated PR-957) [17] (Fig. 4C and D). However, $GP_{118-125}$ presentation was not affected when LMP7 activity was blocked. Additionally, when β5c activity in wild-type or LMP7-deficient macrophages was inhibited with the β5c-selective inhibitor PR-825 [17], $GP_{118-125}$ presentation was not altered (Fig. 4C and D). Activity assays with a fluorogenic substrate for the chymotrypsin-like activity demonstrated that ONX 0914 and PR-825 were active in thioglycolate-elicited macrophages (Supporting Information Fig. 1A). Hence, the chymotrypsin-like activity of the proteasome subunits LMP7 and β5c are not essential for $GP_{118-125}$ processing in macrophages.

To confirm these results in vivo, C57BL/6 wild-type mice were infected with LCMV-WE and treated with ONX 0914 or PR-825. The LCMV-specific T cell response was analysed on day 8 by ICS for IFN-γ (Fig. 5). Neither ONX 0914 (Fig. 5A) nor PR-825 (Fig. 5B) affected the $GP_{118-125}$ response, although both inhibitors were

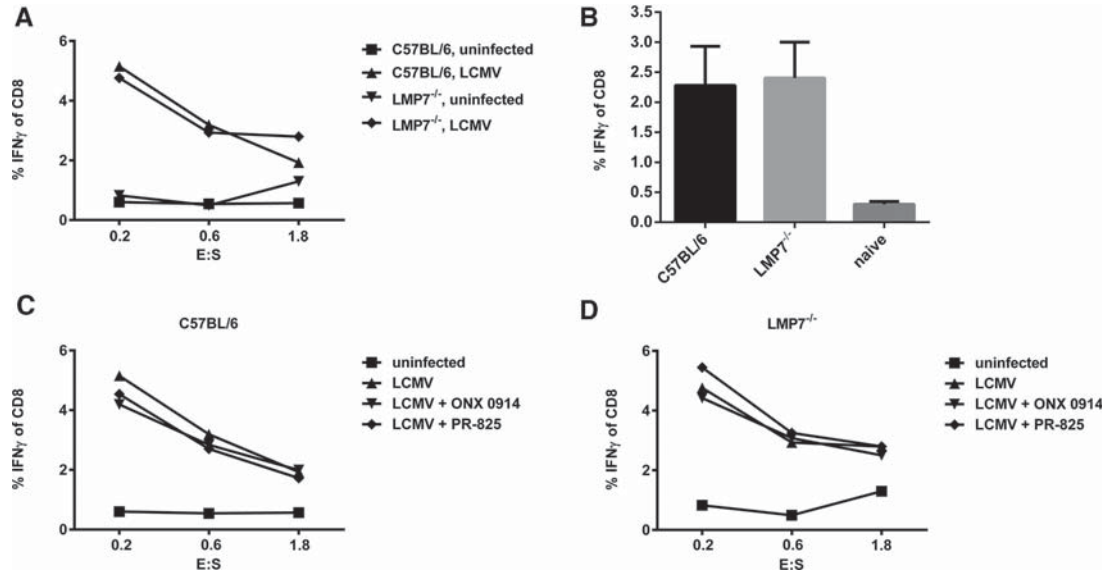

Figure 4. $GP_{118-125}$ presentation is not altered on $LMP7^{-/-}$ macrophages and dendritic cells. (A, C, D) Thioglycolate-elicited macrophages derived from C57BL/6 (A, C) or $LMP7^{-/-}$ (A, D) mice were treated with ONX 0914 (300 nM) or PR-825 (125 nM) (C, D) and infected with LCMV. 24 h post infection, cells were used as stimulators for a $GP_{118-125}$-specific CTL line. Activation of the CTL line was analyzed by staining for CD8 and intracellular IFN-γ. Shown are the percentages of IFN-γ-positive cells of $CD8^+$ cells as determined by flow cytometry. The percentage of IFN-γ^+ of $CD8^+$ T cells (y-axis) is plotted vs the E:S ratio (effector (CTL line) to stimulators (macrophages)). Uninfected macrophages were used as negative controls. The macrophages from two mice were pooled and all samples were measured in duplicates. The experiments have been performed twice, yielding similar results. (B) C57BL/6 or $LMP7^{-/-}$ mice were infected with LCMV-WE. On day 4 post infection, $CD11c^+$ cells were magnetically sorted from the spleen and used as stimulators for a $GP_{118-125}$-specific CTL line. Activation of the CTL line was analyzed by staining for CD8 and intracellular IFN-γ. Shown are the percentages of IFN-γ-positive cells of $CD8^+$ cells as determined by flow cytometry. Naïve C57BL/6 mice were used as negative controls. Data are presented as the mean ± SEM of 12 different mice pooled from 3 independent experiments.

