Epitope-specific in vivo protection against cytomegalovirus disease by CD8 T cells in the murine model of preemptive immunotherapy

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Abstract Preclinical research in murine models as well as subsequent clinical trials have concordantly revealed a high protective potential of antiviral CD8 T cells, of donor-derived ex vivo memory CD8 T cells in particular, in the immunotherapy of cytomegalovirus (CMV) infection in immunocompromised recipients. Although it is generally held view that the observed beneficial effect of the transferred cells is viral epitope-specific, involving the recognition of MHC class-I presented peptides by cognate T cell receptors, this assumption awaits formal proof, at least with regard to the in vivo function of the CD8 T cells. This question is particularly evident for CMV, since the function of viral immune evasion proteins interferes with the MHC class-I pathway of peptide presentation. Alternatively, therefore, one has to consider the possibility that the requirement for epitope recognition may be bypassed by other ligand–receptor interactions between CD8 T cells and infected cells, which may trigger the signaling for effector functions. Clearly, such a mechanism might explain why CD8 T cells are so efficient in controlling CMV infection despite the expression of viral immune evasion proteins. Here we provide direct evidence for epitope-specificity of antiviral protection by employing a recombinant murine CMV (mCMV), namely the mutant virus mCMV-IE1-L176A, in which an immunodominant viral epitope of the regulatory immediate-early protein IE1 is functionally deleted by a point mutation replacing

leucine with alanine at the C-terminal MHC anchor position of the antigenic peptide.

Keywords Antiviral control · BAC mutagenesis · CD8 T cells · Cell sorting · CTL line · Cytomegalovirus · Epitopes · Epitope deletion · Immediate-Early peptide · Immunotherapy · NKG2D receptor · Protection · RAE-1 ligand · T cell receptor

Introduction and rationale of the question addressed

Immunotherapy by adoptive transfer of donor-derived CD8 T cells is a promising option to avoid cytomegalovirus disease in immunocompromised recipients of hematopoietic stem cell transplantation (HSCT), both in the murine model [1, 2] and upon clinical application [3, 4] (see the accompanying review article by Holtappels et al. in this issue of MMI). Notably, in a preemptive therapy regimen tested in the BALB/c mouse model—a regimen that involves HSCT and adoptive T cell transfer on the day of murine CMV (mCMV) infection of immunocompromised recipients—as few as 400 IE1 epitope-specific, T cell receptor (TCR)-sorted memory CD8 T cells (CD8-T_M) derived from latently infected and thus CMV-immune donors inhibited the infection of the recipients' spleen by 3 log₁₀ infectious units compared to a “no therapy” control group. Moreover, 10⁴ of these cells proved to be curative in that they completely prevented viral histopathology and organ disease with no detectable side effect of immunopathology [1] (see also the accompanying review article by Holtappels et al. in this issue of MMI).

Such an impressive dose-effectiveness of CD8 T cell transfer is somewhat surprising in view of the fact that CMV-infected cells express virus-encoded glycoproteins,

