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Immunoproteasomes are essential for survival and expansion of T cells in virus-infected mice

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Immunoproteasomes containing the IFN-inducible subunits β1i (LMP2), β2i (MECL-1) and β5i (LMP7) alter proteasomal cleavage preference and optimize the generation of peptide ligands of MHC class I molecules. Here, we report on an unexpected new function of immunoproteasome subunits for the survival and expansion of $CD4^+$ and $CD8^+$ T cells during viral infection of mice. The effect of immunoproteasome subunit deficiency on T-cell survival upon adoptive transfer was most prominent for the lack of LMP7 followed by MECL-1 and LMP2. The survival of T cells in uninfected mice or the homeostatic expansion after transfer into RAG-$2^{-/-}$ mice was not affected by the lack of the immunosubunits. Lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells lacking LMP7 or MECL-1 started to divide after transfer into LCMV-infected mice but experienced a considerable cell loss within 2 days after transfer. We provide strong evidence that the loss of immunoproteasome-deficient T cells after transfer is not a consequence of graft rejection by the host, but instead is based on the requirement for immunoproteasomes for the survival of T cells in LCMV-infected mice. Therefore, the immunoproteasome may qualify as a potential new target for the suppression of undesired proinflammatory T-cell responses.

Key words: Cytotoxic T lymphocytes · Immunoproteasome · LCMV · MHC class I

Introduction

The proteasome core complex, referred to as 20S proteasome, is a cylinder-shaped structure consisting of 28 subunits, arranged in four stacked rings. The two outer rings, each made up of seven α-type subunits (α_1–α_7) are framing the two inner rings, each composed of seven β-type subunits (β_1–β_7). The catalytic activity is performed by three β-subunits of each inner ring: β1 (δ), β2 (MC14) and β5 (MB1). In the course of an immune response, the constitutive β-subunits are replaced in newly assembled proteasomes by the IFN-γ- and TNF-α-inducible subunits β1i (LMP2), β5i (LMP7) and β2i (MECL-1) [1], thereby building so-called immunoproteasomes [2]. Immunoproteasomes cleave Ag with a different cleavage preference [3, 4], thus optimizing the quantity and quality of the generated peptides for presentation by MHC class I molecules [5–8].

In the previous experiments, designed to characterize MECL-1-deficient mice, we observed that in contrast to WT T cells, MECL-$1^{-/-}$ T cells were unable to expand in lymphocytic choriomeningitis virus (LCMV)-WE infected mice [9]. These find-

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ings were in accordance with the previous experiments performed with LMP2 and LMP7 × MECL-1 gene-targeted mice. After adoptive transfer of these T cells, followed by an influenza virus infection of the recipient WT mice, neither LMP2$^{-/-}$ nor LMP7$^{-/-}$ × MECL-1$^{-/-}$ T cells were able to expand to the same extent as C57BL/6 WT cells [7, 10]. As a possible explanation, the authors suggest rejection of donor T cells by the host immune response because of either reduced surface MHC expression by LMP7$^{-/-}$ T cells [11] or differences in minor histocompatibility Ag (miHAg). However, it was never thoroughly investigated whether the attenuation of immunoproteasome-deficient T cells in virus infected mice was indeed an artifact of the T-cell transfer experiment based on a host *versus* graft reaction or whether a so far unknown function of immunoproteasome subunits for T-cell survival or expansion could underlie this phenomenon.

An independent hint that immunoproteasome subunits may play a so far unappreciated role for T-cell differentiation and/or expansion were the 20–30% reduced number of CD8$^+$ as compared with CD4$^+$ T cells in lymphoid organs of LMP2$^{-/-}$ [12] and MECL-1$^{-/-}$ [9] mice. Reconstitution experiments of irradiated WT mice with BM from WT and LMP7$^{-/-}$MECL-1$^{-/-}$ mice showed that the lower CD8$^+$/CD4$^+$ ratio remained among the LMP7/MECL-1 double-deficient T cells although they were selected in the same thymus of recipient mice as WT cells with a normal CD8$^+$/CD4$^+$ ratio. This result indicated that the selective reduction of CD8$^+$ T cells lacking LMP7 and MECL-1 was a T-cell intrinsic phenomenon not related to altered Ag presentation in the thymus [13].

In this study, we show that a functional requirement for immunoproteasome subunits rather than graft rejection accounts for the loss of LMP2$^{-/-}$, MECL-1$^{-/-}$ and LMP7$^{-/-}$ T cells in virus-infected mice and hence document a novel function of immunoproteasomes which is unrelated to their function in Ag processing.

Results

Immunoproteasome-deficient T cells cannot compete with WT T cells of virus-infected mice

To investigate the proliferative performance of immunoproteasome-deficient T cells elicited by an LCMV-WE infection in a WT environment, we adoptively transferred MECL-1$^{-/-}$-, LMP2$^{-/-}$-, LMP7$^{-/-}$- or C57BL/6- T cells (all of them carrying the Thy1.2 marker) into LCMV-WE-infected Thy1.1 recipient mice. Eight days post-infection, C57BL/6-derived donor T cells proliferated to an extent of 2.55 ± 0.03% of total lymphocytes, whereas mice that received LMP2$^{-/-}$ T cells comprised only 1.29 ± 0.07% donor T cells. In mice having received MECL-1$^{-/-}$ T cells, we could hardly detect any donor cells on day 8 after infection (0.54 ± 0.17% of total lymphocytes) and a similar loss of the graft was observed for mice which had received LMP7$^{-/-}$ T cells (0.18 ± 0.03%) (Fig. 1A and B).

To document the kinetics of donor T-cell expansion, we injected naïve MECL-1$^{-/-}$ or C57BL/6 control T cells into LCMV-WE-infected WT mice and analyzed the presence of donor T cells in blood on several days after transfer (Fig. 1C and D). Since the endogenous LCMV-specific T cells of the host mouse also received an activation stimulus by the viral infection, the percentage of C57BL/6 WT donor T cells diminished in face of a vigorously expanding host population from day 5 (1.39%) to day 8 (0.5%), when 3×10^7 T cells were transferred (Fig. 1C). Briefly, 7×10^7-injected T cells (Fig. 1D) seem to approach the number of endogenous LCMV-specific T cells, as they could successfully compete with them in their proliferative response, visible in an increasing rather than decreasing relative percentage of C57BL/6 donor T cells (day 5: 5.46% and day 8: 6.8%). However, the percentage of MECL-1$^{-/-}$ donor-derived T cells was reduced compared with the WT donor T cells, starting on day 5 or 6, regardless of the number of transferred T cells.

The expression of immunoproteasomes in T cells was verified by Western analysis of T cells derived from naïve C57BL/6, MECL-1$^{-/-}$, LMP2$^{-/-}$ and LMP7$^{-/-}$ mice (Supporting Information Fig. 1). To ensure that T cells lacking immunoproteasome subunits do not suffer from homing failures, we monitored the migration of the LMP7$^{-/-}$ (Supporting Information Fig. 2A) and MECL-1$^{-/-}$ (Supporting Information Fig. 2B) donor-derived T cells to spleen, peritoneum, popliteal LN, medial iliac LN and blood of the LCMV-WE-infected recipient mouse. LMP7$^{-/-}$ and MECL-1$^{-/-}$ T cells transferred into Thy1.1 mice did not display divergent homing characteristics compared with C57BL/6 T cells. But, as anticipated, cells originating from LMP7$^{-/-}$ or MECL-1$^{-/-}$ donors, respectively, were far below the number of WT donor cells in all organs examined.

