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Review Article

Reverse Genetics Modification of Cytomegalovirus Antigenicity and Immunogenicity by CD8 T-Cell Epitope Deletion and Insertion

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The advent of cloning herpesviral genomes as bacterial artificial chromosomes (BACs) has made herpesviruses accessible to bacterial genetics and has thus revolutionised their mutagenesis. This opened all possibilities of reverse genetics to ask scientific questions by introducing precisely accurate mutations into the viral genome for testing their influence on the phenotype under study or to create phenotypes of interest. Here, we report on our experience with using BAC technology for a designed modulation of viral antigenicity and immunogenicity with focus on the CD8 T-cell response. One approach is replacing an intrinsic antigenic peptide in a viral carrier protein with a foreign antigenic sequence, a strategy that we have termed "orthotopic peptide swap". Another approach is the functional deletion of an antigenic peptide by point mutation of its C-terminal MHC class-I anchor residue. We discuss the concepts and summarize recently published major scientific results obtained with immunological mutants of murine cytomegalovirus.

1. Introduction

Cytomegaloviruses (CMVs) are large enveloped DNA viruses with a linear double-stranded genome of more than 200 kbp in size and a correspondingly high coding capacity of ~160–170 open reading frames (ORFs) [1–4]. Human CMV (hCMV) is the prototype virus of the *Betaherpesvirinae*, a subfamily of the *Herpesviridae*, and is one of the eight known human pathogens of the herpesvirus family, namely, Human Herpesvirus 5 (HHV-5), according to virus taxonomy. It is ubiquitous in the human population with a prevalence among adults of about 50%–70% in industrialised and up to 100% in developing countries. Efficient control by the effector mechanisms of both innate and adaptive immunity prevents overt disease in immunocompetent individuals; however, like with all herpesviruses, after resolution of productive infection, the viral genome is maintained lifelong in a nonproductive state of infection known as viral latency (for reviews, see [5–7]). During latency, by definition [7], infectious particles are no longer produced, and viral genes of the productive cycle are mostly silenced. In more recent concepts, CMV latency is regarded as a more dynamic state of gene silencing and desilencing, with episodes of reactivated viral gene expression that are sensed and terminated by effector-memory CD8 T cells before virions can be assembled and released [8]. This "nonproductive" antigenic activity during latency is considered to be the driving force for repetitive T-cell restimulation and clonal expansion, a phenomenon known as "memory inflation" (for reviews, see [9, 10]). Accordingly, interrupting this immune control of latency by immunosuppressive measures allows reactivated viral gene expression to proceed to the

production of infectious virions [11, 12] resulting in recurrent infection and organ disease. Generally, in individuals with an immature or compromised immune system, acute or recurrent hCMV infection can cause severe disease with multiple organ manifestations such as interstitial pneumonia, hepatitis, gastrointestinal diseases, and bone marrow failure. The reactivation of latent hCMV is a frequent complication after iatrogenic immunosuppression in allogeneic solid organ transplantation or hematoablative conditioning in hematopoietic stem cell transplantation (HSCT), resulting in CMV organ disease and graft failure (reviewed in [13]). In particular, the acute congenital, intrauterine hCMV infection of an embryo or fetus is a major clinical problem. It can result in miscarriage and stillbirth or cause multiple immediate or delayed birth defects, including microencephaly/microcephaly, intracranial calcifications, cerebral palsy, epilepsy, vision loss, sensorineural hearing loss, developmental delays, and mental retardation (for impressive case reports, go to http://williamshaffer.org/cmv/). Development of an hCMV vaccine has, therefore, been assigned a high priority by the Institute of Medicine, The National Academies, Washington (see [14], reviewed in [15]). It is thus of utmost importance to better understand the principles of immunity against CMVs in acute and latent infection.

Since betaherpesviruses are strictly host species-specific in their replication [16], hCMV infection and pathogenesis cannot be analysed in animal models, except in humanized mice with human tissue implants, which is highly demanding and has limitations [17–20]. The immune response to hCMV can be inferred from immunological monitoring in hCMV-infected (seropositive) but otherwise healthy volunteers or in patients with acute or reactivated hCMV infection (for a review, see [21]). A problem is, however, that the genetics and hence the proteomes and the repertoire of immunogenic sequences of patients' virus isolates are not known in advance and mostly not even accessible retrospectively. An aimed experimentation with defined virus strains—natural ones or engineered viruses—prohibits, of course. Studying animal CMVs paradigmatically in the corresponding natural host is, therefore, used for identifying principles of predictive value. Among animal models of CMV infection [22–24], of which the rhesus macaque model is most likely closest to the situation in humans [25, 26], the mouse model using murine CMV (mCMV) is currently still most advanced [9, 27, 28], mainly for logistic reasons and because of defined and easily manipulable host genetics and the availability of a wealth of already existing mouse mutants. The probably most relevant contribution of the mouse model so far was the identification of CD8 T cells as the principal antiviral effector cells operative in the cellular immunotherapy of CMV disease in immunocompromised HSCT recipients (see [29–34]; for a recent review, see [35]), a finding that was successfully translated into clinical research, trials, and practice [36–40].