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Fig. 1 Phenotypic and functional characterization of an IE1 epitope-specific CTL line. An IE1 epitope-specific short-term CTLL (briefly IE1-CTLL) was generated from spleen cells of latently infected, “memory” BALB/c mice (at 3 months after intraplantar infection with 10⁵ PFU of mCMV-WT.Smith) by three rounds of in vitro restimulation with the corresponding synthetic antigenic peptide IE1 168-YPHFMPNL-176 at a concentration of 10⁻⁹ M. **a** (top panel) TCR Vβ chain usage in the CTLL was tested by two-color cytofluorometric analysis using FITC-labeled Vβ screening panel antibodies [1] and APC-labeled L^d-IE1 peptide pentamers (ProImmune Limited, Oxford, GB). Bars show the percentages of Vβx (x = 2, 4, 6, 7, 8.1/ 8.2, 8.3, 9, 10^b, 13, 14)-expressing cells among pentamer-stained IE1-TCR⁺ cells. **a** (bottom panel) Two-color cytofluorometric analysis of the same cells by staining with APC-labeled L^d-IE1 peptide pentamers (see above) and PE-labeled rat IgG1 anti-mouse NKG2D (eBioscience, San Diego, California). Control staining was performed with APC-labeled D^d-m164 peptide pentamers specific for unrelated TCRs and PE-labeled rat IgG1 isotype control antibody. Shown are dot plots with 2,000 cells displayed. Scales represent log fluorescence intensities. **b** (left panel) IFN-γ ELISpot assay with the IE1-CTLL as responder cells and with MEF stimulator cells exogenously loaded with synthetic IE1 peptide at the indicated concentrations. Black bars represent the frequencies of responding cells calculated by linear regression analysis, and the error bars indicate the upper 95% confidence limits (see reference [1] for more details of the assay and the calculations). **b** (right panel) IFN-γ ELISpot assay with the IE1-CTLL as responder cells and with MEF as stimulator cells infected at a multiplicity of 4 with BAC-derived mCMV-WT.BAC or the mutant viruses indicated. n.i. Not-infected MEF, UV UV-light inactivated mCMV-WT.BAC

so called immunoevasins, that are specifically dedicated to interfere with the MHC class-I pathway of antigenic peptide presentation to CD8 T cells (for a review, see [5]). As shown for cells infected with wild-type mCMV (mCMV-WT), the concerted action of immunoevasins m04/gp34, m06/gp48, and m152/gp40 completely prevents lysis of infected cells by a large array of CTLs differing in MHC-restriction and in the epitopes recognized [6, 7], although CTL specific for the D^d-restricted m164-epitope represent a reported exception [8]. In principle, these findings also apply to the triggering of a second effector function of CD8 T cells, namely the secretion of interferon (IFN)-γ as measured in an ELISpot assay. Unlike the lysis of infected target cells, the ELISpot assay gives an estimate of the numbers of CD8 T cells that are capable of detecting presented peptide and that receive sufficient signaling required for IFN-γ synthesis and secretion. In the ELISpot assay the inhibition by the concerted action of the three immunoevasins was less complete compared to the

cytolysis assay in that a small proportion of CD8 T cells, most likely those with the highest functional avidity and TCR affinity, were sensitized by cells infected with mCMV-WT [7]. Nevertheless, there is consent between our group and Ann Hill’s group (see the contribution by Doom and Hill in this issue of MMI) that the inhibition exerted by the concerted action of all three immunoevasins is stronger than for any of the immunoevasins expressed alone or for any combination of two of them. Thus, there remains an obvious discrepancy between the almost complete inhibition of antigen presentation observed in vitro for a variety of cell types, including fibroblasts, macrophages, dendritic cells, and the strong protective function of CD8 T cells against mCMV-WT infection in vivo. Apparently, this discrepancy not only applies to adoptive CD8 T cell transfer, but may also explain the very limited impact of immunoevasin gene deletion in recombinant virus mCMV-Δm04 + m06 + m152 on the control of infection in immunocompetent mice [9] (see also the contribution by Doom and Hill in this issue of MMI). Clearly, if mCMV-WT is already almost completely controlled, how can we expect any significant further improvement by the deletion of the immune evasion genes?

Another striking finding revealed by the immunotherapy studies was the apparent irrelevance of the type of epitope; essentially all CTL lines (CTLL) protected in adoptive transfer assays regardless of the immunodominance status