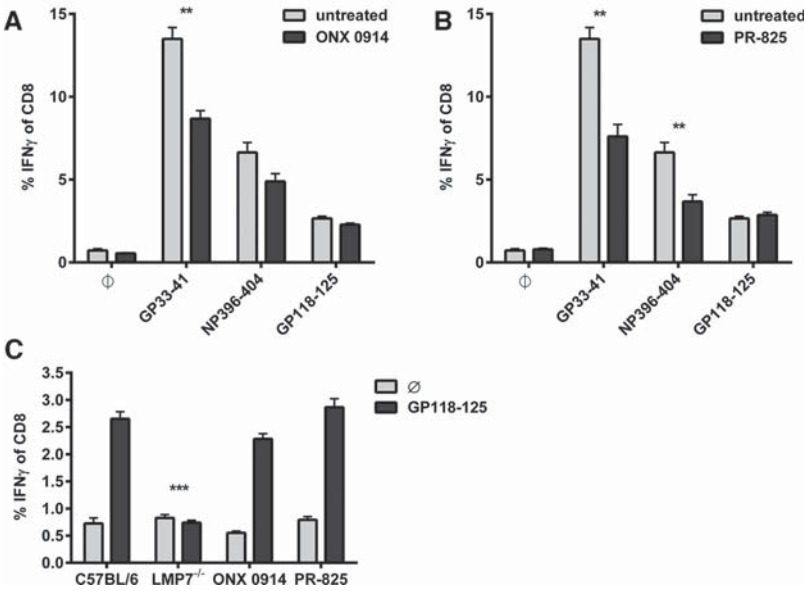

Figure 5. LMP7 and β5c activities are not required for a $GP_{118\text{-}125}$-specific CTL response. C57BL/6 (A, B, C) or $LMP7^{-/-}$ (C) mice were infected with LCMV-WE on day 0. On day −1, 0, 1, 2, and 3, mice were treated with ONX 0914 (A), PR-825 (B) or were left untreated. 8 days post infection, spleen cells were harvested, stimulated in vitro with indicated peptides, and analysed by flow cytometry after staining for CD8 and intracellular IFN-γ. Shown are the mean percentages ± SEM ($n = 5$) of IFN-γ-positive cells of $CD8^+$ cells (y-axis). $^{**}p<0.01$; $^{***}p<0.001$; unpaired Student's *t*-test. Unstimulated cells (Ø) were used as negative controls. Data are from a single experiment representative of two experiments with 5 mice per experiment, yielding similar results.

active in mice (Supporting Information Fig. 1B). As previously reported, ONX 0914 reduced the $GP_{33\text{-}41}$ response [9, 17]. Interestingly, β5c inhibition affected the $GP_{33\text{-}41}$ response at a comparable level (Fig. 5B), indicating that both β5c- and LMP7-activity contribute to the $GP_{33\text{-}41}$ CTL response. Taken together, although a $GP_{118\text{-}125}$-specific CTL response is lacking in LMP7-deficient mice (Fig. 1 and Fig. 5C), the proteolytic activity of LMP7 is not required for the generation of a normal $GP_{118\text{-}125}$-specific response (Fig. 5A and C).