CD8 T cells recognize infected cells through the interaction of their T cell receptor (TCR) with a cell-surface presentation complex formed by an antigenic viral peptide bound to a major histocompatibility complex class I (MHC-I) molecule (see [41]; for a review see [42]). The following presented viral peptides are generated by proteolytic processing of viral proteins, usually in the proteasome, and are transported via the TAP-complex into the lumen of the endoplasmic reticulum, where they bind to nascent MHC-I proteins under the assistance of chaperones. Finally, the peptide-loaded MHC-I (pMHC) complexes travel with the vesicular flow to the cell surface [43–45].

The first antigenic peptide of mCMV to be identified was the IE1 peptide $_{168}$YPHFMPTNL$_{176}$ (IE$_{168-176}$) that is derived from the regulatory IE1 protein pp76/89 (encoded by ORFm123) and is presented by the murine MHC-I molecule H-2-L^{d} [46, 47]. It was only during the past decade that a number of further antigenic peptides of mCMV were identified for MHC haplotypes H-2^{d} (summarized in [35]) and H-2^{b} [48]. It is self-evident that the repertoire of potentially antigenic viral peptides in a certain virus-host combination depends on the sequences of the viral proteins, that is the proteome of the particular virus strain/isolate, as well as on the presenting MHC-I alleles expressed in the individual host. In addition, whether a presented peptide is actually immunogenic also depends on the individual TCR repertoire of the host, which is generated by random gene rearrangements and differs even between genetically identical individuals such as in mouse inbred strains. So, the repertoire of responding CD8 T cells is unique for each individual virus-host pair. That nevertheless certain antigenic peptides elicit a quantitatively higher response than others, a phenomenon known as immunodominance, is not yet fully understood (reviewed in [49]). Whilst overlapping 15-amino acid peptides representing all known ORFs for a pangenomic evaluation of hCMV immunogenicity revealed antigenic peptides for most ORFs with memory CD8 T cells derived from the MHC-I polymorphic test population of 33 hCMV-seropositive volunteers enrolled to represent high MHC allele coverage, individual persons responded only to a limited number of ORFs, namely, between only 1 and 32 ORFs with a median value of 8 ORFs [50]. This is in good accordance with the number of antigenic ORFs identified with an mCMV library of ORF transfectants, with the CD8 T-cell response in C57BL/6 mice, haplotype H-2^{b} [48] being somewhat broader than in BALB/c mice, haplotype H-2^{d} [51, 52]. It is important to note that specificity repertoires differ between acute and memory CD8 T-cell responses [53, 54], since repeated restimulation of only certain specificities by viral gene expression and presentation of antigenic viral peptides/epitopes during latency (for a review, see [9]) is supposed to shape and restrict immunological memory. CD8 T-cell repertoire focusing during selection into memory, concomitant with clonal expansion and increasing oligoclonality, applies not only to different epitopes but also to TCR clonotypes specific for defined individual epitopes [55].

Here, we review our experience and findings using bacterial artificial chromosome (BAC) mutagenesis of the mCMV genome to specifically alter the antigenic potential of the virus, that is, its "immunome", by deleting immunodominant epitopes and by replacement of intrinsic antigenic peptides with foreign antigenic peptides.

2. Manipulation of the CMV Genome

2.1. Genetic Manipulation in the Pre-BAC Era. For a long time, a major limitation in manipulating CMV-genomes has been the cloning and the handling of these large DNA molecules of ~230 kbp in size. So, it was then not possible to clone the complete genomes as single molecules. Instead, a cosmid library was used to clone overlapping fragments of the hCMV genome [56]. This allowed manipulating parts of the viral genome, cloned into plasmids with classical cloning methods, by introducing mutations like deletions or insertions into viral genes. Plasmids carrying the mutated sequences were subsequently cotransfected with the conventionally isolated wild-type (WT) viral genomic DNA to retrieve recombinant genomes by spontaneous homologous recombination [57]. Alternatively, transfection of the complete set of overlapping cosmids was used to produce recombinant viral genomes [58]. Other investigators have used site-specific recombination in mammalian cells that were cotransfected with viral genomes and PCR-fragments containing a selectable marker flanked by sequences determining the target region in the viral genome [59]. However, all these methods have drawbacks. (I) The procedure is very time consuming due to the slow replication kinetics of hCMV in cell culture. (II) After transfection, cells always contain a mixture of viral WT and mutated genomes so that cross-complementation can occur. (III) In order to select successfully mutated viral genomes, viral particles need to be plaque-purified in presence of a drug for selection during at least three passages. (IV) The efficiency of homologous recombination in eukaryotic cells is low compared to bacterial systems. (V) The dependence of these techniques on replicating virus during the selection process does not allow the isolation of mutants defective in replication. The cloning of CMV genomes into BACs allowed to overcome these limitations.

2.2. Entrapping the Viral Genome. The development of BACs from the *E. coli* F-factor allowed cloning of DNA fragments of more than 100 kbp in size in contrast to conventional plasmids and cosmids, which were limited to ~20 kbp and ~40 kbp, respectively [60]. This allowed Messerle and colleagues in their pioneering work [61] to clone the full-length mCMV genome, as the first genome of a herpesvirus, into a single molecule for propagation in bacteria. To achieve this, the BAC-vector was integrated into a nonessential stretch of the mCMV genome, replacing the region ranging from ORFm151 to ORFm158. In comparison with WT virus, however, the virus reconstituted from this BAC prototype was impaired in its replicative fitness *in vivo* due to the deletion of viral immunomodulatory sequences and the integration of BAC vector sequences [62]. For instance, deletion of ORFm152 in the viral genome attenuates the virus by the loss of the most potent dual immunoevasin of mCMV, protein gp40/m152, which simultaneously inhibits the CD8 T-cell response by retaining pMHC in a cis-Golgi compartment and the NK cell response by downmodulating RAE-1 ligands of the activatory NK cell receptor NKG2D [63–66]. In order to reconstitute viral pathogenicity, the deleted genomic region was reinserted and, in addition, the BAC vector sequence was flanked by short identical sequences to allow its elimination by homologous recombination during virus passage in eukaryotic cells [62]. The resulting BAC is larger in size than is the regular viral capsid packaging capacity. Thus, viral genomes that have undergone recombination are preferentially encapsidated during intranuclear capsid assembly, which results in a rapid loss of bacterial sequences from the recombinant BAC genomes during the propagation of the reconstituted virus. The successful cloning of the mCMV genome paved the way for the cloning of other herpesvirus genomes, for example of hCMV [67], HSV-1 [68], EBV [69], and MHV-68 [70].