Fig. 2 Expression of MHC class-I molecules and of NKG2D ligand RAE-1 by infected MEF. (*Left column*) Two-color cytofluorometric analysis of the expression of the ER-resident viral glycoprotein m164/gp36.5 (ordinate log fluorescence scale; Alexa Fluor 488), identifying infected cells, and the MHC class-I molecule L^d (abscissa log fluorescence scale; PE) performed as described in greater detail previously [7]. Shown are *dot plots* with 2,000 cells displayed. (*Right column*) Corresponding analysis, except that abscissa PE fluorescence represents the expression of RAE-1 measured by using PE-conjugated rat anti-mouse RAE-1 γ antibody (CX1, Pharmingen). MEF were infected at a multiplicity of 4 with the viruses indicated. *n.i.* Not-infected MEF, *UV* UV-light inactivated mCMV-WT.BAC

of the respective antigenic peptide (for reviews, see [10] and the accompanying article by Holtappels et al. in this issue of MMI). We therefore began to wonder if the observed protection was based on TCR-mediated recognition of MHC class-I presented antigenic peptide at all. Actually, it is known that at least activated CD8 T cells express NKG2D, better known as an activating natural killer (NK) cell receptor interacting with cellular ligands of the RAE-1

family, as well as with H60 and MULT-1. Furthermore, mCMV-infected cells express the virus-encoded ligand m157 of the activating NK cell receptor Ly49H that is expressed in C57BL/6 mice (for reviews, see [11] and the accompanying article by Lenac in this issue of MMI). Although the latter example applies to NK cell function, still unknown ligand–receptor interactions between infected cells and activated T cells might likewise exist, which could possibly account for a TCR- and epitope-independent triggering of protective effector functions. Such a TCR-bypassing induction of effector function would even be specific for mCMV-infected cells in the case that the putative ligand is virus-encoded or at least selectively expressed.

To our knowledge, epitope-specificity of the CD8 T cell-mediated protection against CMV has never been proven in a formal scientific sense. Here we report on our approach to solve this problem.

Results

NKG2D expression and target cell recognition by an IE1-epitope specific CTLL

As explained in detail in the accompanying review by Holtappels et al. (this issue of MMI), epitope-specific CD8 T cells for adoptive cell transfer can either be generated from a polyclonal and polyspecific pool of CD8- T_M by selection through repeated restimulation with the MHC-presented cognate antigenic peptide, resulting in an epitope-specific CTLL, or can be directly purified from the pool of CD8- T_M by TCR-based epitope-specific cell sorting making use of MHC-peptide multimers as TCR ligands.

With the aim to realize the first strategy, we generated a CTLL specific for the L^d -restricted IE1 peptide 168-YP-HFMPTNL-176 (Fig. 1). After as few as three rounds of in vitro restimulation of CD8- T_M , the resulting short-term CTLL proved to be still polyclonal—as indicated by a broad TCR- β chain usage (Fig. 1a, *top*)—but already epitope-specific, since virtually all cells were stained by an MHC-IE1-peptide pentamer. Notably, most of the cells co-expressed NKG2D (Fig. 1a, *bottom*). It should be noted that all cells also still expressed the co-receptor molecule CD8 (data not shown) [1], which is important for the avidity of target cell recognition.

A polyclonal CTLL usually consists of individual cells differing in TCR affinity and functional avidity. The range of functional avidities represented in the particular IE1-specific CTLL studied herein, was assessed in an IFN- γ secretion-based ELISpot assay using L^d -expressing, BALB/c ($H-2^d$)-derived mouse embryo fibroblasts (MEF)

Fig. 3 Schema of the mutagenesis rationale. The IE1 exon 4 *green* encodes the IE1 peptide 168-YPHFMPTNL-176 that binds to the MHC class-I molecule L^d with anchoring amino acid residues proline (P) in position 2 and leucine (L) in position 9 [27]. For annulling antigenicity, the C-terminal anchor residue leucine is replaced with alanine (L176A) by introducing the codon mutation CTA → GCA

into the BAC-cloned mCMV genome. Revertants A176L and A176L* were generated by replacing alanine with leucine by means of codon mutations GCA → CTA and GCA → CTT, respectively [21]. Mutations are marked in *red*. Reproduced from Ref. [22] with permission by Caister Academic Press

as stimulator cells exogenously loaded with synthetic IE1 peptide in graded concentrations (Fig. 1b, *left panel*). Whereas high-avidity cells in the CTLL population recognized presented IE1 epitope at a peptide concentration of 10^{-10} M, low avidity cells became sensitized only at 10^{-8} M. Higher concentrations did not recruit more CTL for recognition, which implies that all cells that are capable of responding were stimulated.

