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An Altered T Cell Repertoire in MECL-1-Deficient Mice

Michael Basler,* Jacqueline Moebius,* Laura Elenich,† Marcus Groettrup,2* and John J. Monaco†

Immunoproteasome subunits low-molecular mass polypeptide (LMP)2 and LMP7 affect Ag presentation by MHC class I molecules. In the present study, we investigated the function of the third immunosubunit LMP10-multicatalytic endopeptidase complex-like (MECL)-1 in MECL-1 gene-targeted mice. The number of CD8+ splenocytes in MECL-1−/− mice was 20% lower than in wild-type mice. Infection with lymphocytic choriomeningitis virus (LCMV) elicited a markedly reduced cytotoxic T cell (CTL) response to the LCMV epitopes GP276–286/Db and NP205–212/Kb in MECL-1−/− mice. The weak CTL response to GP276–286/Db was not due to an impaired generation of this epitope but was attributed to a decreased precursor frequency of GP276–286/Db-specific T cells. The expansion of TCR-Vα10+ T cells, which contain GP276–286/Db-specific cells, was reduced in LCMV-infected MECL-1−/− mice. Taken together, our data reveal an in vivo function of MECL-1 in codetermining the T cell repertoire for an antiviral CTL response. The Journal of Immunology, 2006, 176: 6665–6672.

Cytotoxic T lymphocytes recognize peptide epitopes presented on MHC class I (MHC-I) molecules, which allows CTL to monitor our body for cells harboring intracellular pathogens. The proteasome is the main protease in the cytoplasm and the nucleus, which is responsible for the generation of most peptide ligands of MHC-I molecules. The proteasome is the main protease in the cytoplasm and the nucleus, which is responsible for the generation of most peptide ligands of MHC-I molecules (1, 2). The proteolytic core complex of the proteasome system is the 20S proteasome, which is constructed like a cylinder of four stacked rings. The outer two rings consist of seven different subunits that bind to regulatory complexes of the 20S core particle, whereas the two inner rings are made up of seven different subunits of the β-type (3). Three of the β subunits designated Y/δ (β1), X/MB1 (β5), and Z/MEC14 (β2) bear the active centers of the 20S proteasome. The catalytic activities of the proteasome have been classified with the help of fluorogenic peptides as chymotrypsin-like (cleavage C-terminal of hydrophobic amino acids), trypsin-like (cleavage C-terminal of basic aa), and caspase-like (also known as peptidyl-glutamyl-peptide hydrolyzing; cleavage C-terminal of acidic aa). Upon stimulation of cells with IFN-γ, these constitutively expressed subunits are replaced by inducible subunits named low-molecular mass polypeptide (LMP)2 (βi), LMP7 (β5i), and multicatalytic endopeptidase complex-like (MECL)-1 (β2i) during the de novo assembly of 20S proteasomes. In contrast to LMP2 and LMP7, the MECL-1 subunit is not encoded in the MHC locus, and the discovery of this third subunit exchange (MECL-1 for MC14) lagged behind several years (4–6). Recently, De et al. (7) reported that, in Con A blasts derived from MECL-1-deficient mice, incorporation of MECL-1 into proteasomes is dependent on LMP2 and to a lesser extent on LMP7, but LMP2 and LMP7 are integrated independently of MECL-1 into proteasomes.

Analysis with small fluorogenic substrates showed that the exchange of LMP2 for δ down-regulates the cleavages C-terminal of acidic residues (caspase-like activity) and favors the cleavage C-terminal of hydrophobic residues (chymotrypsin-like activity). Contradicting results have been obtained for the exchange of LMP7 for MB1 and MECL-1 for MC14 (8, 9). The overexpression of a mutant MECL-1 (T1A) led to a complete loss in trypsin-like activity (10).