Recipient mice do not reject adoptively transferred immunoproteasome-deficient T cells

The fact of a diminished MHC class I surface expression on LMP7 gene-targeted T cells and the potential presence of differing miHAg, that could arise due to altered proteasome compositions, necessitates the exclusion of rejection processes as potential cause for the impaired expansion of adoptively transferred immunoproteasome-deficient donor T cells. It has been shown that the rejection of tg CD4$^+$ T cells carrying miHAg takes approximately 21 days [14] and, to quote a second well-studied miHAg, 40–75% of male hematopoetic cell grafts survive in female recipients at day 10 after transfer [15]. As we are injecting only T cells but no professional APC, we assume that the rejection process would take even longer. But, as shown in Fig. 1, depending on the immunoproteasome subunit missing, most transferred T cells had disappeared by day 8 post-infection.

To further rule out rejection phenomena, we transferred a 1:1 mixture of C57BL/6 WT and MECL-1$^{-/-}$ T cells into naïve Thy1.1 mice. Control- and immunoproteasome-deficient T cells could be discriminated by their CFSE intensity (C57BL/6: CFSE low; MECL-1$^{-/-}$: CFSE high). One day after transfer, we bled the mice

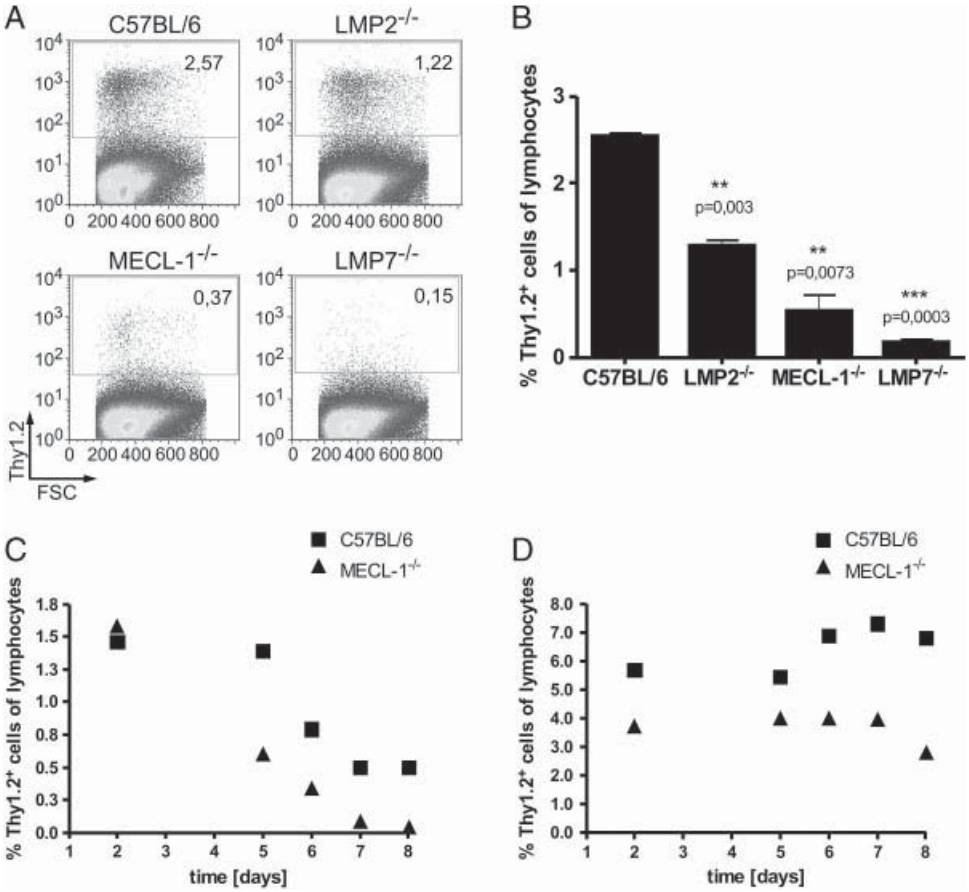

Figure 1. Reduced expansion of adoptively transferred immunoproteasome-deficient T cells in LCMV-WE infected WT mice. (A and B) Magnetically purified Thy1.2$^+$ T cells (2.5×10^7) of naïve LMP2$^{-/-}$, LMP7$^{-/-}$, MECL-1$^{-/-}$ or C57BL/6 control mice were i.v. transferred into different Thy1.1 mice, and at the same time, mice were infected with 200 PFU LCMV-WE. Eight days post-infection, splenocytes were stained for Thy1.2 (transferred T cells) and analyzed by flow cytometry (A). The numbers in the dot plots indicate the percentages of cells. (B) The percentage of Thy1.2$^+$ cells $\pm$ SD, recovered from the spleen of host mice 8 days post-infection/transfer is shown. The experiments, each with two Thy1.1 recipient mice receiving cells from one genotype have been repeated three times, yielding similar results; *p*-values were calculated according to Student's unpaired *t*-test. (C and D) Kinetic of T-cell expansion in blood of LCMV-WE-infected host. (C) 3×10^7 or (D) 7×10^7 magnetically enriched MECL-1$^{-/-}$ or C57BL/6 control T cells were adoptively transferred into naïve Thy1.1 WT mice that were infected with 200 PFU LCMV-WE i.v. immediately after T-cell transfer. On days 2, 5–8 after T-cell transfer and viral infection, the percentages of Thy1.2$^+$ lymphocytes in the blood of recipient mice were determined by staining with anti-Thy1.2 antibodies and flow cytometry analysis. The experiment has been performed three times with three mice each yielding similar results.

to confirm that all animals started with a 1:1 ratio of WT- and MECL-1$^{-/-}$ T cells. The percentage of MECL-1$^{-/-}$ cells remained stable over the whole time period (day 4: 39.8% and day 7: 42.2% CFSE high CD8$^+$ T cells), arguing against the rejection of MECL-1-deficient T cells in uninfected recipient mice (Supporting Information Fig. 3A).

Being aware of the possibility that LMP7 gene-targeted T cells might be rejected by NK cells due to a diminished MHC expression [11], we injected T cells of LMP7$^{-/-}$ or C57BL/6 mice into Thy1.1 mice that were either LCMV-WE infected or remained naïve. Nine days after transfer, the LMP7$^{-/-}$ T cells were hardly detectable in the virus-infected mice, but comparable numbers of WT (1.025% cells) and gene-targeted (0.815% cells) T cells were found in the naïve animals (Supporting Information Fig. 3B). In a further approach to exclude rejection phenomena, we adoptively transferred T cells derived from LMP2$^{-/-}$, LMP7$^{-/-}$, MECL-1$^{-/-}$ and C57BL/6 mice into different naïve Thy1.1 mice and monitored their persistence in blood on day 2 and day 10 and in spleen on day 22 after transfer. There were no statistically significant differences between the various donor T cells on day 2 or day 10, but we noted a reduction in particular of LMP2-deficient donor T cells in spleen 22 days after transfer (Supporting Information Fig. 3C). Whether this was due to rejection of donor cells or failures in homeostatic proliferation or deregulation of some protein factor controlled by the function of immunoproteasomes has not yet been investigated.

In order to directly compare the loss of LMP7 gene-targeted T cells in an LCMV-WE-infected recipient mouse to rejection processes due to miHAg, we injected a 1:1 mixture of female LMP7$^{-/-}$ T cells and female or male Thy1.1 WT T cells into naïve or LCMV-WE-infected female CD45.1 congenic mice. The sex-chromosome encoded HY-Ag of the male Thy1.1 WT donor cells are recognized as foreign in the female recipients and will eventually induce a T-cell response resulting in the rejection of the male T cells [15]. Mice were bled on day 1 and day 4 after

transfer and sacrificed on day 8 after transfer to analyze the $CD8^+$ T-cell population in blood (day 1 and day 4; Fig. 2A and B) or spleen (day 8; Fig. 2C) for the percentage of WT and gene-targeted donor cells. In naïve recipient mice, all donor T cells (female/male WT and $LMP7^{-/-}$) were slightly reduced in number, but were still present at similar levels after 4 and 8 days (Fig. 2D and F). However, in LCMV-WE-infected host mice, LMP7-deficient T cells were substantially decreased already on day 4 and hardly detectable on day 8 after transfer. On the contrary, the percentages of Thy1.1 WT donor T cells in the same recipient mice were maintained from day 1 to day 8 after transfer, regardless of the gender of the T cells and thus regardless of the presence or absence of HY miHAg (Fig. 2E and G). Taken together, these data indicate that the inability of LMP7 gene-targeted T cells to survive in an LCMV-WE-infected recipient is unrelated to miHAg-induced rejection processes.