Most importantly, the cloning of the complete viral genome as a BAC made it accessible to the powerful mutagenesis methods available in the *E. coli* system and thus allowed targeting any site in the viral genome for site-directed mutation (see [71, 72], for a review see [73]). BAC recombination systems have many advantages. (I) Use of the *E. coli* recombination system allows an effective and rapid mutation and selection process compared to the fairly inefficient and slow recombination in eukaryotic cells. (II) It enables the introduction of insertion-, deletion- and point-mutations. (III) The low copy numbers of the BAC per bacterial cell make it possible to isolate genetically defined mutants with only minimal risk of WT contamination and cross-complementation. (IV) Mutated genomes can easily be characterised by restriction enzyme analysis, PCR, and sequencing prior to virus reconstitution. (V) The process of introducing the mutation is absolutely independent of viral replication. Hence, it allows production and isolation of mutants that lack essential functions. (VI) Mutations can be easily reverted to demonstrate their causal involvement in an observed phenotype.

Altogether, the cloning of CMV genomes as BACs opens all possibilities to perform extensive studies of viral gene functions by reverse genetics approaches including single gene deletions and insertions, genome-wide analysis of loss-of-function mutations, as well as a pinpointed analysis of gene function by single amino acid replacement with minimal alteration of the viral genome.

2.3. BAC Mutagenesis for the Introduction of Seamless Point Mutations or Peptide Swaps. Up to date, several methods for the mutagenesis of BACs are established, and they exhibit—based on the type of mutation—specific advantages and disadvantages. The fastest way for generating viral mutants, for example, gene deletion mutants, is the use of linear DNA fragments generated by PCR combined with the *red* recombination enzymes of the bacteriophage λ [71, 74–77]. The major disadvantage of this method, however, is that a selection marker or—after removing the selection marker by Flp recombinase [78, 79]—some nucleotides remain in the viral genome after mutagenesis. The removal of any unwanted surplus nucleotides is indeed essential for the generation of viral mutants with changes in single nucleotides or several codons for single amino acid or peptide sequence replacements, for which maintenance of the respective viral

ORF is mandatory. Three methods of choice exist to avoid surplus nucleotides: (I) BAC recombination using the *gal*K selection marker [80], (II) markerless recombination using the *red* recombinase system in combination with homing endonuclease I-*Sce*I cleavage [81], and (III) allelic exchange using a shuttle plasmid [61, 62, 67, 82–84]. In contrast to the single-step method using linear DNA fragments, all three methods require two recombination steps, which is somewhat more time consuming. Whilst the *gal*K selection and the cleavage by I-*Sce*I involve special bacteria, the allelic exchange requires the cloning of a shuttle plasmid. The most common shuttle plasmid is the suicide vector pst76-KSR [67, 85] that offers a temperature-sensitive replication and contains positive as well as negative selection markers. Into this vector, the mutation, usually generated by PCR, is integrated flanked by homologous regions of about 2 kbp specific for the intended integration site in the CMV BAC [61]. For constructing a functional shuttle vector several cloning steps might be necessary, but once the plasmid is cloned, it is widely applicable for introducing the respective mutation into a range of BAC backbones that already carry other mutations of interest (Figure 1).

3. Manipulation of Viral CD8 T-Cell Epitopes

Reverse genetics using BAC mutagenesis can be used to engineer viruses with altered immunological properties, for instance by physical or functional deletion of intrinsic antigenic peptides/epitopes or by insertion of foreign epitopes, thereby modulating the viral "immunome" as a part of its proteome. Deletion of intrinsic epitopes allows to evaluate the contribution of the respective native sequences to the antiviral immune response and to predict the consequences of epitope loss as it might occur in natural virus variants [88]. On the other hand, insertion of a foreign epitope in a replicating vector virus can be used as a research tool providing an "immunological tag" or as a vaccine approach for improving the immune response against a pathogen from which the "transgenic epitope" is derived. Table 1 compiles both types of mutant viruses and corresponding revertant viruses produced in our lab and made available upon request. Figures 2 and 4 illustrate these two concepts of CMV immunomutagenesis.