LMP2- and LMP7-deficient mice have been analyzed intensively. LMP2 gene-targeted mice displayed no change in MHC-I cell surface expression, but the numbers of CD8+ cells were reduced in blood, spleen, and thymus. LMP7-deficient mice showed a reduced MHC-I surface expression. Some T cell epitopes are affected by LMP2 and LMP7, but the bulk of MHC-I ligands can still be generated in LMP2- and LMP7 gene-targeted mice (11, 12). LMP2-deficient mice had an altered immunodominance hierarchy in the TCD8+ response to influenza virus infection. TCD8+ responses to two dominant determinants dropped markedly, whereas responses to two subdominant epitopes were enhanced. The altered immunogenicity of some epitopes could be attributed to changes in epitope presentation, whereas the CTL response to one determinant (NP366–374) was reduced due to alterations in the TCD8+ repertoire (13). Transplantation experiments with LMP7 gene-targeted mice revealed that also these mice produce a different spectrum of peptides and hence possess an altered T cell repertoire (14). As immunoproteasome subunits are expressed in the thymus (15–17), positive and negative selection via the TCR could be altered in the absence of immunoproteasome subunits due to differences in the expression of positively and negatively selecting peptides in the thymus. In this work, we used the infection with lymphocytic choriomeningitis virus (LCMV) to study the characteristics of Ag processing and presentation in MECL-1 gene-targeted mice. The cytotoxic immune response to LCMV is essential for elimination of the virus from infected mice. In C57BL/6 mice, this response is shaped by CTL specific for the dominant GP-derived epitopes.
GP33–41/Db, GP34–41/Kb, GP276–286/Db, and NP-derived NF396–404/Db, as well as the subdominant epitopes GP92–101/Db, GP118–125/Kb, and NP205–212/Kb (18–20). Analysis of splenocytes derived from LCMV-infected MECL-1/−/− mice revealed a markedly reduced CTL response to GP276 and NP205. The reduction of GP276 was not due to an impaired Ag presentation of this epitope, but we could attribute these results to a change of the T cell repertoire in MECL-1-deficient mice.

Materials and Methods

Library screening

A genomic 129/SvJ DNA library was a gift from M. Shull (University of Cincinnati, Cincinnati, OH). Liver DNA was digested partially with SacI and cloned into the BamHI site of the λ DASH II vector (Stratagene). The library was screened with a human MECL-1 cDNA probe (gift from M. Cruz, University of Cincinnati) using standard techniques. Hybridizing clones were isolated, plaque purified, expanded, and characterized.

Targeting construct and generation of MECL-1-deficient mice

The targeting construct was assembled in pBlueScript KS+ using convenient restriction enzyme sites. The neomycin expression cassette (from pMC1NeoPolyA: Stratagene) was flanked by homology arms to replace exons 5–7 and disrupt splicing to exon 8 at the targeted allele. The long arm was a 2.4-kb fragment that extends from the HindIII site 2 kb upstream of exon 1 to the KpnI site at the beginning of exon 5. The short arm was an 840-bp XbaI fragment carrying exon 8 and 3′ sequences. A thymidine kinase expression cassette was included next to the long arm to allow positive-negative selection.

D3 ES cells were transfected, and G418-resistant clones were screened for the proper integration of the targeting construct. Properly targeted ES cell clones were injected into C57BL/6 blastocysts for the generation of chimeric mice. Genotyping was performed by Southern blotting with 5′ probes. The neomycin expression cassette was included next to the long arm to allow positive-negative selection.

D3 ES cells were transfected, and G418-resistant clones were screened for the proper integration of the targeting construct. Properly targeted ES cell clones were injected into C57BL/6 blastocysts for the generation of chimeric mice. Genotyping was performed by Southern blotting with 5′ probes and 3′ probes and then by PCR. All mice were backcrossed onto the C57BL/6 background.

Mice, viruses, cells, and media

C57BL/6 mice (H-2b) were obtained originally from Charles River Laboratories. B6.PL (Thy1.1) mice were obtained from The Jackson Laboratory. Mice were kept in a specific pathogen-free facility and used at 6–10 wk of age. Animal experiments were approved by the review board of Regierungspräsidium Freiburg.

LCMV-WE was obtained originally from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Mice were infected with 200 pfu (low dose) of LCMV-WE i.v., and the specific CTL response was analyzed at day 8 postinfection. To determine the amount of Ag presented in spleens derived from C57BL/6 and MECL-1/−/− mice, were infected with 200 pfu (low dose) or 104 pfu (high dose) LCMV-WE i.v. and analyzed on day 4 or day 2, respectively.

DC2.4 (H-2b) is a mouse dendritic cell line (a gift from Dr. K. Rock, University of Massachusetts Medical School, Worcester, MA). All media were purchased from Invitrogen Life Technologies and contained GlutaMAX, 10% FCS, and 100 U/ml penicillin/streptomycin.