In an attempt to better define the reason why immunoproteasome-deficient T cells cannot survive in the LCMV-infected host, we monitored the expression of a panel of cluster of differentiation molecules on different hematopoetic cells and at different time points of the antiviral immune response to identify potential deregulations regarding homeostatic proliferation, homing to peripheral lymphoid organs, activation, migration to the source of infection or apoptosis. In none of the analyzed tissues and at no time point, significant differences in the expression of the indicated marker molecules between C57BL/6 WT and immunoproteasome deficient mice were detectable (Supporting Information Table 1).

Unaltered homeostatic and mitogen-induced T-cell expansion

Next, we investigated whether the homeostatic expansion of MECL-1, LMP2 and LMP7 single knockout T cells was disturbed. To this aim, we monitored the reconstitution of the T-cell repertoire in RAG-2-deficient mice, after injection of a 1:1 mixture of WT ($Thy1.1^+$) and either $LMP2^{-/-}$ or $LMP7^{-/-}$ or $MECL\text{-}1^{-/-}$ or C57BL/6 T cells (Supporting Information Fig. 4). The development of $Thy1.1^+$ WT donor cells and the corresponding $Thy1.2^+$ immunosubunit-deficient T cells in one $RAG\text{-}2^{-/-}$ recipient was monitored from day 2 to 2 months after transfer (Supporting Information Fig. 4A–D). There were no differences detectable in the homeostatic expansion of single knockout T cells compared with WT T cells.

Caudill *et al.* reported on hyperproliferating $CD4^+$ and $CD8^+$ $MECL\text{-}1^{-/-} \times LMP7^{-/-}$ but not single knockout T cells in response to anti-CD3/CD28 or PMA/ionomycin stimulation as

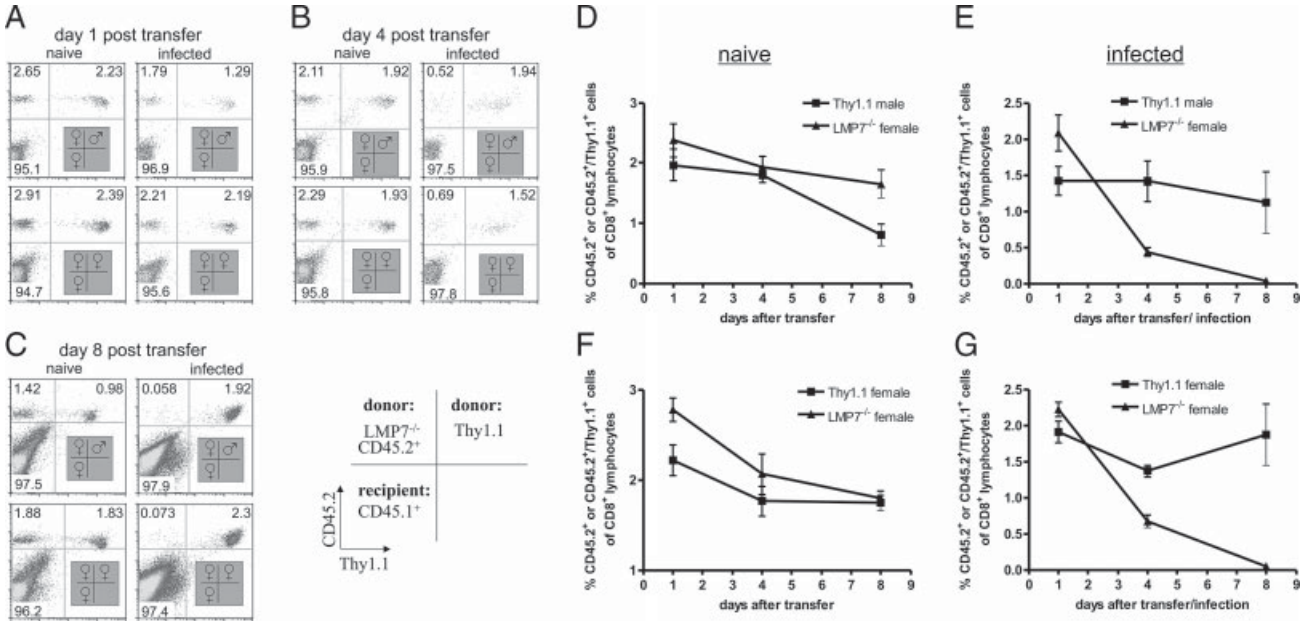

Figure 2. HY miHAg do not cause T-cell rejection within 8 days. T cells of female $LMP7^{-/-}$ and both female and male Thy1.1 WT mice were magnetically enriched by depletion of non-T cells. Briefly, 1.5×10^7 cells of a 1:1 mixture of $LMP7^{-/-}$ and Thy1.1 female or $LMP7^{-/-}$ and Thy1.1 male T cells were i.v. injected into female CD45.1 congenic mice. The recipient mice were infected with 200 PFU LCMV-WE i.v. immediately after T-cell transfer or remained naïve. In total, (A) 1 and (B) 4 days after transfer, blood of the recipient mice was analyzed for the percentage of $LMP7^{-/-}$ ($CD45.2^+$, $Thy1.1^-$) and Thy1.1 ($CD45.2^+$, $Thy1.1^+$) donor $CD8^+$ T cells by flow cytometry. (C) On day 8 after transfer, mice were sacrificed and splenocytes were analyzed as described for blood. Dot plots in the upper row represent the transfer of mixtures of WT male+$LMP7^{-/-}$ cells; dot plots in the row below represent the transfer of WT female+$LMP7^{-/-}$ cells. Gated $CD8^+$ lymphocyte populations are shown in dot blots, showing staining for Thy1.1 (*x*-axis) *versus* CD45.2 (*y*-axis). The experiment was performed with two naïve and three infected CD45.1 recipient mice receiving a mixture of WT male+$LMP7^{-/-}$ or WT female+$LMP7^{-/-}$ cells, respectively. The numbers indicate the percentage of cells in the respective quadrants. (D–G) The figures sum up the data acquired from all recipient mice and all time points analyzed. Data points represent mean ± SD ($n = 3$). (D) Survival of male Thy1.1 and female $LMP7^{-/-}$ T cells in the same naïve or (E) LCMV-WE-infected recipient. (F) Survival of female Thy1.1 and female $LMP7^{-/-}$ T cells in the same naïve or (G) LCMV-WE-infected recipient.

well as during mixed lymphocyte reactions [16]. To address the mitogen-induced T-cell expansion, we stimulated CFSE-labeled splenic T cells from LMP7$^{-/-}$ × MECL-1$^{-/-}$ mice, for 48 h (data not shown), 72 and 96 h (data not shown) *in vitro* with either plate-bound anti-CD3/CD28 (Supporting Information Fig. 5A) or PMA/ionomycin (Supporting Information Fig. 5B). Neither CD4$^+$ nor CD8$^+$ LMP7$^{-/-}$ × MECL-1$^{-/-}$ T cells showed a significant hyperproliferation at any time point and activating signal used. In accordance with this, in mixed BM chimeric mice it was shown that LMP7$^{-/-}$ × MECL-1$^{-/-}$ T cells expanded to the same extent as immunoproteasome-expressing T cells in response to bacterial infections [13]. A mitogen-induced hyperproliferation is therefore unlikely to be the underlying mechanism why T cells lacking single immunoproteasome subunits do not persist in the LCMV-infected host.