Altered $GP_{118\text{-}125}$-specific T cell repertoire in $LMP7^{-/-}$ mice

It has been reported that immunoproteasome-deficient mice might harbour an altered T cell repertoire [6, 13, 34, 43]. Since $GP_{118\text{-}125}$ presentation is not affected by LMP7-deficiency in macrophages, we intended to investigate whether LMP7-deficient mice have an altered $GP_{118\text{-}125}$-specific T cell repertoire. To investigate whether LMP7 expression alters the T cell repertoire in the thymus, CD4 and CD8 single positive (SP) cells in the thymus were screened with a panel of antibodies specific for different Vβ-segments of the TCR (Supporting Information Fig. 2). When comparing SP thymocytes from wild-type and LMP7-deficient mice, only minor alterations in Vβ-segment usage were recorded for TCR-Vβ2 and TCR-Vβ3 in CD8 SP thymocytes and no alteration in TCR usage of CD4 SP T cells was observed. Hence, TCR-Vβ screening did not provide evidence that bulk T cell selection in the thymus is affected by LMP7. Similar findings were found by Osterloh et al. in the periphery in the spleen of LMP7-deficient mice [43].

To test whether there is an impact of LMP7 on the generation of $GP_{118\text{-}125}$-specific T cell precursors, we transferred wild-type $Thy1.1^+/Thy1.2^-$ $CD8^+$ cells into wild-type C57BL/6 ($Thy1.1^-/Thy1.2^+$) or $LMP7^{-/-}$ ($Thy1.1^-/Thy1.2^+$) mice and infected these mice with LCMV. On day 8 post infection, we analysed the $GP_{118\text{-}125}$-specific $CD8^+$ response of the transferred T cells in wild-type and LMP7-deficient mice. However, in contrast to wild-type mice, we were not able to recover transferred $Thy1.1^+$ cells from LMP7-deficient mice (Supporting Information Fig. 3). It seems that the transferred cells have been rejected upon LCMV infection in LMP7-deficient mice. A similar result was observed when wild-type skin was transplanted to LMP7-deficient mice [7]. Conversely, a T cell transfer of LMP7-deficient T cells into wild-type mice is also not feasible to investigate the $GP_{118\text{-}125}$-specific T cell repertoire since adoptively transferred immunoproteasome-deficient cells do not expand upon LCMV infection [14]. We decided to use bone marrow chimeras as an alternative approach. Wild-type or LMP7-deficient mice were irradiated and reconstituted with either wild-type or LMP7-deficient bone marrow. 8 weeks post reconstitution, mice were infected with LCMV-WE and the $GP_{33\text{-}41}$- and $GP_{118\text{-}125}$-specific T cells response in the spleen was analysed 8 days later (Fig. 6). No difference in the $GP_{33\text{-}41}$-specific CTL response was observed for all the analysed bone marrow chimeras. In contrast, irrespective whether $LMP7^{-/-}$ mice were reconstituted with wild-type or $LMP7^{-/-}$ bone marrow, these mice were not able to mount a $GP_{118\text{-}125}$-specific T cell response suggesting that the expression of LMP7 in a radioresistant cell type is necessary for the generation of $GP_{118\text{-}125}$-specific CTLs (Fig. 1). Since professional antigen presenting cells (APCs) are inducing the LCMV-specific CTL response [44], we assume that an influence of LMP7 on priming of $GP_{118\text{-}125}$-specific T cells can be excluded in $LMP7^{-/-}$ mice reconstituted with wild-type bone marrow. Additionally, wild-type mice reconstituted with LMP7-deficient bone marrow mounted a normal $GP_{118\text{-}125}$ response, indicating that LMP7-deficient $GP_{118\text{-}125}$-specific T cells are able to expand and LMP7-deficient APCs can process and present this peptide. These data strongly argue for an altered precursor frequency of $GP_{118\text{-}125}$–specific T cells in LMP7-deficient mice.