Synthetic peptides


Intracellular staining (ICS) for IFN-γ

Splenocytes (2 × 106) were incubated in round-bottom 96-well plates with 10−7 M of the specific peptide in 100 μl IMDM 10% and brefeldin A (10 μg/ml) for 5 h at 37°C. The staining, fixation, and permeabilization of the cells were performed exactly as previously detailed (24).

Flow cytometry

Vα staining of TCRs was performed exactly as described previously (24). Differences between groups were assessed by unpaired t test ((www.graphpad.com)). Values of p < 0.05 are considered to be statistically significant.

Western blotting

Proteins from two-dimensional gels were blotted onto nitrocellulose (Schleicher & Schuell BioSciences), blocked with PBS/5% (w/v) low-fat dry milk/0.2% Tween for 1 h, and agitated overnight at 4°C with a rabbit polyclonal Ab recognizing MECL-1 (23). The blots were washed three times with TBS-i and incubated for 1 h with HRP-conjugated goat anti-rabbit Ab (DakoCytoMation). After extensive washing with PBS/0.2% Tween 20, proteins were visualized on x-ray films by ECL.

Peptide-specific CTL lines

Splenocytes from LCMV-WE memory mice (at least 1 mo postinfection with 200 pfu of LCMV-WE i.v.) were restimulated with irradiated (10,000 rad) and peptide-loaded (10−7 M) (GP33–41 (KAVYNFATC, Db) or GP376–286 (SGVENPGGYCL, Db)) DC2.4 in the presence of 40 U/ml IL-2. Cultures were restimulated weekly as described above at a ratio of 10:1. An additional density centrifugation step was conducted 1–2 days before using CTL in in vitro Ag presentation experiments. CTL were used in ICS at an E:S ratio of 0.2 in the first dilution, and serial 3-fold dilution of stimulators were performed.

Adoptive transfer

T cells from splenocytes of Thy1.2-positive mice (C57BL/6, MECL-1/−/−) or Thy1.1-positive mice were isolated with anti-CD90 (Thy1.2)-coated microbeads (Miltenyi Biotec) or with the Pan T cell isolation kit (Miltenyi Biotec). Purified T cells (2.5 × 107) were transferred i.v. into naive mice. After transfer, mice were immediately infected with 200 pfu of LCMV-WE i.v. Eight days later, splenocytes were analyzed as described in Intracellular staining (ICS) for IFN-γ with anti-Thy1.1-PE (clone OX-7; BD Pharmingen) and anti-Thy1.2-PE (clone 53-2.1; BD Pharmingen) instead of anti-CD8-Cy5.

Results

Generation of MECL-1-deficient mice

To investigate the physiological role of the IFN-γ-inducible proteasome subunit MECL-1, mice lacking this subunit were generated by means of homologous recombination in embryonic stem (ES) cells. Thereby, the exons 5–7 of the targeted mecl-1 allele were replaced with a neomycin (neo) resistance gene (Fig. 1A). ES cells containing the desired homologous recombination were injected into C57BL/6 blastocysts for the generation of chimeric mice. Chimeras were bred to C57BL/6 mice to establish C57BL/6 or MECL and LCMV-infected (8 days postinfection with 200 pfu of LCMV-WE i.v.) mice were performed exactly as described previously (21).

To investigate the physiological role of the IFN-γ-inducible proteasome subunit MECL-1, mice lacking this subunit were generated by means of homologous recombination in embryonic stem (ES) cells. Thereby, the exons 5–7 of the targeted mecl-1 allele were replaced with a neomycin (neo) resistance gene (Fig. 1A). ES cells containing the desired homologous recombination were injected into C57BL/6 blastocysts for the generation of chimeric mice. Chimeras were bred to C57BL/6 mice to establish
germline transmission of the targeted allele, and progeny were intercrossed to generate MECL-1<sup>−/−</sup> mice. The absence of the disrupted exons 5–7 was confirmed by Southern blotting (Fig. 1B). The mice showed no visible abnormalities, were fertile, and lived to at least 1 year of age.