The recognition of endogenously processed and presented IE1 peptide by the so characterized CTLL was tested with MEF as stimulator cells (Fig. 1b, *right panel*). Absence of CTL sensitization by uninfected MEF (*n.i.* not infected) verified the virus-specificity of the CTLL, which implies that cross-recognition of cellular peptides by IE1-specific TCRs played no role. Absence of sensitization by MEF exposed to UV light-inactivated virus showed that viral gene expression is required, thus excluding a possible recognition of virion protein-derived peptides [12, 13] as well as of cellular peptides induced by the signaling and the resulting global change of the cellular transcriptome that is associated with the viral entry process (for reviews see [14, 15]). In accordance with all what we have learnt about immunoevasins encoded by mCMV (for reviews see [5, 10, 16, 17], as well as the article by Doom and Hill in this issue of MMI), MEF infected with mCMV-WT.BAC stimulated only a minor fraction of the IE1-specific CTL in this T cell line, most likely those with the highest avidity. Absence of viral immunoevasins m04/gp34 and m06/gp48 in MEF infected with the respective deletion mutant mCMV- Δ m04 + 06 improved peptide presentation and thus

recruited also CTL with somewhat lower avidity. Stimulation of all cells that are capable of responding (recall Fig. 1b, *left panel*) required the additional deletion of the immunoevasin m152/gp40 in MEF infected with the respective deletion mutant mCMV- Δ m04 + 06 + 152 (Fig. 1b, *right panel*).

RAE-1 ligands of NKG2D do not mediate the sensitization of an IE1-specific CTLL

So far so good, but do we really have definite proof for TCR-mediated IE1-epitope specificity of the CTL sensitization? Alternatively, one might have speculated that other receptor–ligand pairs formed between the CD8 T cells and infected stimulator cells might account for triggering IFN- γ synthesis in the activated CD8 T cells also in a TCR-independent manner, with NKG2D-RAE-1 interaction being a candidate. Clearly, such a mechanism can hardly explain the peptide-dose dependence of the recognition of uninfected cells exogenously loaded with antigenic peptide (see above), but the situation is less obvious for infected cells.

As shown above, cells of the IE1-specific CTLL express NKG2D. For interaction with RAE-family members to occur, namely RAE-1 α , β , and γ in the case of BALB/c mice [18], these molecules must be expressed by the stimulator or target cells. It is known, however, that the immunoevasin m152/gp40 simultaneously down-regulates MHC class-I and RAE-1 cell surface expression (for a review, see [11]), a finding reproduced by us in the

◀ **Fig. 4** Verification of the immunological “loss of function” phenotype of mutant mCMV-IE1-L176A. **a** Loss of target cell recognition by the mutation L176A measured with an IFN- γ ELISpot assay using the IE1-CTL as responder cells, and MEF as stimulator cells that were infected at a multiplicity of four with the viruses indicated. To enhance *ie1* gene expression and avoid the action of immunoevasins, infection was performed in the presence of cycloheximide for 3 h followed by blocking early phase transcription with actinomycin D [12]. Grey bars represent the frequencies of sensitized responder cells, and error bars indicate the 95% confidence intervals. **b** Loss of CD8 T cell priming and memory by the mutation L176A. Shown are IFN- γ ELISpot assays measuring the frequencies of epitope-specific CD8-T_M present in a spleen-derived immunomagnetically-purified CD8 T cell population of “memory” BALB/c mice at 5 months after intraplantar infection with 10^5 PFU of the viruses indicated. Stimulator cells were P815 (*H-2^d*) mastocytoma cells exogenously loaded with synthetic IE1-peptides corresponding to the mutations A176L and L176A, and for control with the D^d-restricted m164 peptide that is unrelated to the mutations. Ø no peptide added. Reproduced from reference [21] with the permission by the Journal of Virology (American Society for Microbiology)