3.1. Replacement of Single Amino Acid Residues in an Antigenic Peptide. Single amino acid substitutions at either the TCR contact sites or the MHC-I contact sites of an antigenic peptide can be used to modulate or destroy the antigenicity and immunogenicity of an epitope [10, 90–92]. Such mutations can have a profound influence on the CD8 T-cell response with an as minimal as possible influence on protein function and viral biology, in particular when substitutions are "conservative" avoiding replacement of positively charged with negatively charged residues, of charged with uncharged residues, of hydrophobic with hydrophilic residues, or of conformation compatible with conformation breaking residues and vice versa. In our lab, we followed the established strategy of deleting one or more epitopes functionally by loss-of-presentation point mutagenesis replacing the respective C-terminal "anchor" amino acid residue, which is usually a tailed hydrophobic residue binding deeply into a hydrophobic pocket of the peptide-presenting MHC-I molecule [93–97], with alanine (A, Ala). This was achieved by BAC mutagenesis of the mCMV genome, using the two-step replacement method with shuttle vectors [8, 51] (Figure 1). The rationale of this strategy is illustrated in Figure 2 for the example of replacing the MHC-I H-2-L^d-binding C-terminal amino acid leucine (L, Leu) in the immunodominant IE1 peptide YPHFMPTNL (see the Introduction) with Ala, yielding the mutation L176A in recombinant virus mCMV-IE1-L176A [8] (Table 1). For this peptide, early studies [98] have mapped TCR contact residues, MHC contact residues, and neutral "spacer" residues by using the prototypic clonal cytolytic T lymphocyte (CTL) line IE1, the clone with which the IE1 peptide was originally identified [47], and a set of synthetic peptides in which systematically each position in the sequence was replaced with Ala, thereby identifying proline (P, Pro) in position 2 and Leu in position 9 of the peptide as the two residues that preferentially bind to the presenting MHC-I molecule L^d. Since "spacer" residues can potentially acquire an MHC binding function compensating for an outage of a "budgetary" MHC binding residue [98], loss of MHC-I binding by the point mutation must be verified experimentally and for each peptide under study. In the specific case of using clone IE1 as the probe, substitution of Leu with Ala reduced the antigenicity of exogenously peptide-loaded target cells by $\sim 6 \log_{10}$ grades of molar peptide concentration whereas the reduction was equivalent with Val, less complete with Met, Ile, and Tyr, but more complete with Asn and Trp [99]. We, nevertheless, chose the replacement with Ala, since the small biochemical difference of just an isopropyl group in the hydrophobic side chain promised an only minimal impact, if any, on the regulatory functions of the IE1 protein. This assumption was confirmed experimentally in a number of functional assays, including viral replicative fitness, IE1's transcriptional transactivator activity, and its capacity to dissociate repressive nuclear domains ND10 [8]. It is important to note that the reduction in the recognition of the mutated peptide is also influenced by the functional avidity distribution of the CD8 T-cell population used for the assay in that high-avidity effector cells can be triggered even by trace amounts of presented peptide whereas low-avidity effector cells may then fail. For peptide SIINFEKA, we have indeed seen an only ~10-fold reduction of antigenicity compared with SIINFEKL when high-avidity OT-I cells, carrying a transgenic K^b-SIINFEKL-specific TCR [100], were stimulated with peptide-loaded target cells (Gergely and Lemmermann, unpublished data). Such a reduction may nevertheless be already critical for the endogenous generation and presentation of pMHC complexes, in particular when viral immunoevasins inhibit their transport to the cell surface (see [89], reviewed in [15, 52]). It is important to note that replacement of the C-terminal residue of an antigenic peptide also impacts its generation by proteasomal cleavage. This effect of the mutation can be predicted by *in silico* analysis with NetChop