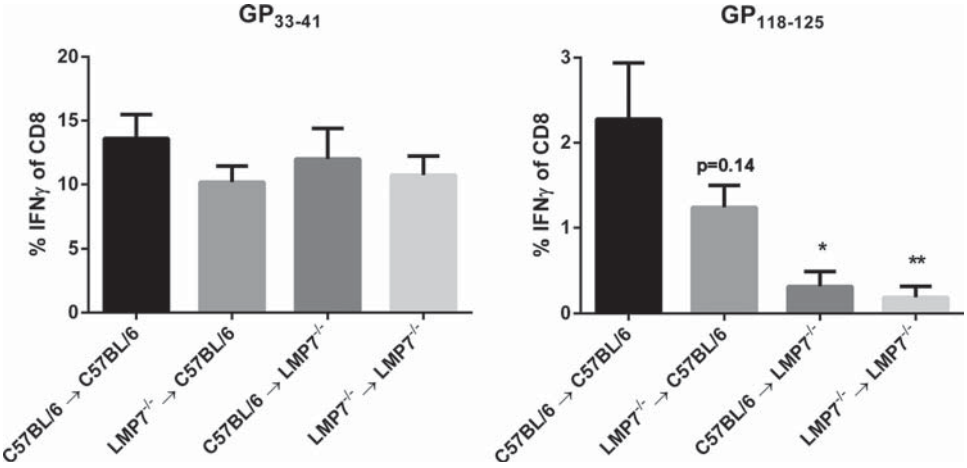

Figure 6. No $GP_{118\text{-}125}$-specific CTL response in LMP7-deficient bone marrow chimera recipients. C57BL/6 or $LMP7^{-/-}$ mice were irradiated and reconstituted with bone marrow derived from C57BL/6 or $LMP7^{-/-}$ mice. 8 weeks post irradiation, mice were infected with LCMV-WE (day 0). On day 8, spleen cells were harvested, stimulated in vitro with $GP_{33\text{-}41}$ (left panel) or $GP_{118\text{-}125}$ (right panel) peptides, and analysed by flow cytometry after staining for CD8 and intracellular IFN-γ. Shown are the mean percentages ± SEM ($n = 7$) of IFN-γ-positive cells of $CD8^+$ cells (y-axis). $^*p<0.05$; $^{**}p<0.01$; unpaired Student's *t*-test. Data are from a single experiment representative of two experiments with seven mice per experiment, yielding similar results.

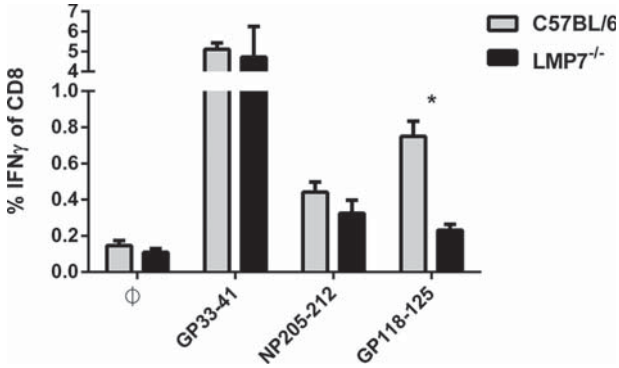

Figure 7. LMP7-deficient mice lack $GP_{118\text{-}125}$-specific T cell precursors. Magnetically enriched $CD8^+$ cells derived from C57BL/6 or $LMP7^{-/-}$ mice were transferred into $RAG1^{-/-}$ mice. Three weeks post transfer, the mice were infected with LCMV-WE and the $GP_{33\text{-}41}$, and $GP_{118\text{-}125}$-specific T cell response was analysed by ICS on day 8 post infection. Shown are the mean percentages ± SEM ($n = 4$) of IFN-γ-positive cells of $CD8^+$ cells (y-axis) as determined by flow cytometry. $^*p<0.05$; unpaired Student's *t*-test. Unstimulated cells (Ø) were used as negative controls. Data are from a single experiment representative of two experiments with four mice per experiment, yielding similar results.

To address this question, magnetically sorted $CD8^+$ T cells from naïve LMP7-deficient or wild-type mice were adoptively transferred into $RAG1^{-/-}$ mice. Three weeks after homeostatic expansion of the transferred $CD8^+$ T cells, the mice were infected with LCMV-WE and the $GP_{33\text{-}41}$, $NP_{205\text{-}212}$ and $GP_{118\text{-}125}$-specific T cell response was analysed by ICS for IFN-γ (Fig. 7). Both, transferred wild-type and LMP7-deficient $CD8^+$ cells, mounted a comparable $GP_{33\text{-}41}$- or $NP_{205\text{-}212}$-specific T cell response. However, $GP_{118\text{-}125}$-specific $CD8^+$ T cells derived from $LMP7^{-/-}$ mice, in contrast to wild-type mice, were not able to expand upon LCMV infection. This result indicates that LMP7-deficient mice lack $GP_{118\text{-}125}$-specific precursor $CD8^+$ T cells.