**Mutant mice lack MECL-1**

To verify the absence of MECL-1 protein expression in MECL-1-deficient mice, purified spleen proteasomes were separated by two-dimensional gel electrophoresis (IEF/SDS-PAGE). MECL-1 is expressed constitutively in spleens of C57BL/6 wild-type mice (Fig. 1C) but completely absent in MECL-1-deficient mice. Western blot analysis of two-dimensional gels from LCMV-WE-infected liver (Fig. 1D) confirmed the lack of MECL-1. Additionally, these Western blot analyses revealed several so far uncharacterised isoforms of MECL-1, with the same molecular mass but different isoelectric points.

**LMP2 incorporation into immunoproteasomes of LCMV-infected livers is slightly reduced in MECL-1-deficient mice**

To analyze the incorporation of LMP2 and LMP7 in MECL-1-deficient mice, MECL-1<sup>−/−</sup> and C57BL/6 mice were infected with 200 pfu of LCMV-WE. Eight days later, the proteasome subunit composition of infected livers was analyzed by two-dimensional PAGE (Fig. 2A). Incorporation of LMP7 into proteasomes was not affected in MECL-1<sup>−/−</sup> mice compared with wild-type mice. In contrast, LMP2 was slightly but consistently reduced in MECL-deficient mice. It seems that LMP2 incorporation into proteasomes in LCMV-infected livers occurs in the absence of MECL-1 but less efficiently.

**MECL-1-deficient proteasomes have an enhanced caspase-like activity**

To investigate the role of MECL-1 in peptide degradation, the peptide hydrolyzing activities of purified proteasomes from LCMV-infected liver of C57BL/6 and MECL-1<sup>−/−</sup> mice were compared with help of fluorogenic peptides. No differences in hydrolyzing Bz-VGR-AMC (trypsin-like activity) and (Z)-GGL-AMC (chymotrypsin-like activity) were observed (Fig. 2B). In contrast, Z-LLE-β NA (caspase-like activity) was cleaved more efficiently by MECL-1-deficient proteasomes than by proteasomes from wild-type mice. This is in agreement with an enhanced caspase-like activity in proteasomes of LMP2-deficient mice (11) because LMP2 incorporation in MECL-1-deficient mice was reduced slightly (Fig. 2A).

**MECL-1 gene-targeted mice have reduced numbers of CD8<sup>+</sup> T cells**

To determine whether MECL-1-deficient mice have a reduced number of T cells, splenocytes from naïve MECL-1<sup>−/−</sup> and C57BL/6 control mice were stained for CD4 and CD8 and analyzed by flow cytometry. Splenocytes of MECL-1<sup>−/−</sup> mice showed a 20% reduction of CD8<sup>+</sup> cells (Fig. 3), whereas the number of CD4<sup>+</sup> cells was not affected (data not shown). The numbers of CD4<sup>+</sup> and CD8<sup>+</sup> cells in the thymus were not altered in MECL-1-deficient mice compared with wild-type mice (data not shown). The MHC-I surface expression on lymphocytes and dendritic cells in spleens was not reduced significantly compared with wild-type C57BL/6 mice (data not shown).

**An impaired CTL response against GP276 in LCMV-infected MECL-1<sup>−/−</sup> mice**

To characterize MECL-1-deficient mice in a model system of viral infection, we infected these mice with LCMV-WE and determined virus titers in spleens 4 days later. No significant differences in virus titers were obtained in control and knockout mice (data not shown). The CTL response in C57BL/6 mice after LCMV-WE infection is directed against three dominant (GP33–41<sup>/Db</sup>, GP34–41<sup>/Kb</sup>, and NP396–404<sup>/D<sub>B</sub></sup>) and several subdominant epitopes

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**FIGURE 1.** Disruption of the MECL-1 gene by homologous recombination. A, Targeting strategy. The genomic organization of the psmb10 locus and the targeting strategy is shown. Exons are depicted as □, the thymidine kinase cassette as □, and the neomycin resistance cassette as □. Relevant restriction sites and probes are indicated. B, Southern blot analysis of tail DNA from a representative litter. DNA was digested with EcoRI and probed with the 3′ probe. The wild-type allele generates a 15-kb band, and the targeted allele yields an 8.7-kb band. C, IEF/SDS-PAGE analysis of two-dimensional gels from LCMV-WE-infection (day 8 postinfection with 200 pfu) infected C57BL/6 (upper panel) and MECL-1<sup>−/−</sup> (lower panel) mice. The proteins were visualized by Coomassie blue staining. The position of the proteasome subunit MECL-1 is indicated. D, An IEF/SDS-PAGE of 20S proteasome (70 μg) purified from the liver of LCMV-WE (WT 15 kb).
The generation of V_{P10}1b-specific CTLs is impaired in LCMV-infected MECL-1-deficient mice