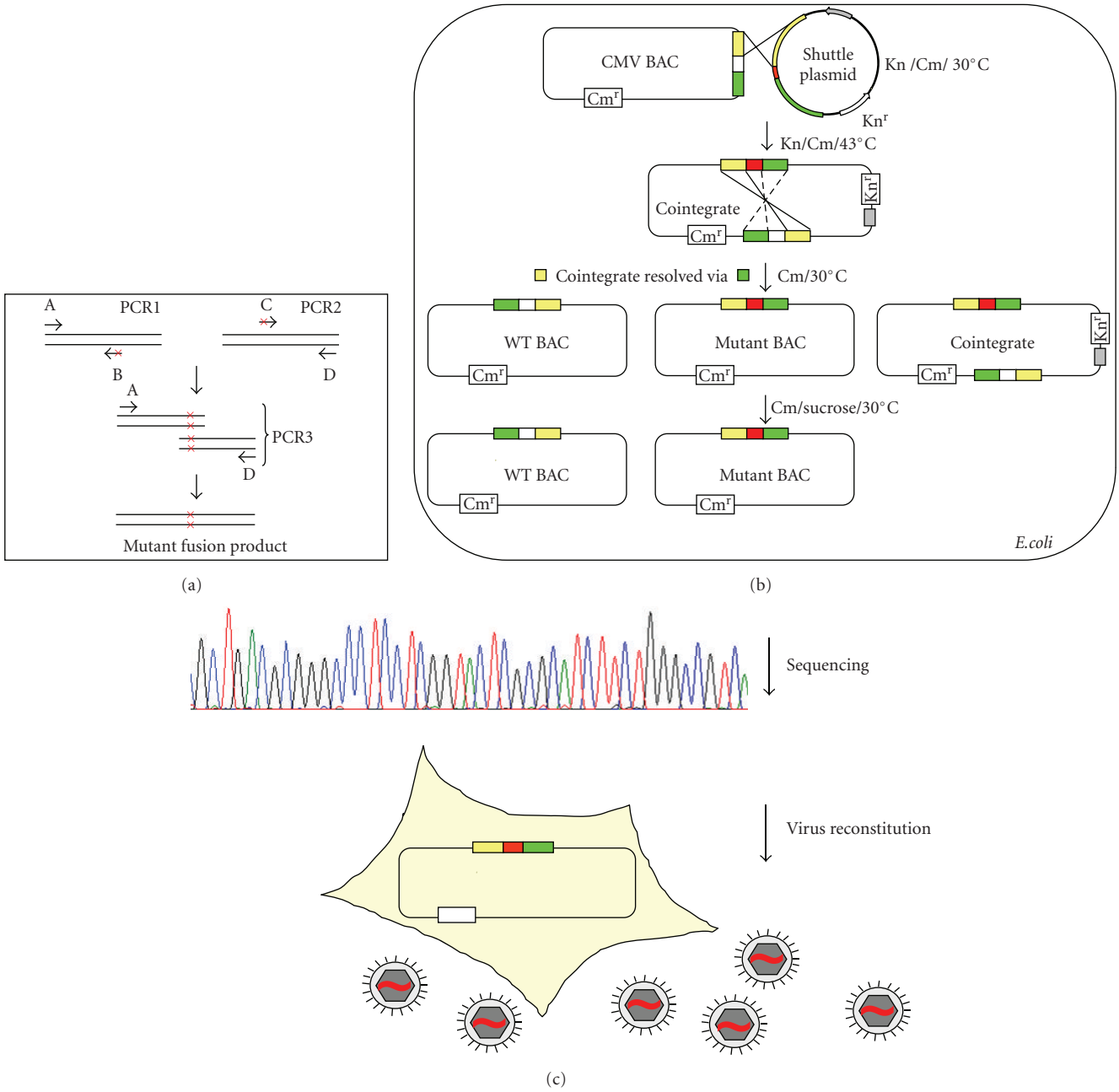

Figure 1: Method of herpesvirus point mutagenesis. (a) Site-directed mutagenesis by overlap extension. The dsDNA and primers are represented by double lines and arrows, respectively. The direction of the arrows is indicating the 5′-to-3′ orientation of primers, which are denoted by capital letters. The site of mutagenesis is marked with red symbol X. PCR amplificates yielded by PCR1 and PCR 2 served as templates in the combination PCR (PCR3) performed with primers A and D to generate the final amplificate that includes the intended point mutation. (b) Shuttle-plasmid allelic exchange in *Escherichia coli*. A shuttle plasmid harbouring the point mutation (red box) flanked by homologous viral sequences (green and yellow boxes) is transformed into bacteria that contain the CMV BAC. The first homologous recombination leads to formation of cointegrates due to recombination via one homology arm. Cointegrates are selected by chloramphenicol (Cm) and kanamycin (Kn). Nonintegrated shuttle plasmids are removed at 43°C. In the second recombination step, cointegrates are resolved to generate either a WT viral BAC (via yellow box) or a mutant viral BAC (via green box). Clones still containing cointegrates are eliminated by sucrose counterselection against SacB (gray box), and finally, bacterial clones containing either mutated or WT CMV BACs are isolated (see [61, 62], for detailed description of the method, see [82]). (c) DNA of selected bacterial clones was tested for genomic structural integrity, and the presence of the mutation was confirmed by sequencing. Reconstitution of BAC-derived recombinant virus was achieved by transfection of mutated BAC DNA into permissive eukaryotic cells. After at least four viral passages and a second round of plaque purification, PCRs were performed to verify the absence of BAC vector sequences [8].