Discussion

The immunoproteasome is critically involved in the generation of MHC-I restricted epitopes [11, 12, 24]. Infection of LMP7-deficient mice with LCMV showed a reduced $GP_{118\text{-}125}$-specific CTL response (Fig. 1). Interestingly, a similar result was observed in LCMV-infected triply immunoproteasome-deficient mice [11]. In contrast, the $GP_{118\text{-}125}$-specific CTL response in LMP2- (Fig. 2A) or MECL-1-deficient [13] mice was comparable to wild-type mice, indicating that LMP2 and MECL-1 do not influence the $GP_{118\text{-}125}$-specific CTL response. Furthermore, LCMV infection of $LMP2^{-/-}$/$MECL\text{-}1^{-/-}$ double-deficient mice treated with the well-defined LMP7-selective inhibitor ONX 0914 to generate mice lacking the activity of all immunoproteasome subunits induced a normal $GP_{118\text{-}125}$-specific CTL response [9]. This result suggests that the proteolytic activity of LMP7 during LCMV infection is dispensable to elicit a $GP_{118\text{-}125}$-specific CTL response. Indeed, we could confirm this result in wild-type mice treated with the LMP7-selective inhibitor ONX 0914 (Fig. 5). It has been shown that structural features rather than the proteolytic activity of an immunoproteasome subunit are needed for the generation of certain epitopes [4, 42]. Recently, we provided a rationale why the structural property of an immunoproteasome subunit rather than its activity is required for the generation of a CTL epitope [10]. Namely, CTL epitopes, which are destroyed by constitutive proteasome subunits, are simply protected from destruction by the incorporation of their homologous immunoproteasome subunit, which do not exert the destructive cleavage. To test whether the constitutive β5c proteasome subunit destroys the $GP_{118\text{-}125}$ CTL epitope, macrophages or mice were treated with the β5c-selective inhibitor PR-825 [17]. Neither in vitro (Fig. 4C and D) nor in vivo (Fig. 5) a destruction of $GP_{118\text{-}125}$ by β5c could be observed. $GP_{118\text{-}125}$ presentation by LMP7-deficient macrophages and dendritic cells was unaltered (Fig. 4A and B), indicating that LMP7 is not required

for the processing of this CTL epitope. Taken together, $GP_{118-125}$ presentation in LMP7-deficient cells is not altered, the catalytic activity of LMP7 is not required for the generation of this epitope, β5c does not destroy $GP_{118-125}$, and the processing of this epitope is not influenced by other immunoproteasome subunits.