Expansion of T cells in *Listeria monocytogenes*- or vaccinia virus-infected host mice

To examine whether we are facing a pathogen-specific effect, we also transferred T cells of the different immunoproteasome subunit deficient and WT mice in naïve Thy1.1 mice that were either infected with vaccinia Virus (VV-WR) or with recombinant *Listeria monocytogenes* expressing OVA (rLM-OVA). There were no differences in T-cell expansion between the different mouse strains in rLM-OVA-infected recipient mice (Supporting Information Fig. 6C) and only slightly reduced numbers of LMP2$^{-/-}$ (0.59 ± 0.06%), LMP7$^{-/-}$ (0.36 ± 0.04%) and MECL-1$^{-/-}$ (0.55 ± 0.02%) derived CD8$^+$ T cells compared with the CD8$^+$ T-cell population of the WT donors (0.73 ± 0.04%) in VV-WR-infected mice (Supporting Information Fig. 6A). However, the percentage of LMP7$^{-/-}$-derived CD4$^+$ T cells (3.89 ± 0.21%) was clearly decreased in VV-WR-infected WT mice, compared with immunoproteasome expressing CD4$^+$ T cells (7.62 ± 0.4%), LMP2$^{-/-}$ or MECL-1$^{-/-}$ CD4$^+$ T cells (Supporting Information Fig. 6B).

Both LMP7-deficient CD8$^+$ and CD4$^+$ T cells vanish in LCMV-infected mice

So far, we had mainly used CD8$^+$ T cells to study a requirement of immunoproteasomes during antiviral immune responses. To investigate other leukocyte populations, we investigated the development of adoptively transferred LMP7$^{-/-}$ CD4$^+$ T cells (CD4$^+$), B cells (CD19$^+$), DC (CD11c$^+$) and NK cells (NK1.1$^+$) in naïve and LCMV-WE infected WT hosts compared with the corresponding endogenous cell types. Six days after transferring total splenocytes of LMP7$^{-/-}$ (CD45.2$^+$) or C57BL/6 mice (CD45.2$^+$), the numbers of donor-derived CD4$^+$, CD8$^+$, CD19$^+$, CD11c$^+$ and NK1.1$^+$ cells in CD45.1 recipient mice were determined. In the absence of LCMV infection, the numbers of cells lacking or expressing LMP7 were equal for all cell types analyzed (Fig. 3A). On the contrary, in LCMV-WE-infected host mice, the percentage of LMP7$^{-/-}$ cells was markedly reduced compared with C57BL/6 cells with CD4$^+$, CD8$^+$ and CD11c$^+$ cells being hardly detectable (Fig. 3B). The loss of CD11c$^+$ cells does most likely not represent a loss of DC but rather T cells which have been shown to upregulate CD11c expression during LCMV infection [17]. Almost all remaining donor LMP7$^{-/-}$-derived cells were B cells and also these were significantly reduced compared with WT donor B cells.

The almost complete loss of LMP7-deficient CD4$^+$ and CD8$^+$ T cells in the infected mice in face of a relative persistence of B cells argues by itself against an MHC class I-dependent rejection phenomenon being the cause of the loss of LMP7$^{-/-}$ T cells because flow cytometric analysis of transferred B cells and CD8$^+$ T cells showed a similar cell surface expression of H-2K^b and a slightly higher expression of H-2D^b on B cells. To better document this finding, we simultaneously transferred sorted B220$^+$ B cells and CD8$^+$ T cells from CD45.2$^+$ WT or LMP7$^{-/-}$ donor mice into CD45.1$^+$ WT recipient mice and monitored the survival of B cells and T cells up to day 8 post-transfer. Although the LMP7$^{-/-}$CD8$^+$ T cells had almost completely disappeared by day 8, LMP7$^{-/-}$ B cells survived in the same mouse (Fig. 3C) which is inconsistent with a rejection based on different peptide/MHC I complexes displayed on the surface of LMP7$^{-/-}$ T cells. Instead, this finding points at a function of immunoproteasomes for the expansion and/or survival in the virus-infected host which is particularly crucial for T cells.

Monitoring the proliferation and apoptosis of LCMV-WE-specific LMP7$^{-/-}$ and MECL-1$^{-/-}$ gene-targeted T cells *in vivo*

As immunoproteasome-compromised T cells fail to expand in response to LCMV-WE infections, we crossed LMP7$^{-/-}$ and MECL-1$^{-/-}$ mice with P14 mice, which are TCRtg for the LCMV-WE MHC class I epitope GP33 (glycoprotein derived, aa 33–41). With these mice, we were able to track the *in vivo* expansion of virus-specific CD8$^+$ T cells that lack LMP7 or MECL-1, respectively. CFSE-labeled T cells from P14, P14 × LMP7$^{-/-}$ and P14 × MECL-1$^{-/-}$ mice were analyzed 16 and 40 h after transfer for the ag-induced T-cell proliferation and for apoptotic/necrotic events within the donor population. Already 16 h after transfer, the average percentage of CD8$^+$CFSE$^+$ T cells in infected Thy1.1 mice receiving P14 T cells was only 47% of the percentage of CD8$^+$CFSE$^+$ T cells in the corresponding naïve recipient (Fig. 4A). This decrease by more than 50% was probably due to proliferating host cells, which had already been infected for 24 h when the donor cells encountered Ag for the first time. Nevertheless, in mice receiving P14 × LMP7$^{-/-}$ T cells only 24.7% (of the percentage in naïve mice) and in mice receiving P14 × MECL-1$^{-/-}$ T cells only 33.7% could be recovered 16 h after transfer (Fig. 4A), pointing to either selective loss or impaired expansion of these cells. The differences were even more prominent 40 h after transfer. Although immunoproteasome compromised T cells did proliferate, as apparent from the different CFSE dilution steps, proliferating P14 CFSE$^+$CD8$^+$ T cells reached up to 92% of the CFSE$^+$CD8$^+$