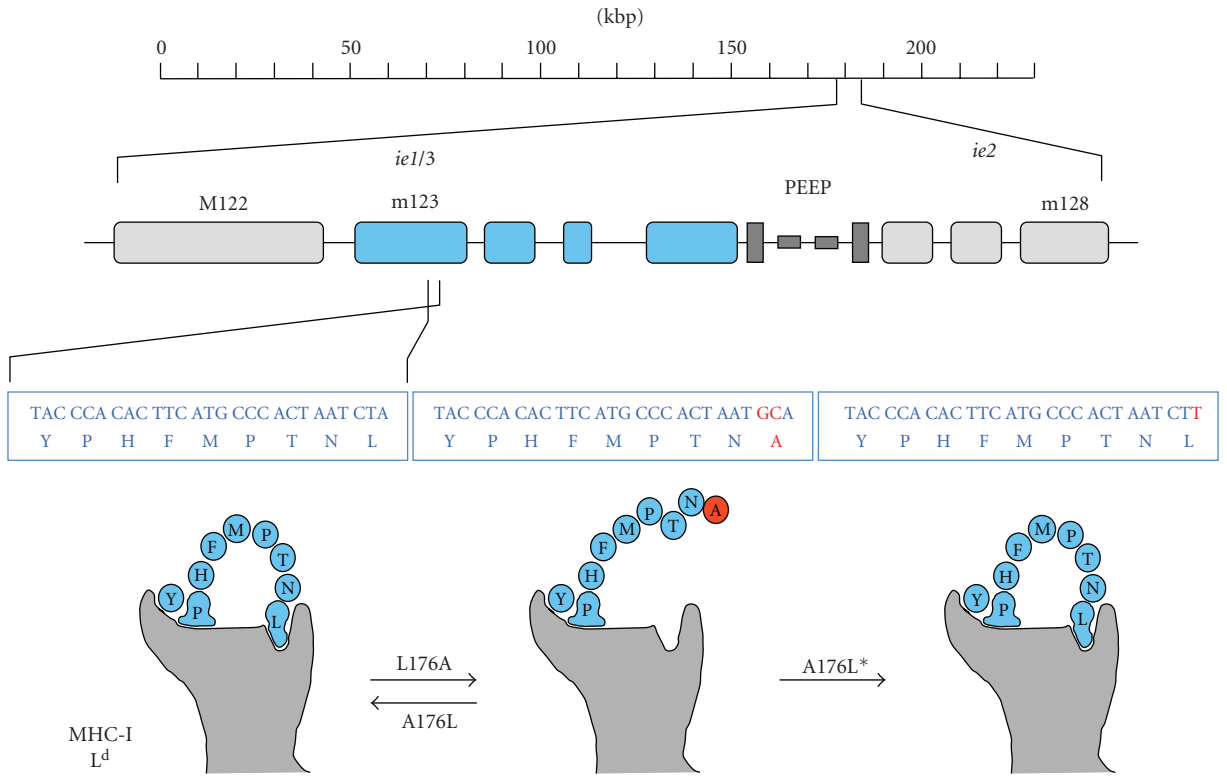

Figure 2: Loss-of-presentation mutagenesis of antigenic peptides. (Top) Genome size scale of mCMV and bidirectional gene pair architecture of the major immediate-early (MIE) locus, with the promoter-enhancer-enhancer-promoter (PEEP) region flanked to the left by the *ie1/3* transcription unit (ORFs m123/M122) and to the right by gene *ie2* (ORFm128) [86]. Exons are symbolized by boxes, with blue boxes representing the four exons specifying the IE1 mRNA. The coding sequence for the antigenic IE1 peptide is located in exon 4. (Center) Nucleotide and corresponding amino acid sequences for the authentic and mutated IE1 peptides, with mutations being highlighted in red. (Bottom) Artwork models illustrating the binding of authentic and mutated IE1 peptides to the presenting MHC class-I molecule H-2-L^d through the C-terminal anchor residue. The mutation is highlighted in red. L176A: mutant; A176L: authentic revertant; A176L*: "wobble" revertant maintaining a single nucleotide polymorphism as a genetic marker distinguishing it from WT and authentic revertant.

Table 1: Recombinant mCMVs with mutated CD8 T cell epitopes.

Virus mCMV-X	Protein	Replacement	Resulting peptide	Ref.
IE1-L176A	IE1	L176A	$_{168}$YPHFMPTNA$_{176}$	[8]
IE1-A176L	IE1	A176L$_{[CTA]}$	$_{168}$YPHFMPTNL$_{176}$	[8]
IE1-A176L*	IE1	A176L$_{[CTT]}$	$_{168}$YPHFMPTNL$_{176}$	[8]
m164-I175A	m164	I175A	$_{167}$AGPPRYSRA$_{175}$	[51]
m164-A175I	m164	A175I	$_{167}$AGPPRYSRI$_{175}$	[51]
[IE1 − L176A + m164 − I175A]	IE1+m164	L176A I175A	$_{168}$YPHFMPTNA$_{176}$ $_{167}$AGPPRYSRA$_{175}$	[51]
[IE1 − A176L + m164 − A175I]	IE1+m164	A176L A175I	$_{168}$YPHFMPTNL$_{176}$ $_{167}$AGPPRYSRI$_{175}$	[51]
SIINFEKL	m164	$_{167}$AGPPRYSRI$_{175}$ → $_{167}$SIINFEKL$_{174}$	$_{167}$SIINFEKL$_{174}$	[89]
SIINFEKA	m164	$_{167}$AGPPRYSRI$_{175}$ → $_{167}$SIINFEKA$_{174}$	$_{167}$SIINFEKA$_{174}$	[89]
Δm06m152-SIINFEKL	m164	$_{167}$AGPPRYSRI$_{175}$ → $_{167}$SIINFEKL$_{174}$	$_{167}$SIINFEKL$_{174}$	[89]
Δm06m152-SIINFEKA	m164	$_{167}$AGPPRYSRI$_{175}$ → $_{167}$SIINFEKA$_{174}$	$_{167}$SIINFEKA$_{174}$	[89]