Why then is the $GP_{118-125}$-specific CTL response altered in LMP7-deficient mice? The normal processing of $GP_{118-125}$ (Fig. 4) suggests that LMP7-deficient mice lack $GP_{118-125}$ precursor T cells. Immunoproteasome subunits are constitutively expressed in the thymus [34, 35]. According to Nil et al., immunoproteasome subunits are expressed in thymic DCs, thymic macrophages, and mTECs. No mRNA expression, neither for MECL-1, LMP2, nor LMP7, could be detected in cTECs. In contrast, Murata et al. found MECL-1 and LMP2 expression in cTECs, but only a weak LMP7 expression [35]. Instead of LMP7, β5t is incorporated into MECL-1 and LMP2 containing thymoproteasomes. β5t-deficient mice have a disturbed development of $CD8^+$ T cells in the thymus [35] leading to a markedly altered T cell receptor repertoire that is defective in both allogeneic and antiviral responses [36]. It is assumed that thymoproteasomes support positive selection by generating peptides optimized for the selection of weakly self-reactive, naïve T cell clones [37]. Since immunoproteasome subunits are also expressed in thymic cells they might influence positive and negative selection processes by producing an altered MHC-I peptidome. Indeed, several studies have demonstrated that immunoproteasomes determine the CTL repertoire [6, 13, 34, 43, 45, 46]. Osterloh et al. found that one of the self-peptides responsible for positive selection of transgenic ovalbumin-specific OT-I T cells, which is derived from the F-actin capping protein (Cpalpha1), is efficiently generated only by immunoproteasomes [43]. Furthermore, OT-I mice lacking LMP7 expression showed a 50% reduction of SIINFEKL-specific T cells. This data indicate that LMP7 can influence the naïve T cell repertoire. Nevertheless, since cTECs are mediating positive selection [47] and express β5t [35] it remains elusive how LMP7 can influence positive selection. Adoptive transfer experiments in LMP2-deficient mice revealed that the reduced immunogenicity of the influenza A virus-derived epitope $NP_{366-374}$ can be attributed to alterations in the CTL repertoire and not to the decreased capacity of $LMP2^{-/-}$ APCs to generate this CTL epitope. The frequency of influenza $PA_{224-233}$-specific CTLs is intact in $MECL1^{-/-}$ but impaired in $LMP2^{-/-}$ and $LMP7^{-/-}/MECL1^{-/-}$ mice [45]. In the LCMV system, an alteration in the T cell repertoire was described by the means of two different T cell epitopes [13, 34]. The number of $CD4^+8^+$ GP_{33-41}-specific transgenic P14-T cells was markedly reduced in the thymus of P14-tg $LMP2^{-/-}$ mice compared to P14-tg LMP2-proficient mice [34]. In MECL-1-deficient mice, the reduced $GP_{276-286}$-specific T cell response could be attributed to an altered T cell repertoire in these mice [13]. On a first glance, the reduced number of $CD8^+$ cells found in the spleen of MECL-1-deficient mice [13] might be attributed to an altered thymic T cell selection, but experiments with bone marrow chimeras demonstrated this to be a T cell intrinsic phenomenon due to altered homeostatic proliferation of T cells [48]. Recently, Kincaid et al. crossed mice lacking all three immunoproteasome subunits with mice lacking the thymosubunit β5t to generate mice lacking all four specialized proteasome β-subunits (4KO mice) [46]. These mice, expressing only constitutive subunits in all cells, had a profound defect in the generation of $CD8^+$ T cells. Analysis of T cell development in the 4KO mice revealed an impaired positive and negative selection in the thymus. This is consistent with a peptide-switching model wherein developing $CD8^+$ T cells need to be positively and negatively selected on different peptides for the establishment of a broad TCR repertoire. Thus, thymoproteasomes in cTECs produce unique peptides for positive selection, whereas negative selection occurs on peptides produced by constitutive, immunoproteasomes, or mixed proteasomes.

Using bone marrow chimeras (Fig. 6) and adoptive T cells transfer (Fig. 7) we could show that LMP7-deficient mice lack $GP_{118-125}$-specific T cell precursors. How can LMP7 affect the $GP_{118-125}$-specific T cell repertoire? Since LMP7 in the thymus is mainly expressed in cells mediating negative selection, we speculate that proteasomes lacking LMP7 in these cells generate a self-peptide mediating negative selection of $GP_{118-125}$-specific T cells. TCRs specific for a viral MHC-I-peptide complex can cross-react with multiple other MHC-I-peptide complexes, including self-peptides [49]. The cross-reactivity rather depends on similarities in the three-dimensional structure of the MHC-I-peptide complex and can be unrelated to the primary sequence [50]. Immunoproteasomes are induced during inflammation. Thus, the expression of immunoproteasomes in cells mediating negative selection helps to eliminate potential autoreactive T cells recognizing self-antigen in inflamed tissues. Nevertheless, since minute LMP7 expression was detected in cTEC preparations [35, 37, 51] we cannot completely rule out that LMP7 affects also positive selection of $GP_{118-125}$-specific T cells.

Our data support the idea that LMP7 is an important factor for filling up holes in the spectrum of T cell receptor specificities. A "hole" in the T cell repertoire may represent an important mechanism of viral persistence. Indeed, hepatitis C virus (HCV) immune escape can occur via exploitation of a hole in the T cell repertoire [52]. Although HCV elicits a substantial virus-specific immune response, HCV frequently persists. Wölfl et al. report for a naturally occurring mutation in a human $CD8^+$ T cell epitope that only affects TCR contact without altering peptide processing or MHC affinity. Due to a hole in the naïve T cell repertoire, $CD8^+$ T cells recognizing this escape variant peptide are lacking in most individuals.