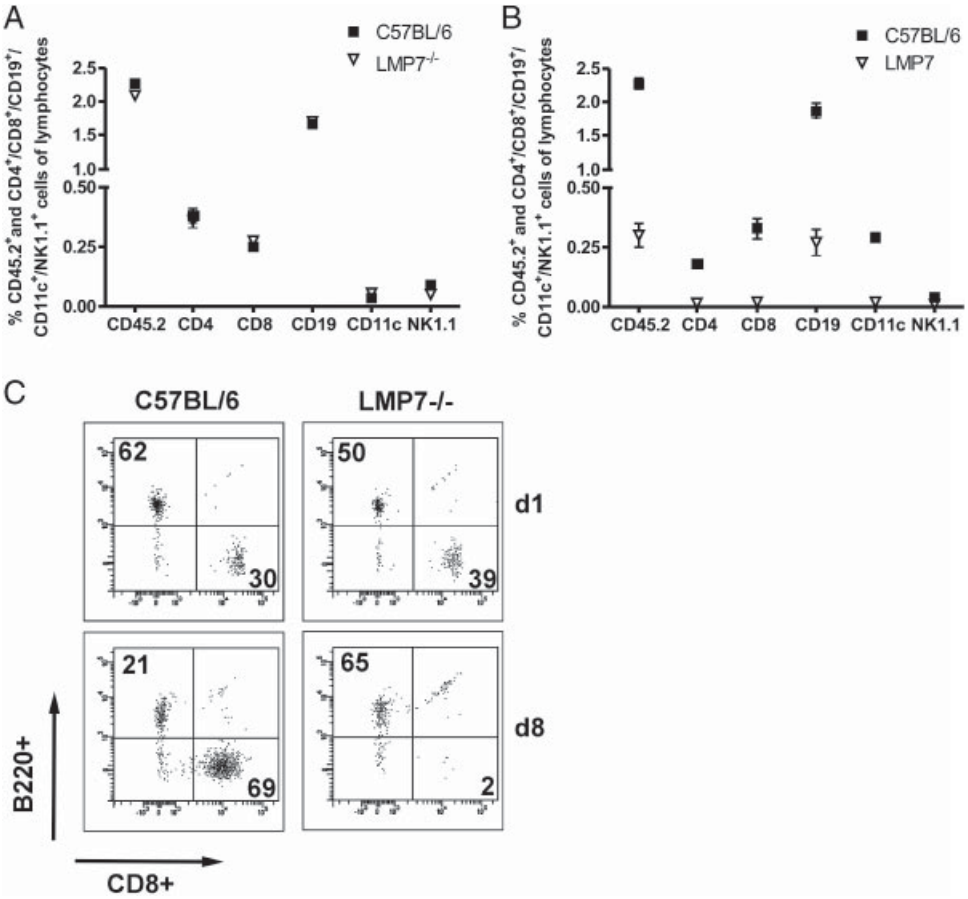

Figure 3. Impact of immunoproteasome deficiency on different leukocyte populations during an antiviral immune response. In brief, 3×10^7 total splenocytes of C57BL/6 WT or LMP7$^{-/-}$ mice were i.v. transferred into different (A) naïve or (B) LCMV-WE-infected (200 PFU i.v., immediately after cell transfer) CD45.1 congenic mice. Six days after transfer and LCMV-WE infection, recipient mice were sacrificed and splenocytes were costained for CD45.2$^+$ cells (to identify the donor-derived cells) and for CD4, CD8, CD19, CD11c and NK1.1 surface markers followed by flow cytometric analysis. One out of the two representative experiments, with two (or one) naïve and four (or three) infected recipient mice *per* genotype, is shown. Data points represent mean $\pm$ SD. (C) B220$^+$ B cells and CD8$^+$ T cells from either WT (left) or LMP7-deficient (right) CD45.2$^+$ donor mice were magnetically sorted and transferred into CD45.1$^+$ recipient mice which were simultaneously infected with 200 PFU LCMV-WE. On days 1 and 8 after transfer, blood cells of recipient mice were stained for CD45.2, CD8 and B220 surface expression and analyzed by flow cytometry. Shown are dot plots of B220 *versus* CD8 expression of donor cells (gated for CD45.2$^+$ cells). The experiment has been reproduced three times with two mice each.

cells in the corresponding naïve recipient, whereas P14 $\times$ LMP7$^{-/-}$ and P14 $\times$ MECL-1$^{-/-}$ T cells added up to only 51.72 and 50%, respectively (Fig. 4B). To test if evidence for hyperproliferation of donor P14 $\times$ LMP7$^{-/-}$ and P14 $\times$ MECL-1$^{-/-}$ cells can be obtained, we analyzed the percentage of CD8$^+$ donor cells passing the different cell division steps 40 h after transfer (Fig. 4C). P14 and P14 $\times$ LMP7$^{-/-}$ CD8$^+$ cells were distributed very similarly between the different cell division steps. The proliferation of P14 $\times$ MECL-1$^{-/-}$ T cells was lagging behind, since about 45% of all CFSE$^+$CD8$^+$ cells did not divide at all at this time point, but the ones dividing were doing it with a similar kinetic like P14 or P14 $\times$ LMP7$^{-/-}$ T cells. Taken together, we did not obtain any evidence for hyperproliferation of Ag-stimulated CD8$^+$ T cells lacking either LMP7 or MECL-1 *in vivo*. However, whether a possible episode of hyperproliferation is followed by immediate apoptosis cannot be ruled out by these experiments.

Accordingly, we investigated whether or not immunoproteasome-compromised T cells display irregularities in the controlling and timing of apoptotic events after TCR stimulation. For this purpose, the percentage of apoptotic and dead donor-derived CD8$^+$ cells was determined in parallel to the T-cell expansion studies. The percentage of apoptotic (Annexin$^+$/To-Pro-3$^-$) cells in the population of P14 $\times$ LMP7$^{-/-}$ donor T cells exceeded that of P14 WT and non-TCRtg LMP7$^{-/-}$-derived donor cells by approximately 40% 16 h after transfer in LCMV-WE-infected mice (Fig. 5A and B). If the recipient mice were left uninfected, the different donor genotype-derived cells did not differ in the percentage of apoptotic cells 16 and 40 h after transfer (Fig. 5A–D) and the same was true for all donor cells analyzed in LCMV-WE-infected recipients 40 h after transfer. LCMV-WE-infected and naïve host cells and the corresponding donor TCRtg CD8$^+$ T cells were analyzed for the expression of a panel of cluster of differentiation molecules known to be involved in T-cell activation, migration, downregulation and homeostasis. However, immunoproteasome compromised donor T cells