No alteration of GP276 epitope presentation in MECL-1-deficient mice

Recently, we have shown that overexpression of immunoproteasome subunits in MECL-1-deficient mice leads to a diminished presentation of GP276 (24). To investigate the contribution of Ag presentation to the reduced GP276-specific CTL response observed in MECL-1 gene-targeted mice (Fig. 4), thioglycolate-elicited peritoneal macrophages from MECL-1-deficient as well as C57BL/6 control mice were infected in vitro for 20 h with LCMV-WE and analyzed for GP276 presentation with GP276-specific T cell hybridomas (Fig. 5A). GP276 presentation was not affected in LCMV-infected MECL-1-deficient macrophages. To determine the amount of Ag presented directly in vivo, we i.v. infected MECL-1^{−/−} and control C57BL/6 mice with a high dose (2 × 10^7 pfu) or low dose (200 pfu) of LCMV-WE and analyzed the status of GP33 and GP276 presentation in the spleen on day 2 or 4 after infection, respectively. Splenocytes from these mice were used as stimulators for IFN-γ production (ICS) by mono-specific CTL lines specific for GP33 or GP276 (for CTL specificity analysis see Fig. 5D). To exclude that activated T_{CD8}^{+} in infected spleens falsify the result, an intracellular IFN-γ staining (ICS) with infected splenocytes but without monospecific CTL was performed. No IFN-γ-producing CTLs were detected on day 2 and day 4 after LCMV-WE infection in these spleens, thus indicating that they did not contribute to the number of IFN-γ-staining cells (data not shown). As shown in Fig. 5, B and C, neither GP33 nor GP276 is presented differently in spleens on day 2 (high dose) or day 4 (low dose) post-LCMV-WE infection. Hence, cells deficient for MECL-1 are able to produce and present GP276 in similar amounts as wild-type cells.
peptide stimulation; intracellular IFN-γ was measured using FACS analysis after stimulation with indicated peptides for 5 h, and screened by flow cytometry after staining for CD8 and intracellular IFN-γ. The values represent the mean of three different mice. One of four representative experiments is shown. B, FACS plots of splenocytes from LCMV-infected C57BL/6 (wild type) and MECL-1−/− mice after 5 h in vitro stimulation with GP276 or NP205. Ø represents LCMV-infected mice without in vitro peptide stimulation; intracellular IFN-γ is plotted vs CD8. C, CTL response in LCMV-infected C57BL/6 (wild type) and MECL-1−/− mice was analyzed by staining with GP276-H-2Db-specific tetramers and flow cytometry. The y-axis shows the percent tetramer positive of all CD8+ cells. The values represent the mean of three different mice.

mice showed no difference in Vα10 usage compared with C57BL/6 (Fig. 6). After LCMV infection, however, the extent of CTL using Vα10 was significantly increased in C57BL/6 mice compared with MECL-1-deficient mice. The numbers of Vα6+ and Vα9+ cells did not differ between wild-type and MECL-1−/− T cells neither in naive nor in LCMV-infected mice (data not shown).

The repertoire of GP276 specific T cells is altered in MECL-1-deficient mice

To address whether the T cell repertoire is altered in MECL-1-deficient mice, we adoptively transferred magnetically enriched T cells derived from Thy1.2 into host C57BL/6 wild-type mice after 5 h in vitro peptide stimulation; intracellular IFN-γ is plotted vs CD8. C, CTL response in LCMV-infected C57BL/6 (wild type) and MECL-1−/− mice was analyzed by staining with GP276-H-2Db-specific tetramers and flow cytometry. The values represent the mean of three different mice.

To directly show that MECL-1−/− mice have indeed a reduced GP276-specific CTL precursor frequency, the adoptive transfer experiment shown in Fig. 7A was performed vice versa. We adoptively transferred magnetically enriched T cells derived from MECL-1−/− (Thy1.2) or C57BL/6 (Thy1.2) control mice into different Thy1.1 recipient mice and infected these mice with LCMV-WE. As APC in Thy1.1 mice present GP276 similarly to MECL-1−/− or C57BL/6 T cells, the transferred T cells should expand accordingly to their precursor frequency. Eight days postinfection, T cells were analyzed in spleens. C57BL/6 donor T cells expanded to a level of 1.7% of total lymphocytes (Fig. 7B), whereas MECL-1-deficient T cells could not be detected in spleens by staining for Thy1.2. A similar phenomenon has been reported previously for adoptively transferred T cells from LMP2−/− mice into influenza virus-infected wild-type mice (13). These data could indicate that immunoproteasomes fulfill—apart from a role in processing epitopes for MHC-I presentation—another so far unknown function in T cell expansion in virus-infected mice.