Taken together, the lack of immunoproteasome in the thymus can lead to the elimination of virus-specific T cell precursors. Hence, we describe the immunoproteasome as a crucial factor in determining and completing the CTL repertoire.

Materials and methods

Mice, viruses, and media

C57BL/6 mice ($H\text{-}2^b$) were originally purchased from Charles River, Germany. MECL-1 [13], LMP2 [53], and LMP7 [32]

gene-targeted mice were kindly provided by Dr. John J. Monaco (Department of Molecular Genetics, Cincinnati Medical Center, Cincinnati, OH, USA). B6.PL (Thy1.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). PA28$\alpha^{-/-}/\beta^{-/-}$ [40] mice were contributed by T. Chiba (Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). RAG1$^{-/-}$ mice were provided by the Swiss Immunological Mutant Mouse Repository (SwIMMR). Mice were bred and kept in a specific pathogen-free facility and used at 6–10 weeks of age [54]. Animal experiments were approved by the Review Board of Governmental Presidium Freiburg of the State of Baden-Württemberg. All methods were carried out in accordance with the approved guidelines and regulations. Recombinant vaccinia virus encoding the LCMV glycoprotein (VVG2) was obtained from D. Bishop (Institute of Virology, Oxford, U.K.) and was propagated on BSC40 cells [55]. LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Mice were infected with 200 pfu LCMV-WE i.v. or 2×10^6 pfu VVG2 i.p. All media were purchased from Invitrogen-Life Technologies (Karlsruhe, Germany) and contained GlutaMAX, 10% FCS, and 100 U/mL penicillin/streptomycin.

Synthetic peptides

The synthetic peptides GP_{33-41} (KAVYNFATC), $GP_{276-286}$ (SGVENPGGYCL), $NP_{396-404}$ (FQPQNGQFI), $NP_{205-212}$ (YTVKYPNL), GP_{92-101} (CSVNNSHHYI), and $GP_{118-125}$ (LNHNFCNL) were obtained from P. Henklein (Charité, Berlin, Germany).

Proteasome inhibitor

Proteasome inhibitors were used as previously described [22]. Shortly, inhibitors specific for β5i (ONX 0914; formerly called PR-957) and β5c (PR-825) were obtained from Onyx Pharmaceuticals. Both inhibitors were dissolved at a concentration of 10 mM in DMSO and stored at −20°C [17]. For proteasome inhibition in mice, ONX 0914 was formulated in an aqueous solution of 10% (w/v) sulfobutylether-ß-cyclodextrin and 10 mM sodium citrate (pH 6) and administered to mice as an s.c. bolus dose of 10 mg/kg (in a volume of 100 μL). PR-825 was dissolved in 2% ethanol in saline and administered as an s.c. bolus dose of 2 mg/kg (in a volume of 100 μL) in mice.

Intracellular cytokine staining (ICS)

Analysis of T cell responses was performed as previously detailed [2, 56]. Briefly, splenocytes (1×10^6) were incubated in round-bottom 96-well plates with 10^{-6} M of the specific peptide in 100 μL IMDM 10% FCS + brefeldin A (10 μg/mL) for 5 h at 37°C. A neubauer chamber was used for counting. The staining, fixation, and permeabilization of the cells was performed exactly as detailed previously [8]. Cells were acquired with the use of the BD Accuri™ C6 flow cytometer system or BD FACSCalibur™ and analyzed with the FlowJo software (Tree Star). 100 000 lymphocytes (gated according to FSC/SSC) were acquired. Gating strategy is indicated in Fig. 1. Background IFN-γ production (without peptide stimulation) was between 0.1 and 0.6 as indicated. Raw data can be provided per request. The ICS was performed using well established laboratory protocols.