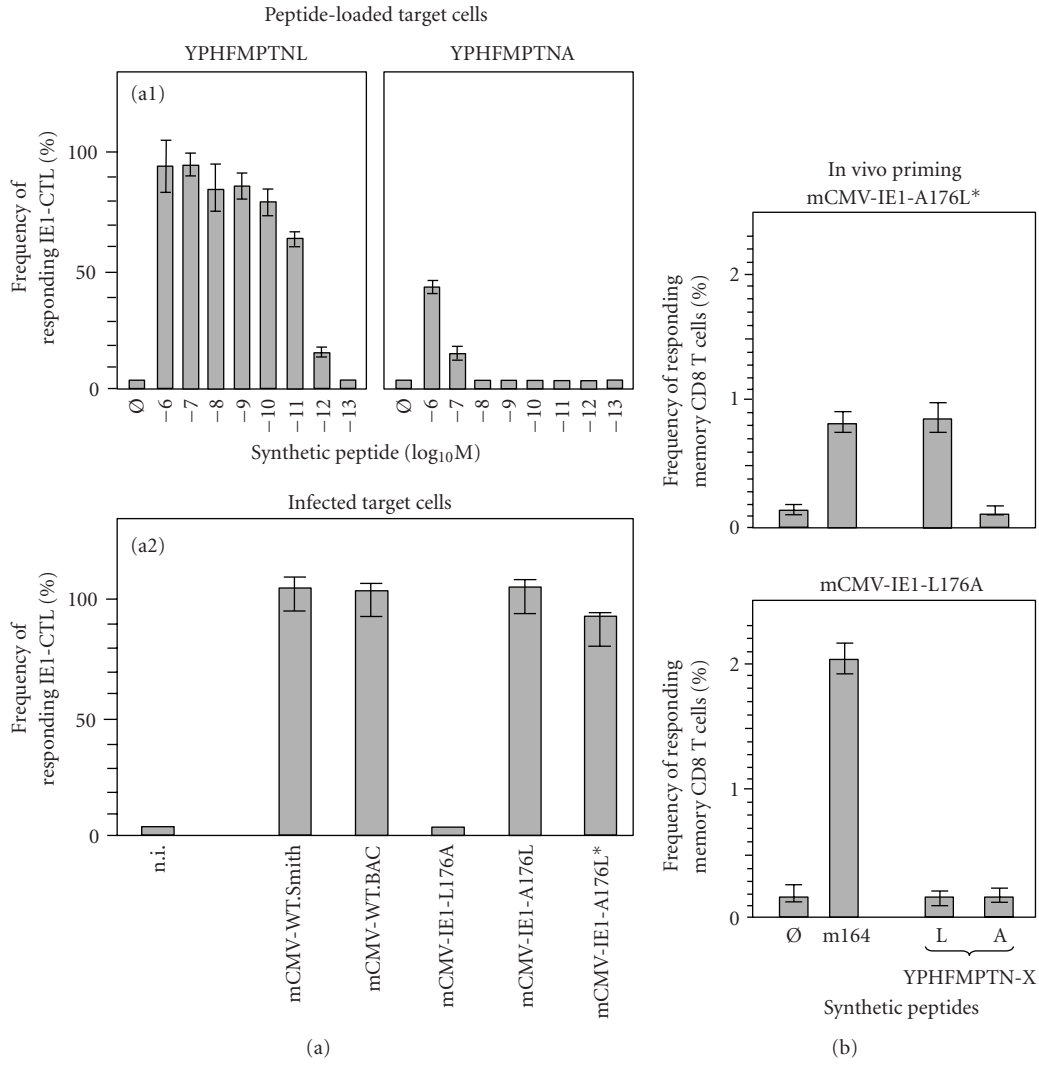

Figure 3: Verification of the loss-of-presentation phenotype. (a1) Loss of MHC-I H-2-L^d binding by replacement of the C-terminal anchor residue Leu with Ala in the IE1 peptide. The response of a polyclonal IE1-specific CTLL, representing a broad TCR affinity repertoire, was measured in an IFN-γ-based ELISpot assay performed with L^d-expressing stimulator cells loaded exogenously with the indicated synthetic peptides at graded molar concentrations. Recognition of pMHC complexes by CTL serves as an indirect measure for pMHC formation by peptide binding to the presenting MHC-I molecule ∅, no peptide added. Note that presented IE1-Ala analog is recognized only by high-avidity CTL after high-dose peptide loading. (a2) Loss of endogenous peptide presentation by the mutation L176A. The response of an IE1-CTLL (see above) was measured with L^d-expressing stimulator cells that were infected with the indicated WT, mutant, or revertant viruses (see also Table 1). n.i., not infected as a negative control. Note the selective lack of recognition of stimulator cells infected with mutant virus mCMV-IE1-L176A. (b) Loss of *in vivo* immunogenicity by the mutation L176A. BALB/c (H-2^d haplotype) mice were infected with "wobble" revertant virus or with mutant virus (see Figure 2), and *ex vivo* isolated memory CD8 T cells were used as effector cells in an IFN-γ-based ELISpot assay performed with L^d-expressing stimulator cells that were loaded exogenously with saturating doses of the indicated synthetic peptides ∅, no peptide added. Note the selective loss of IE1-specific CD8 T-cell priming after infection with mutant virus mCMV-IE1-L176A. See Simon et al. [8] for further explanation and experimental details. Reproduced in modified arrangement from reference [8] with permission by the Journal of Virology (American Society for Microbiology).

3.1 (http://www.cbs.dtu.dk/services/NetChop/) [101] as well as with MAPPP (http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html) [102] and experimentally determined by *in vitro* proteasomal digestion of ~25 amino acid-long substrates (comprising the peptide of interest with its authentic flanking residues), followed by mass spectrometric identification and quantitation of processed fragments [103]. As a consequence, even if the reduction of MHC-I binding of the mutated peptide is incomplete, a complete block of endogenous pMHC formation and thus of peptide presentation in infected cells can result from the combination of inefficient proteasomal cleavage and low affinity of MHC-I binding.