expression was most strongly reduced in the presence of all three immunoevasins after infection with mCMV-WT.BAC and still significantly but less completely reduced in the selective presence of m152/gp40 after infection with mCMV- $\Delta m04 + 06$. This is in good accordance with the corresponding CTL sensitization data shown in Fig. 1b (right panel). Yet, since RAE-1 was found to be reduced simultaneously after infection with these two viruses (Fig. 2, right column), the CTL sensitization data would be compatible also with NKG2D-RAE-1 interaction.

What clearly argues against epitope-independent CTL sensitization through NKG2D-RAE-1 interaction is the fact that uninfected cells as well as cells mock-infected with UV-inactivated virus failed to stimulate the CD8 T cells, although a significant proportion of them expressed NKG2D (Fig. 2). In addition, as we have shown previously, IE1-CTL completely failed in being sensitized by infected MEF derived from BALB/c-H-2^{dm2} mice in which the IE1-epitope presenting MHC class-I molecule L^d is not expressed due to a deletion in the MHC locus [7]. Another argument against a critical role for NKG2D-RAE-1-mediated sensitization of CD8 T cells is provided by recent data from Ann Hill’s group demonstrating that blockade of this interaction with anti-NKG2D antibody had for some epitopes a detectable but altogether little impact on CTL activity [19]. This is in accordance with the current view that NKG2D-RAE-1 interaction does not independently lead to signaling in NKG2D-expressing T cells, but that NKG2D may act as a costimulatory molecule enhancing TCR-mediated, epitope-specific sensitization [20]. This applies then also to other NKG2D ligands, namely MULT-1 and H60 that are down-regulated by the virally-encoded proteins m145 and m155, respectively.

experiment shown in Fig. 2. As in our experience cultures of infected MEF always contain cells that are refractory to infection [7], a phenomenon that is independent of the multiplicity of infection (data not shown), it is essential to restrict the analysis of MHC class-I and of RAE-1 expression to infected cells. This was achieved by two-color cytofluorometric analysis of the expression of the viral ER-resident glycoprotein m164/gp36.5 (S.A.O.-K., manuscript in preparation) and MHC class-I glycoprotein L^d (Fig. 2, left column) [7] or of RAE-1 (Fig. 2, right column), respectively. In accordance with the literature, L^d

Fig. 5 Epitope-specificity of antiviral protection by an IE1 epitope-specific CTLL. (*Top panel*) schema of the experimental protocol for preemptive immunotherapy of CMV infection in the immunocompromised host (see also the accompanying review article by Holtappels et al. in this issue of MMI). BALB/c recipients were infected with the viruses symbolized and received graded cell numbers of an IE1-CTLL by intravenous administration. *Flash symbol* total-body γ irradiation with a single dose of 6.5 Gy. (*Bottom panel*) resulting data. Virus titers are shown as plaque-forming units (PFU) per organ; infected cells in the liver refer to a representative tissue section area of 10 mm². *Symbols* represent individual recipients, with *closed* and *open circles* indicating infection with revertant and mutant virus, respectively. The *horizontal bars* mark the median values

Universal approach to test for epitope-specificity in vitro and in vivo

Although we have now excluded a TCR-independent, epitope-unspecific activation of NKG2D-expressing CD8 T cells through interaction of NKG2D with its ligands expressed on target cells in vitro, the reasoning was complicated and was limited to a particular receptor–ligand interaction, not knowing whether other interactions might exist between CD8 T cells and mCMV-infected cells. Expression of putative virally-encoded ligands for such interactions appears to be plausible in the light of the fact that CMVs in general have a high coding capacity with many genes that are not essential for virus replication but may play a role in virus–host interaction.