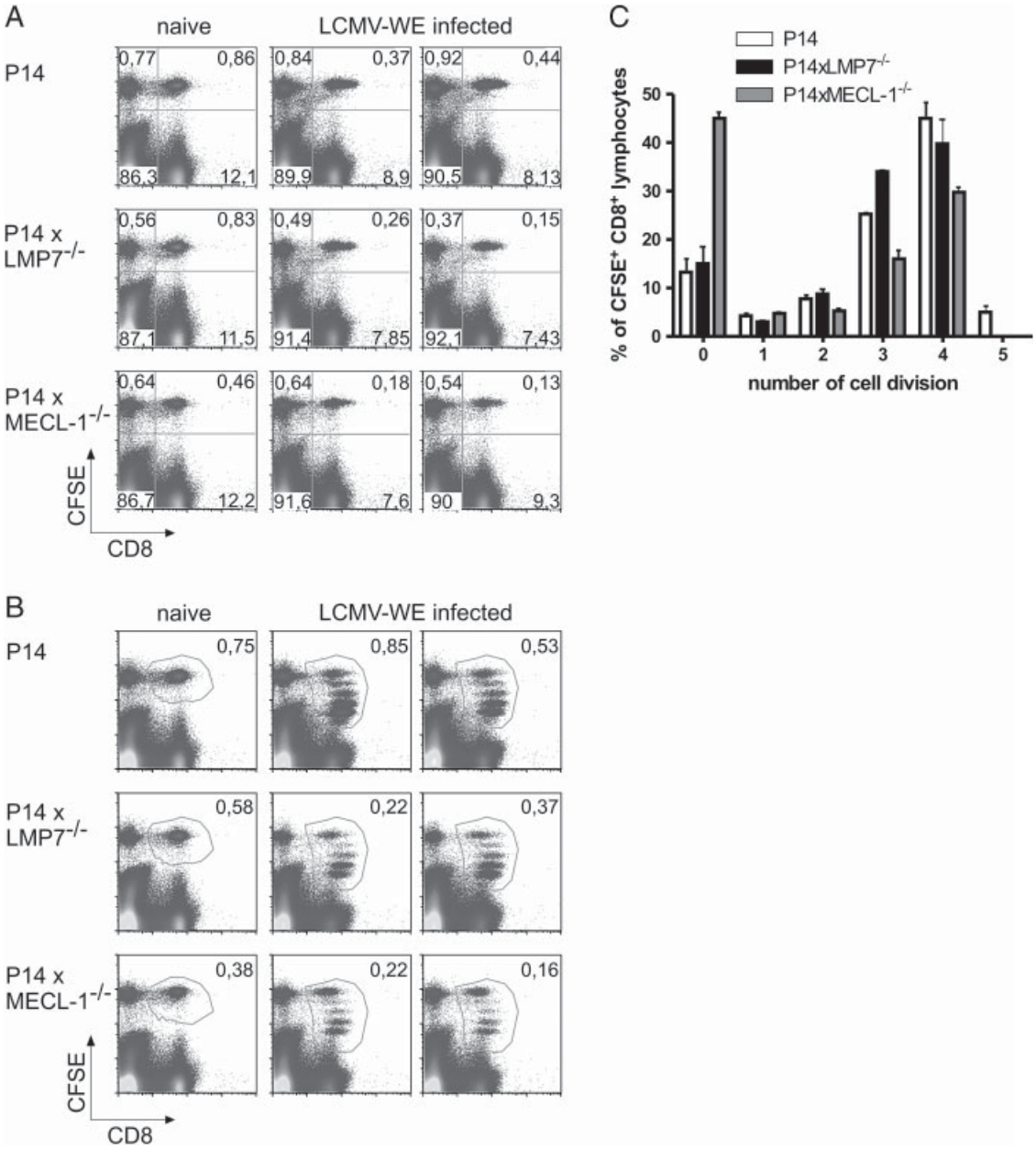

Figure 4. Analysis of adoptively transferred LMP7$^{-/-}$ and MECL-1$^{-/-}$ TCRtg T cells. (A) In total, 2×10^7 magnetically purified T cells of naïve P14, P14 × LMP7$^{-/-}$ and P14 × MECL-1$^{-/-}$ mice were fluorescently labeled (CFSE) and i.v. transferred into different naïve or LCMV-WE infected (2×10^4 PFU i.v., 24 h before T-cell transfer) Thy1.1 mice. Totally, 16 h (A) and 40 h (B) after transfer, recipient mice were sacrificed, splenocytes were stained for CD8 (*x*-axis) and proliferation of transferred TCRtg T cells was analyzed by means of CFSE dilution (*y*-axis) with the help of flow cytometry. (C) The percentage ± SD of transferred CD8$^+$ cells that are passing the indicated number of cell division; the sum of all CFSE$^+$CD8$^+$ cells of one donor genotype being distributed between the different cell division states is set to 100%. The results are representative of four separate experiments, each with two infected mice and one naïve recipient mouse *per* time point and genotype.

displayed no altered expression levels for any of the listed molecules compared with WT donor T cells (Supporting Information Table 2).

In summary, only TCRtg donor cells in infected host mice displayed enhanced levels of apoptotic cells at very early time points, leading to the presumption that either the TCR stimulation or the cytokine storm induced by the high quantity of LCMV-specific donor cells deliver signals which can only be accommodated in the presence of functional immunoproteasomes very early after infection.

Discussion

Mice lacking the immunoproteasome subunits LMP2, LMP7 and MECL-1 are known to have mild phenotypes. Although clear

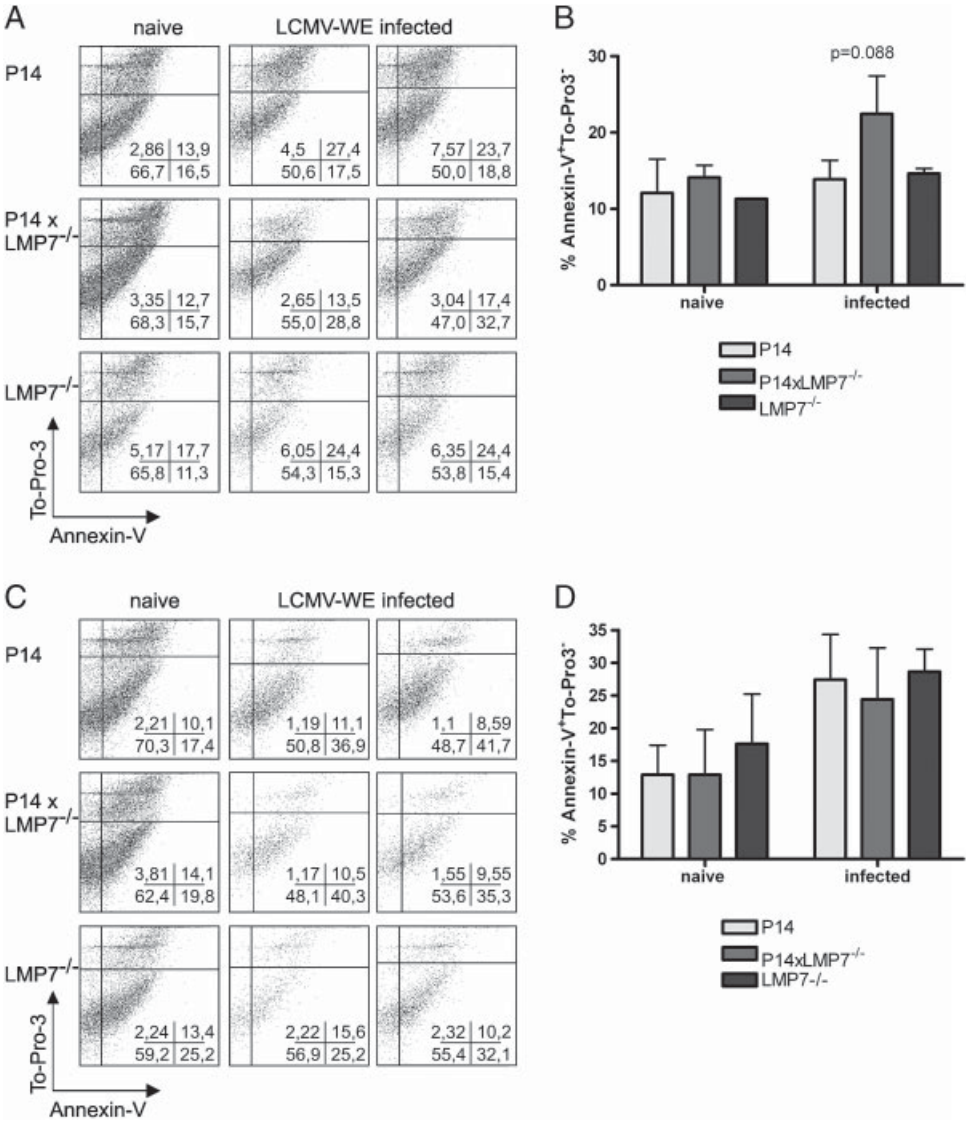

Figure 5. P14 × LMP7$^{-/-}$ T cells undergo apoptosis early after transfer in LCMV-WE-infected Thy1.1 WT mice. The experiment was performed as described in Fig. 4, with the exception of replacing P14 × MECL-1$^{-/-}$ donors by non-TCRtg LMP7$^{-/-}$ mice. Briefly, 16 h (A and B) and 40 h (C and D) after transfer, all CFSE-positive cells (donor derived) were subjected to an annexin (*x*-axis) and dead cell (To-Pro-3, shown on *y*-axis) staining. Dot plots represent CD8$^+$CFSE$^+$ cells gated as shown in Fig. 4B. Three additional experiments with similar outcomes (shown in (B and D); mean ± SEM) have been performed; *p*-values were calculated according to Student's unpaired *t*-test.

differences in the generation of selected CTL epitopes have been documented, the mice could readily cope with a whole array of viruses and bacteria including LCMV, VV and listeria with similar efficiency as WT control mice. It was only after transfer of LMP2$^{-/-}$, LMP7$^{-/-}$ and MECL-1$^{-/-}$ T cells into a virus-infected WT host that a deficiency of these cells to expand and survive was noted [7, 9]. Recently, Hensley *et al.* observed a partial loss of transferred LMP2$^{-/-}$ cells even in naïve mice [18]. A trivial explanation for the loss of transferred immunoproteasome-deficient cells would be that the transferred cells were specifically recognized and rejected by host T cells. In this study, we investigated the fate of immunoproteasome-deficient CD4$^+$ and CD8$^+$ T cells in LCMV-infected mice and came to the conclusion that the rapid loss of these cells cannot be attributed to graft rejection but that it identifies the requirement for immunoproteasomes for the persistence of leukocytes in an LCMV-infected mouse in which WT recipient cells mount a fulminant innate as well as adaptive CTL response associated with a vigorous storm of proinflammatory cytokines.