Discussion

To date, the reason for the expression of the three cytokine-inducible proteasome subunits, LMP2, LMP7, and MECL-1 replacing their constitutive counterparts, is still a matter of debate. It is probably only a part of MHC-I ligands that is affected by the immunoproteasome subunits, but the bulk of MHC-I ligands can still be produced in the absence of LMP2 and LMP7 (27, 28). The subunit exchange of MECL-1 was discovered several years after LMP2 and LMP7 (4–6), and the in vivo function of MECL-1 has remained poorly characterized. In this study, we analyzed the immune response to the well-characterized model virus LCMV in MECL-1 gene-targeted mice. Proteasomes isolated from livers of LCMV-WE-infected MECL-1−/− mice displayed an only slightly reduced incorporation of LMP2 (Fig. 2A). Similar results were obtained with LPS blasts from MECL-1-deficient mice showing no major reduction in LMP2 and LMP7 incorporation in the absence of MECL-1 (7). In contrast, LMP2-deficient mice display an impaired MECL-1 incorporation, and the absence of LMP7 reduced the level of both LMP2 and MECL-1 and resulted in an accumulation of their precursors (7). Therefore, MECL-1-deficient mice are compared with LMP2−/− and LMP7−/− mice the only knock-out mice that permit to essentially study the function of a single immunosubunit. Hence, defects in MECL-1-deficient mice are probably attributed to a loss in MECL-1 and not to the minor reduction in LMP2 incorporation. However, it is noteworthy that LMP2-deficient mice have a similar phenotype as MECL-1−/− mice with respect to reduced CD8+ counts in spleen, unchanged MHC-I expression in lymphocytes and splenocytes, and a change in T cell repertoire. It may hence be possible that these phenotypes are linked to the low incorporation of MECL-1 into proteasome precursors of LMP2−/− mice rather than to the lack of LMP2 itself. With respect to the presentation of the GP276 epitope, our data suggest that the exact composition of active site proteasome subunits is pivotal. While we observed an enhanced presentation of GP276 in LCMV-infected macrophages from LMP2−/− and LMP7−/− mice (24), GP276 presentation by MECL-1-deficient
macrophages or splenocytes was at the same level as in wild-type cells (Fig. 5, A–C).

Analysis of proteasomes from LCMV-infected liver by Western blot revealed three different isoforms of MECL-1 with similar molecular weights but different isoelectric points, suggesting post-translational modifications as a cause for this polymorphism. Kuckelkorn et al. (29) found that MECL-1 is expressed as different isoforms in liver, thymus, small intestine, and colon, but the molecular basis for these differences remains to be elucidated.

The analysis of cleavage specificity of MECL-1-deficient proteasomes isolated from LCMV-WE-infected livers with the help of small fluorogenic substrates showed no difference in cleavage after basic amino acids (trypsin-like activity) when compared with MECL-1-proficient proteasomes (Fig. 2B). Given that immunoproteasomes have been reported to possess a higher trypsin-like activity than constitutive proteasomes (30, 31) and given that overexpression of a catalytically inactive \(/H_9252/\2 subunit suppressed the trypsin-like activity (10), one may have predicted a reduction in the trypsin-like activity for MECL-1-deficient proteasomes. However, an enhancement of the trypsin-like activity was not observed with immunoproteasomes by others (21, 32). Our data are more compatible with MC14 and MECL-1 contributing equally to the trypsin-like activity, which is in accordance with structure predictions for the P1 binding pockets for MC14 and MECL-1 (3). Apparently, the enhanced incorporation of the constitutive MC14 subunit can compensate for the loss of MECL-1 with respect to the trypsin-like activity.