For testing epitope-specificity of target cell recognition in vitro, and, more importantly, of protective antiviral function in vivo rigorously and unambiguously, we have chosen the universal genetic approach of selectively deleting an epitope by a point mutation of its C-terminal MHC anchor residue that binds to a hydrophobic pocket in the class-I molecule. This strategy has a reasonably good chance to selectively annul the recognition of the epitope of interest without

affecting any other known as well as unknown interactions between CD8 T cells and infected target cells. The strategy is sketched in Fig. 3 for the case of the IE1 epitope. BAC mutagenesis was used to replace codon CTA with GCA leading to an amino acid replacement of leucine with alanine in mutant virus mCMV-IE1-L176A. For control and testing of unintended alterations in the genome, two revertant viruses were produced, namely mCMV-IE1-A176L by back-mutating codon GCA to CTA, as well as a revertant mCMV-IE1-A176L* in which a single nucleotide marker (like a single nucleotide polymorphism; SNP) is left in the wobble position of the Leu codon CTT [21, 22].

As demonstrated in greater detail previously [21], the L176A mutation did not interfere with any of the known regulatory functions of the IE1 protein but showed the intended immunological phenotype of preventing the recognition of infected target cells by IE1-specific CTL in vitro (Fig. 4a), as well as the priming of an IE1 epitope-specific CD8 T cell response in vivo (Fig. 4b). Thus, apparently, no other known or unknown receptor–ligand interaction between CD8 T cells and target cells was able to substitute for the specific recognition of L^d-presented IE1 peptide by cognate TCRs.

Fig. 6 Epitope-specificity of antiviral protection by TCR-sorted IE1 epitope-specific CD8-T_M. Legend as for Fig. 5 except that IE1 epitope-specific CD8-T_M were transferred for preemptive immunotherapy. The CD8-T_M were isolated from the spleens of latently infected BALB/c mice (at 3 months after infection with 10⁵ PFU of mCMV-WT.Smith) by immunomagnetic purification of CD8 T cells followed by cytofluorometric sorting of cells expressing IE1 epitope-specific TCRs. For details of cell sorting, see reference [1]. Asterisks mark the individual mice used for the liver histology shown in Fig. 7

Eminent epitope-specificity of antiviral CD8 T-cell function in adoptive transfer

Infection in vivo is a condition that is only imperfectly represented by in vitro findings; therefore, only adoptive transfer of epitope-specific CD8 T cells can give a definite answer.

In a first approach, we used the preemptive therapy protocol (see also the review article by Holtappels et al. in this issue of MMI). Immunocompromised BALB/c recipients were infected with the WT-like virus revertant mCMV-IE1-A176L or with the epitope mutant mCMV-IE1-L176A and, on the same day, received graded numbers of IE1-CTL. After 12 days, viral load in the lungs and spleen was determined by virus plaque assay, and infection of the liver was quantitated by immunohistology detecting intranuclear IE1 protein in infected liver cells (Fig. 5, *schema*). The data were unequivocal in that the transferred IE1 epitope-specific effector cells controlled the in vivo replication of the epitope-expressing revertant virus in a dose-dependent manner, whereas infection with the mutant virus progressed uninfluenced (Fig. 5, *bottom*). In a second approach we asked if ex vivo TCR-sorted IE1-specific CD8-T_M would behave somewhat differently (Fig. 6). Except the known fact that CD8-T_M are much more

efficient than cells of a CTL (see the review article by Holtappels et al. in this issue of MMI), the epitope-specificity was fully reproduced. For the liver, one might discuss a tendency towards control of the epitope mutant virus at higher cell numbers, but this did not reach statistical significance (comparison between 2,000 and 10,000 cells; $P = 0.4$; Wilcoxon–Mann Whitney rank sum test, two-sided). For visualizing antiviral control by 10⁴ transferred CD8-T_M directly in liver tissue, a two-color immunohistology was performed that identified infected liver cells—which in their majority are hepatocytes—by red staining of intranuclear IE protein and T cells by black staining of cell membrane-bound CD3ε (Fig. 7). Whereas many plaque-like foci of infection were found in livers infected with the epitope mutant virus (Fig. 7, *panels a1 and a2*), infection with the revertant virus was confined to focal T cell infiltrates and largely controlled (Fig. 7, *panels b1 and b2*).