Several observations argue against the possibility of a differential homing or graft rejection phenomenon. First, the loss of immunoproteasome-compromised T cells was not limited to T lymphocytes in the spleen but was also confirmed in blood, peritoneum and different LN and hence excluding homing failures of LMP7 and MECL-1-deficient T cells (Supporting Information Fig. 2). Second, the rejection of transferred LMP7$^{-/-}$ cells by host NK cells due to reduced surface levels of MHC class I molecules is unlikely since adoptively transferred LMP7$^{-/-}$

T cells survived to the same extent as C57BL/6 cells up to day 10 after transfer in naïve recipients (Supporting Information Fig. 3). Nevertheless, LCMV acts as a potent activator of NK cells, but LMP2- and MECL-1-deficient T cells suffer from impaired expansion after transfer into LCMV-WE-infected recipients as well (Fig. 1B) although they are known to express normal MHC class I surface levels [9, 12]. Third, the transfer of $CD8^+$ T cells and $B220^+$ B cells into the same LCMV-infected mouse led to the complete disappearance of $CD8^+$ T cells, whereas the B cells persisted (Fig. 3C). As B cells expressed the same (H-$2K^b$) or slightly higher (H-$2D^b$) levels of MHC class I molecules on the cell surface, this experiment rules out that differences in the peptide repertoire presented on class I proteins by LMP7-deficient and -proficient cells are causing a rejection within 8 days after transfer. Finally, the cotransfer of T cells from male WT and female $LMP7^{-/-}$ donor mice into female recipients showed the loss of $LMP7^{-/-}$ T cells by day 4, whereas the T cells expressing HY miHAg persisted for 8 days (Fig. 2).

An obvious question raised by our findings is toward the mechanism how immunoproteasomes may be involved in the control of T-cell expansion. We have recently observed that the treatment of mouse splenocytes with an LMP7-specific inhibitor reduces the production of IL-6 after LPS stimulation and the production of IFN- after anti CD3/CD28 stimulation [19]. The same effects were not observed with splenocytes from $LMP7^{-/-}$ mice but we did find an enhanced IL-4 production by $LMP7^{-/-}$ cells after stimulation with anti CD3mAb (Basler, M., Kalim, K., Groettrup M., unpublished data). It is hence possible that a deregulated cytokine profile in immunoproteasome-deficient cells causes the loss of these cells in an LCMV-infected WT mouse.

Another link between immunoproteasomes and the propensity of cells to undergo apoptosis has been proposed to rely on NF-κB processing. A link to immunoproteasomes was first provided by a publication reporting that a lack of LMP2 in NOD mice leads to reduced processing of NF-kB p105–p50 [20] but two laboratories refuted this notion shortly after publication [21, 22]. Very recently, however, Yewdell and colleagues found a minor reduction in the extent of IkB degradation, following the stimulation of $LMP2^{-/-}$ B cells with LPS *in vitro* [18]. We have ourselves monitored p105, p50 and IkB levels in $LMP2^{-/-}$, $LMP7^{-/-}$MECL-$1^{-/-}$ and WT T cells after stimulation with anti CD3 or TNF- and failed to find significant differences compared with WT controls (data not shown). Nevertheless, the limited proteolysis of p105–p50 by the constitutive proteasome is well documented [23], and it could be possible that immunoproteasomes selectively process another factor which may be required for T-cell expansion and survival.

Initial functional and phenotypic analyses of immunoproteasome-deficient mice were rather disappointing (discussed in [2]). Infection of the knockout mice with LCMV induced a strong virus-specific CTL response that eliminated the virus comparable to WT mice [24]. No defect in T-cell proliferation could be observed in these mice. Therefore, it is intriguing that a reduced expansion and survival of immunoproteasome-deficient T cells becomes only apparent after adoptive transfer into an infected WT host. It seems that the requirement for immunoproteasomes for the expansion and survival of T cells is critical under certain circumstances. Indeed, in mouse models of rheumatoid arthritis [19] and colitis [25], the lack of a functional immunoproteasome subunit protected mice from autoimmune diseases. Therefore, the data provided in this manuscript support the conception of the immunoproteasome as a potential new target for the suppression of undesired proinflammatory T-cell responses.

Materials and methods

Mice, viruses, bacteria, cells and media

C57BL/6 mice (H-2^b) mice as well as B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ (also referred to as "CD45.1-" or "Ly5.1 congenic mice") were originally obtained from Charles River, Germany. B6.PL (Thy1.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). MECL-1 [9], LMP2 [12] and LMP7 [11] gene-targeted mice were kindly provided by Dr. John J. Monaco (Department of Molecular Genetics, Cincinnati Medical Center, Cincinnati, OH, USA); these mice have been bred onto the C57BL/6 background for at least ten generations. TCRtg P14 mice (tg line 318) [26], specific for aa 33–41 (= gp33 epitope, presented on MHC I) of the LCMV glycoprotein were obtained from Dr. Oliver Planz, Tübingen University. RAG-2-deficient mice bred onto C57BL/6 background were originally obtained from The Jackson Laboratory and bred in individually ventilated cages. Mice were kept in a specific pathogen-free facility and used at 6–12 wk of age. Experimental groups were age and sex matched and the review board of Regierungspräsidium Freiburg has approved experiments.

LCMV-WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and propagated on the fibroblast line L929. VV-WR was obtained from Professor Hans Hengartner, University Hospital Zurich, Switzerland. The virus was propagated on BSC 40 cells. Mice were infected with 200 PFU or 2×10^4 PFU LCMV-WE i.v. or with 2×10^6 PFU VV-WR i.p. BSC 40 is an African green monkey kidney-derived cell line. All cells were grown in MEM 5% FCS.

rLM-OVA was kindly provided by Professor Dirk Busch, Technische Universität München, Munich, Germany. The injection cultures were prepared by inoculation of 10 mL Brain–Heart Infusion Broth with 100 μL of the frozen (−70°C) stock culture. After growing overnight on a shaker at 37°C, the Listeria titer in the culture was estimated by spectrophotometry: 1 $OD_{600\,nm}$ unit = 10^9 cfu/mL. The mice were immunised with 2×10^4 CFU rLM-OVA in 200 μL PBS i.v. To quantify the injection dose, estimated by spectrophotometry, 100 μL of tenfold dilutions of the injection culture were plated on agar plates made of Brain–Heart Agar. Briefly, 24 h after incubation at 37°C, the injection dose was determined by counting the colonies that were growing. All media were purchased from Invitrogen-Life Technologies; Karlsruhe, Germany, supplemented with GlutaMAX, 5 or 10% FCS and 100 U/mL penicillin/streptomycin.

T-cell isolation and adoptive T-cell transfer

T cells from splenocytes of naïve Thy1.2$^+$ mice (C57BL/6, LMP2$^{-/-}$, LMP7$^{-/-}$, MECL-1$^{-/-}$, MECL-1$^{-/-}\times$LMP7$^{-/-}$, P14, P14$\times$LMP7$^{-/-}$ and P14$\times$MECL-1$^{-/-}$) were isolated with anti-CD90.2 (Thy1.2)-coated microbeads (Miltenyi Biotec, Germany). T cells from Thy1.1 mice were isolated with the Pan T Cell Isolation Kit (Miltenyi Biotec). In experiments involving the transfer of Thy1.1 T cells, all donor T cells were isolated with the Pan T Cell Isolation Kit. For adoptive transfer experiments, 1–3$\times 10^7$ T cells were i.v. transferred into recipient mice.

CFSE labeling

In brief, 5×10^7 cells were incubated in 1 mL of 10 μM 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE, Sigma) in PBS, 0.1% FCS for 10 min at 37°C. Labeling of cells was stopped by adding five volumes of ice-cold IMDM 10% FCS and washing three times with IMDM 10% FCS.