Infection of MECL-1 gene-targeted mice with LCMV-WE proceeded normally, yielding the same virus titers on day 4 of infection as in control mice (data not shown). The analysis of a panel of LCMV-specific CTL epitopes revealed an impeded CTL response to GP276 and NP205 (Fig. 4). To investigate whether the reduced CTL response to GP276 in MECL-1-deficient mice is due to a presentation defect, fragmentation of a 25mer containing GP276 by MECL-1\(/H_{11002}/\2 or C57BL/6 immunoproteasomes was analyzed by

FIGURE 5. GP276 Ag presentation is not affected by MECL-1 deficiency. A, Presentation of LCMV-GP276–286 epitopes by LCMV-infected macrophages from C57BL/6 and MECL-1\(/H_{11002}/\2 mice. Peritoneal macrophages were infected with LCMV-WE in vitro 1 day before addition of T cell hybridomas specific for GP276. Uninfected macrophages are marked with Ø. The y-axis shows absorbance of enzymatically converted chromogen at 570 nm in lacZ assays. The values are the means ± SD of three replicate cultures. The experiment has been repeated twice, giving similar results. B and C, LCMV-infected splenocytes derived from C57BL/6 or MECL-1\(/H_{11002}/\2 mice similarly present the LCMV GP-derived CTL epitopes GP33 and GP276 ex vivo. MECL-1\(/H_{11002}/\2 and C57BL/6 mice were infected i.v. with 10^7 pfu (B) or 200 pfu (C) of LCMV-WE. Their splenocytes were isolated at day 2 or day 4 postinfection, respectively, and were used as APC for GP33–41- or GP276–286-specific CTL lines. Activation of CTL lines 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 intracellular IFN-γ (y-axis) produced by CTL lines is plotted vs E:S ratio (effector (CTL lines) to stimulators (splenocytes)). One of three experiments is shown. D, Specificity of GP33- and GP276-specific CTL lines. GP33-specific (upper panel) or GP276-specific (lower panel) CTL lines were incubated with splenocytes pulsed with GP33 or GP276 (x-axis). Activation of CTL lines was analyzed by staining for CD8 and intracellular IFN-γ (y-axis).

FIGURE 6. Analysis of TCR-V₆₉₁₀ usage by CD8+ T cells from C57BL/6 and MECL-1\(/H_{11002}/\2 mice. Splenocytes from naive or LCMV-infected (8 days postinfection with 200 pfu of LCMV-WE i.v.) mice were stained for CD8 and V₆₉₁₀ and analyzed by flow cytometry. Values are the means ± SD of 6 (naive) or 10 (infected) mice. Values of p were determined by unpaired t test and are considered to be statistically significant when p < 0.05.
HPLC-electron spray ionization-mass spectroscopy (data not shown). No significant difference in GP276 production could be observed between the two types of proteasomes. Additionally, neither in vitro-infected thioglycollate-elicited macrophages derived from MECL-1−/− nor ex vivo analysis of Ag presentation by splenocytes of MECL-1−/− revealed an alteration in GP276 presentation in MECL-1 gene-targeted mice (Fig. 5, A–C). These findings suggested that the loss of MECL-1 has an effect on the T<sub>CD8</sub><sup>+</sup> repertoire, as it has been reported for LMP2 gene-targeted mice (13). Yewdell and colleagues (13) showed that the CTL response to several influenza epitopes was changed in LMP2−/− mice, which was due to both, changes in epitope presentation as well as to differences in the CTL precursor frequency. Graft transplantation and adoptive transfer experiments from wild-type mice to LMP7-deficient mice revealed that the CTL repertoire was altered in LMP7−/− mice, probably due to changes in peptide presentation in the thymus (14). Because MECL-1, as well as the other immunoproteasome subunits, are expressed in the thymus (15–17), one can assume that MECL-1 can shape the T cell repertoire by influencing positive and negative selection. T<sub>CD8</sub><sup>+</sup> cells in splenocytes of naive MECL-1-deficient mice were reduced by ~20% compared with wild-type mice, similar to what has been reported for LMP2−/− mice (11).