Concluding remarks

In this work we have asked the question if the protective antiviral effect of CD8 T cells in the preemptive therapy of CMV infection is strictly epitope-specific, involving the recognition of MHC class-I-presented antigenic peptide by

Fig. 7 Epitope-specific formation of focal infiltrates confining the infection. Two-color immunohistological visualization of liver infection (red staining of intranuclear IE1 protein pp76/89 in infected liver cells) and of T cell infiltration (black staining of cell membrane-bound CD3 ϵ). The analysis corresponds to the experiment shown in Fig. 6, specifically to the asterisk-marked mice having received 10^4 IE1 epitope-specific CD8-T_M. **a1, a2** Infection of the adoptive transfer recipient with mutant virus mCMV-IE1-L176A. **b1, b2** Infection of the adoptive transfer recipient with revertant virus mCMV-IE1-

A176L. **a1** Liver tissue section showing multiple, plaque-like foci of infection in the absence of T-cell infiltration. The *arrow* points to a focus shown in greater detail in panel **a2**. Note the intranuclear inclusion bodies, which are pathognomonic of CMV disease and represent the site of viral nucleocapsid formation and DNA packaging. **b1** Liver tissue section showing rare foci of infection in the presence of focal T cell infiltration. The *arrow* points to a focus shown in greater detail in **b2**. Note the encirclement of an infected hepatocyte by many T cells. Bar markers represent 50 μ m

cognate TCRs, or if CD8 T cells can exert a beneficial function also by alternative receptor–ligand interactions. This question cannot be addressed in clinical research, but the murine model can provide “proof of concept”. The answer given by the murine model was pretty clear: *protection is strictly epitope-specific*.

This finding has an additional implication. Besides alternative receptor–ligand interactions between CD8 T cells and infected tissue cells, an antiviral function of epitope-specific CD8 T cells that is unrelated to the recognition of the cognate epitope might have resulted from a “degeneracy” of epitope-specific TCRs (for classical and more recent reviews, see [23–26]). In essence, “degeneracy” means that TCRs are sufficiently flexible to

tolerate mutations at the TCR contact site of cognate epitopes. TCRs may even recognize completely unrelated epitopes differing from the cognate epitope in amino acid sequence of the antigenic peptide as well as in the presenting MHC class-I molecule, most likely based on an analogous shape acquired by cognate and unrelated MHC–peptide complex. An early study identifying the TCR contact sites of the IE1 peptide has indeed revealed that replacements with alanine were critical for an IE1-specific monoclonal CTLL, whereas substitutions were tolerated by IE1-specific polyclonal CTLL ([27] and M.J.R., unpublished data). Thus, our present study has shown that even for polyclonal IE1 epitope-specific CTLL as well as CD8-T_M, cross-recognition of unrelated

epitopes presented by infected cells is not an issue in both target cell recognition in vitro and protection against infection in vivo.

Another important aspect of our work concerns the somewhat astounding finding that focal T cell infiltrates, which develop in liver tissue infected with the IE1 peptide-encoding revertant virus mCMV-IE1-A176L, are missing in liver tissue infected with the epitope mutant virus mCMV-IE1-L176A (Fig. 7). So, the formation of T cell infiltrates is apparently epitope-specific; but how can T cells sense their cognate epitope from a distance to become recruited specifically to infected cells? In fact, it is more conceivable that T cells are attracted to the foci of infection by chemokines, as it was recently suggested from the finding that recruitment of antigen-specific T cells to the mCMV-infected liver involved the chemokines CXCL9 and CXCL10 as well as the expression of the corresponding chemokine receptor CXCR3 by the T cells [28]. Whether epitope-specificity of focal infiltrate formation relates to attraction of CD8 T cells or to local clonal expansion upon antigen encounter is an obvious question to be addressed next.

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