In vivo proliferation assay

Briefly, 2–3$\times 10^7$ Thy1.2-sorted splenocytes from P14 TCRtg, P14$\times$LMP7$^{-/-}$ TCRtg or P14$\times$MECL-1$^{-/-}$ TCRtg mice were CFSE labeled and transferred i.v. into either naïve Thy1.1 mice or Thy1.1 mice that had been infected with 2×10^4 PFU LCMV-WE 24 h earlier. In total, 16 and 40 h after transfer, splenocytes were analyzed with a FACSCalibur^{TM} flow cytometer after RBC-lysis with 1.66% NH_4Cl w/v and staining for CD8$^+$ cells (APC rat anti-mouse CD8a, clone 53–6.7, BD Pharmingen). To determine the percentage of transferred cells currently undergoing apoptosis *versus* T cells that are already dead, the splenocytes have been stained with PerCP rat anti-mouse CD8a (clone 53–6.7, BD Pharmingen), Annexin-V-Pacific Blue (Molecular Probes) and To-Pro-3 (Molecular Probes) after RBC-lysis. In this case, acquisition was done with the LSRII^{TM} flow cytometer (BD Biosciences).

Statistical analysis

To statistically assess differences between groups, Student's unpaired *t*-test was performed using the GraphPad software. A *p*-value<0.05 was considered statistically significant for all analyses.

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References

1 **Khan, S., van den Broek, M., Schwarz, K., de Giuli, R., Diener, P. A. and Groettrup, M.,** Immunoproteasomes largely replace constitutive proteasomes during an antiviral and antibacterial immune response in the liver. *J. Immunol.* 2001. **167**: 6859–6868.

2 **Groettrup, M., Kirk, C. J. and Basler, M.,** Proteasomes in immune cells: more than peptide producers? *Nat. Rev. Immunol.* 2010. **10**: 72–77.

3 **Groettrup, M., Ruppert, T., Kuehn, L., Seeger, M., Standera, S., Koszinowski, U. and Kloetzel, P. M.,** The interferon-γ-inducible 11S regulator (PA28) and the LMP2/LMP7 subunits govern the peptide production by the 20S proteasome *in vitro*. *J. Biol. Chem.* 1995. **270**: 23808–23815.

4 **Toes, R. E. M., Nussbaum, A. K., Degermann, S., Schirle, M., Emmerich, N. P. N., Kraft, M., Laplace, C. *et al.*,** Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products. *J. Exp. Med.* 2001. **194**: 1–12.

5 **Schwarz, K., van den Broek, M., Kostka, S., Kraft, R., Soza, A., Schmidtke, G., Kloetzel, P. M. and Groettrup, M.,** Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1 but not PA28α/β enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. *J. Immunol.* 2000. **165**: 768–778.

6 **Morel, S., Levy, F., BurletSchiltz, O., Brasseur, F., ProbstKepper, M., Peitrequin, A. L., Monsarrat, B. *et al.*,** Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity* 2000. **12**: 107–117.

7 **Chen, W. S., Norbury, C. C., Cho, Y. J., Yewdell, J. W. and Bennink, J. R.,** Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. *J. Exp. Med.* 2001. **193**: 1319–1326.

8 **Sijts, A. J. A. M., Standera, S., Toes, R. E. M., Ruppert, T., Beekman, N. J. C. M., vanVeelen, P. A., Ossendorp, F. A. *et al.*,** MHC class I antigen processing of an Adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. *J. Immunol.* 2000. **164**: 4500–4506.

9 **Basler, M., Moebius, J., Elenich, L., Groettrup, M. and Monaco, J. J.,** An altered T cell repertoire in MECL-1-deficient mice. *J. Immunol.* 2006. **176**: 6665–6672.

10 **Pang, K. C., Sanders, M. T., Monaco, J. J., Doherty, P. C., Turner, S. J. and Chen, W. S.,** Immunoproteasome subunit deficiencies impact differentially on two immunodominant influenza-virus-specific CD8(+) T cell responses. *J. Immunol.* 2006. **177**: 7680–7688.

11 **Fehling, H. J., Swat, W., Laplace, C., Kuehn, R., Rajewsky, K., Mueller, U. and von Boehmer, H.,** MHC class I expression in mice lacking proteasome subunit LMP-7. *Science* 1994. **265**: 1234–1237.

12 **Van Kaer, L., Ashton-Rickardt, P. G., Eichelberger, M., Gaczynska, M., Nagashima, K., Rock, K. L., Goldberg, A. L. *et al.*,** Altered peptidase and viral-specific T cell response in LMP 2 mutant mice. *Immunity* 1994. **1**: 533–541.

13 **Zaiss, D. M. W., deGraaf, N. and Sijtsi, A. J. A. M.,** The proteasome immunosubunit multicatalytic endopeptidase complex- like 1 is a T-cell-intrinsic factor influencing homeostatic expansion. *Infect. Immun.* 2008. **76**: 1207–1213.

14 **Duffy, D., Sparshott, S. M., Yang, C.-p. and Bell, E. B.,** Transgenic CD4 T cells (DO11.10) are destroyed in MHC-compatible hosts by NK cells and CD8 T cells. *J. Immunol.* 2008. **180**: 747–753.

15 **Robertson, N. J., Chai, J. G., Millrain, M, Scott, D., Hashim, F., Manktelow, E, Lemonnier, F. *et al.*,** Natural regulation of immunity to minor histocompatibility antigens. *J. Immunol.* 2007. **178**: 3558–3565.

16 **Caudill, C. M., Jayarapu, K., Elenich, L., Monaco, J. J., Colbert, R. A. and Griffin, T. A.,** T cells lacking immunoproteasome subunits MECL-1 and LMP7 hyperproliferate in response to polyclonal mitogens. *J. Immunol.* 2006. **176**: 4075–4082.

17 **Lin, Y., Roberts, T. J., Sriram, V., Cho, S. and Brutkiewicz, R. R.,** Myeloid marker expression on antiviral CD8+T cells following an acute virus infection. *Eur. J. Immunol.* 2003. **33**: 2736–2743.

18 **Hensley, S. E., Zanker, D., Dolan, B. P., David, A., Hickman, H. D., Embry, A. C., Skon, C. N. *et al.*,** Unexpected role for the immunoproteasome subunit LMP2 in antiviral humoral and innate immune responses. *J. Immunol.* 2010. **184**: 4115–4122.

19 **Muchamuel, T., Basler, M., Aujay, M. A., Suzuki, E., Kalim, K. W., Lauer, C., Sylvain, C. *et al.*,** A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat. Med.* 2009. **15**: 781–787.

20 **Hayashi, T. and Faustman, D.,** NOD mice are defective in proteasome production and activation of NF- kappa B. *Mol. Cell. Biol.* 1999. **19**: 8646–8659.

21 **Kessler, B. M., LennonDumenil, A. M., Shinohara, M. L., Lipes, M. A. and Ploegh, H. L.,** LMP2 expression and proteasome activity in NOD mice. *Nat. Med.* 2000. **6**: 1064.

22 **Runnels, H. A., Watkins, W. A. and Monaco, J. J.,** LMP2 expression and proteasome activity in NOD mice. *Nat. Med.* 2000. **6**: 1064–1065.

23 **Sears, C., Olesen, J., Rubin, D., Finley, D. and Maniatis, T.,** NF-kappa B p105 processing *via* the ubiquitin-proteasome pathway. *J. Biol. Chem.* 1998. **273**: 1409–1419.

24 **Basler, M., Youhnovski, N., van den Broek, M., Przybylski, M. and Groettrup, M.,** Immunoproteasomes down-regulate presentation of a subdominant T cell epitope from lymphocytic choriomeningitis virus. *J. Immunol.* 2004. **173**: 3925–3934.

25 **Basler, M., Dajee, M., Moll, C., Groettrup, M. and Kirk, C. J.,** Prevention of experimental colitis by a selective inhibitor of the immunoproteasome1. *J. Immunol.* 2010. **185**: 634–641.

26 **Pircher, H., Burki, K., Lang, R., Hengartner, H. and Zinkernagel, R. M.,** Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* 1989. **342**: 559–561.

Abbreviations: **LCMV:** lymphocytic choriomeningitis virus · **miHAg:** minor histocompatibility Ag · **rLM-OVA:** recombinant *Listeria monocytogenes* expressing OVA · **VV:** vaccinia virus

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