Adoptive transfer

Adoptive transfer was performed as previously described [13]. Shortly, CD8$^+$ T cells from splenocytes of Thy1.1-positive mice were isolated with the CD8a$^+$ T Cell Isolation Kit (Miltenyi Biotec). Purified CD8$^+$ T cells (1×10^7) were transferred i.v. into naive mice on day 1. On day 0, mice were infected with 200 pfu of LCMV-WE i.v. 8 days later, splenocytes were stained with anti-Thy1.1 or anti-Thy1.2 antibodies and analysed by flow cytometry. CD8$^+$ T cells from splenocytes of C57BL/6 or LMP7$^{-/-}$ mice were isolated with CD8a (Ly-2) MicroBeads (Miltenyi Biotec). 2×10^7 purified CD8$^+$ T cells were transferred i.p. into naive RAG1$^{-/-}$ mice. 4 weeks later, mice were infected with 200 pfu of LCMV-WE i.v. 8 days later, the CD8$^+$ T cell response in the spleen was analysed by intracellular cytokine staining for IFN-γ.

Antigen presentation assays

Antigen presentation assay and generation of LCMV-specific CTL-lines were performed as previously described [13, 55]. An additional density centrifugation step was conducted 1–2 days before using CTLs in antigen presentation experiments. For thioglycolate-elicited macrophages, CTLs were used in ICS at an E:S ratio of 0.2 in the first dilution and subsequent serial 3-fold dilution of stimulators.

CD11c$^+$ dendritic cells from splenocytes of LCMV-infected C57BL/6 or LMP7$^{-/-}$ mice were magnetically isolated with CD11c MicroBeads (Miltenyi Biotec). CTLs were used in ICS at an E:S ratio of 1.

Proteasome immunoprecipitation

Immunoprecipitation was performed as previously described [57]. Shortly, after extensive washing, equal amounts of macrophages or splenocytes were lysed and incubated with three microliters of polyclonal rabbit anti-mouse proteasome Ab [58] and 50 μL protein A microbeads (Miltenyi Biotec) for 30 min on ice. The lysate was applied to the microcolumn (Miltenyi Biotec), and the column was washed five times. Then, 50 μL fluorogenic peptide substrate (Suc-LLVY-AMC; Bachem) was applied to the column at a concentration of 100 μM. The μ column was incubated at 37°C for 30 min. A total of 100 μL lysis buffer was added, and the fluorescence intensity in the eluate was measured at an excitation

wavelength of 360 nm and emission wavelength of 465 nm on a fluorescence plate reader (Infinite M200 pro, TECAN).

Generation of bone marrow chimeras

Generation of bone marrow chimeras was performed as previously described [22]. Shortly, age- and sex-matched LMP7$^{-/-}$ or C57BL/6 recipient mice were lethally irradiated with 9.8 Gy and received 3×10^6 bone marrow cells from age- and sex-matched LMP7$^{-/-}$ or C57BL/6 donor mice on the same day by i.v. injection. Mice were rested 8 weeks before use in experiments.

TCR Vβ expression

TCR Vβ expression analysis was performed as previously described [59]. Shortly, thymocytes were screened for TCR Vβ expression by flow cytometry using PE-conjugated CD4 (GK1.5) and APC-conjugated CD8 (53-6.7) antibodies (both eBioscience, Frankfurt, Germany) in conjunction with a Vβ TCR screening panel (Becton Dickinson, Heidelberg, Germany) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed as previously described [56]. Shortly, the statistical significance was determined using unpaired Student t test. All statistical analyses were performed using GraphPad Prism Software (version 4.03) (GraphPad, San Diego, CA). Statistical significance was achieved when $p<0.05$. $*p<0.05$; $**p<0.01$; $***p<0.001$. If not indicated, data sets did not show significant differences.

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Abbreviations: **APC**: antigen presenting cell · **cTEC**: cortical thymic epithelial cells · **CTL**: cytotoxic T lymphocyte · **E:S**: effector to stimulator · **GP**: glycoprotein · **ICS**: intracellular cytokine staining · **IFN**: interferon · **LCMV**: lymphocytic choriomeningitis virus · **LMP**: low molecular mass polypeptide · **MECL-1**: multicatalytic endopeptidase complex-like–1 · **mTEC**: medullary thymic epithelial cells · **NP**: nucleoprotein · **TCR**: T cell receptor · **TNF**: tumor necrosis factor

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