To address whether the GP276-specific T cell repertoire was indeed altered in MECL-1-deficient mice, purified T cells from MECL-1−/− (Thy1.2) were adoptively transferred into Thy1.1 mice. Unexpectedly, these transferred cells, in contrast to transferred wild-type T cells, were unable to expand after LCMV-WE infection (Fig. 7B). It is noteworthy to point out, that not only GP276-specific T cells but all transferred MECL-1-deficient T cells irrespective of epitope specificity were unable to expand in the host mice, although a vigorous response by T cells of the host occurred. A similar intriguing phenomenon has been observed previously for adoptively transferred LMP2-deficient T cells into wild-type mice that were infected with influenza virus (13). Adoptive transfer experiments with fluorescently labeled T cells from MECL-1-deficient mice into uninfected wild-type host mice revealed that these T cells persisted in host mice at levels similar to transferred wild-type cells (data not shown). Therefore, the transferred MECL-1-deficient T cells were not eliminated by the host due to an altered set of peptides presented on MHC-I of MECL-1-deficient T cells. As MECL-1−/− T cells can readily expand to several LCMV-derived epitopes in MECL-1-deficient mice (Fig. 4), the diminished expansion capacity of adoptively transferred MECL-1−/− T cells is caused by the failure of these cells to compete against wild-type T cells of the recipient during an antiviral immune response.

How can immunoproteasomes influence the expansion capacity of a T cell? A small number of proteins (such as NF-κB p105) have been shown to be processed by the proteasome through limited proteolysis rather than being entirely degraded. Hence, there might exist a factor, exerting an important role in T cell expansion, that is either differentially processed or selectively degraded by immunoproteasomes but not by constitutive proteasomes. Taken together, this result could be a hint that immunoproteasome subunits do not only function to change the processing of MHC-I ligands, but in addition possess a so far uncharacterized function in controlling T cell expansion that may be linked to the proliferation, apoptosis, or homing of T cells.

The altered T cell repertoire for GP276 was finally confirmed with transferring T cells from Thy1.1 mice into either MECL-1−/− or C57BL/6 mice (Fig. 7A). The transferred GP276-specific T cells expanded similarly in MECL-1−/− and wild-type mice, whereas the endogenous T cells displayed a reduced response to GP276. As the transferred T cells can respond normally to GP276, the presentation of this epitope is not altered in MECL-1−/−-deficient mice. Hence, the frequency of GP276-specific T cell precursors must be selectively reduced in MECL-1-deficient mice, strongly suggesting an altered T cell repertoire in these mice.

At a first glance this conclusion might appear contradictory. How can a foreign T cell epitope influence the repertoire of MECL-1-deficient mice? The highly diverse pool of naive precursor T cells reflects a wide range of TCR developed in the thymus by random rearrangements of variable (V), diversity (D), and joining (J) gene segments and the addition and/or deletion of additional nucleotides at the V-D-J junctions. It is generally accepted that low-affinity self-peptides promote positive selection of T cells in the thymus, whereas high-affinity ligands are responsible for negative selection, leading to clonal deletion of self-reactive T cells (33). Recently, Nil et al. (17) reported that immunoproteasome subunits are expressed in thymus-derived dendritic cells, macrophages, and medullary thymic epithelial cells that can induce negative selection. In contrast, RT-PCR analysis of cortical epithelial cells, which are responsible for positive selection, revealed that immunoproteasome subunits are not present in these cells (17). Therefore, the defect in the GP276-specific T cell repertoire in MECL-1-deficient mice is most likely caused by elimination of GP276-specific CTL through negative selection in the thymus. It has been reported that TCR specific for a viral MHC-I-peptide complex can cross-react with multiple other MHC-I-peptide complexes, including self-peptides, a phenomenon called molecular mimicry (34,
35). The cross-reactive peptide can be unrelated to the primary sequence because the cross-reactivity rather depends on similarities in the three-dimensional structure of the MHC-I-peptide complex (36–38). Using a TCR-transgenic model specific for LCMV-GP33, Saibil et al. (40) identified several peptides that were able to cross-react with TCR-transgenic virus-specific T cells in vitro. One peptide was derived from the self-protein dopamine β-monooxygenase (DBM), an enzyme expressed in the adrenal medulla (39, 40). Crystal structure analysis of the ratDBM-H-2D^b complex and the GP33-H-2D^b complex revealed no significant differences on the surface areas of both MHC complexes, although the two peptides share only 56% sequence identity (41). Assuming that GP276-specific CTL are negatively selected in the thymus, a self-peptide, mimicking GP276, must be expressed in the thymus of MECL-1 gene-targeted mice, which is absent in wild-type mice and thus leads to a clonal deletion of GP276-specific T cell in the thymus of MECL-1−/− mice.

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References