The ultimate functional consequences of the L176A mutation in the IE1 peptide [8] are recapitulated in Figure 3. Whilst exogenous loading of cell surface MHC-I L^d with the

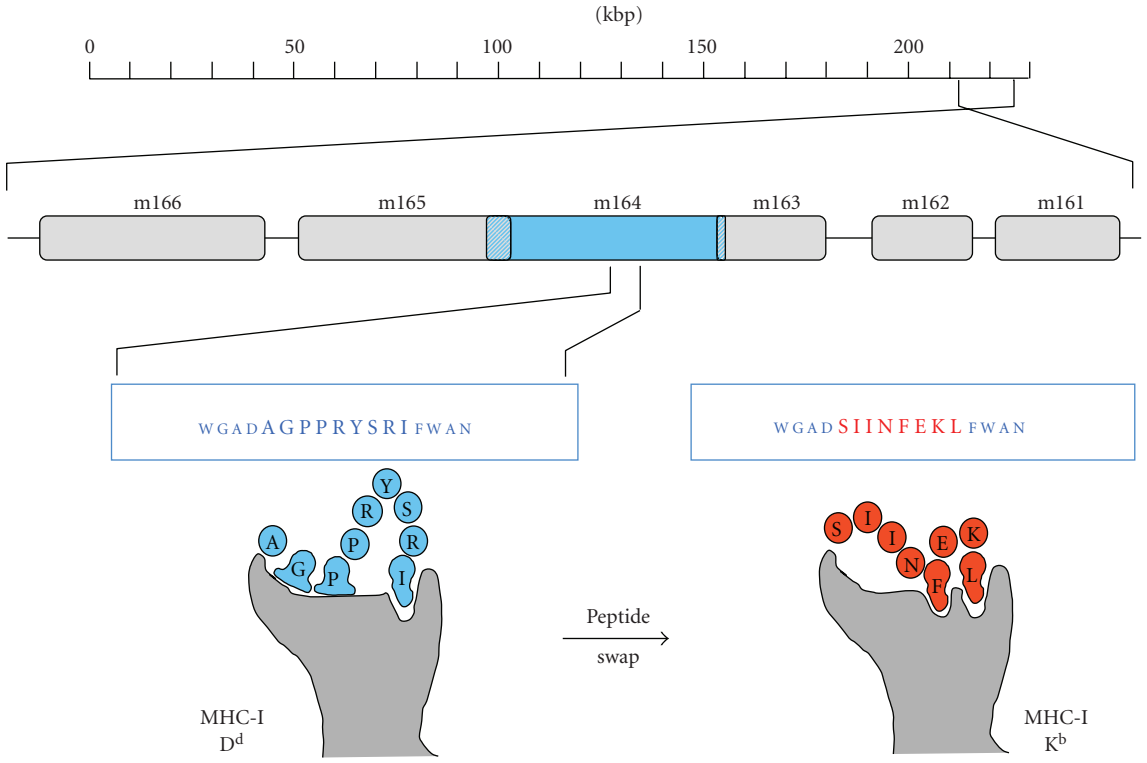

Figure 4: Principle of "orthotopic peptide swap" mutagenesis. (Top) Genome size scale of mCMV showing the genomic region of ORFs m161-m166 enlarged. ORFs are symbolized by boxes, with the blue box representing ORFm164 encoding the viral carrier protein gp36.5 [87] that includes the coding sequence for an intrinsic antigenic peptide that is immunodominant in the MHC haplotype H-2^d [53]. Overlaps with neighboring ORFs are indicated by blue-striped areas. (Center) Amino acid sequences of the intrinsic peptide to be replaced (blue letters with large type size) and the replacing foreign peptide (red letters with large type size). Epitope-flanking residues in the host protein sequence are shown in blue letters with small type size. (Bottom) Artwork models illustrating the binding of the intrinsic antigenic peptide (blue chaplet) and of the inserted foreign antigenic peptide (red chaplet) to their respective presenting MHC-I molecules. Note that in the documented case, peptide swap also leads to a change in MHC-restriction.

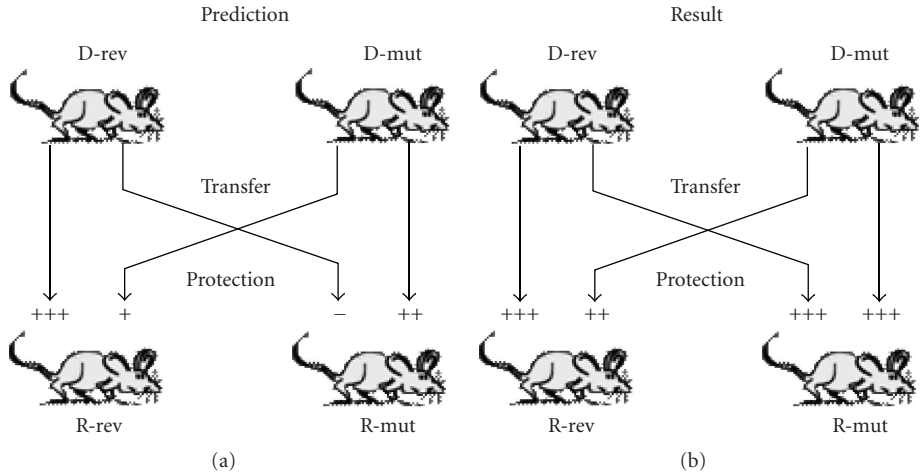

Figure 5: Sketch of a criss-cross adoptive CD8 T-cell transfer experiment for evaluating the role of viral epitope mismatch in protection against CMV disease by cellular immunotherapy. (a) Predicted result. (b) Actual result. Memory CD8 T-cell donors D-rev and D-mut were immunocompetent BALB/c mice infected 6 months earlier with mCMV-rev and mCMV-mut, respectively, expressing or lacking two immunodominant epitopes (IE1 and m164). Recipients R-rev and R-mut were immunocompromised BALB/c mice acutely infected with the respective viruses shortly after the cell transfer. Arrows indicate the direction of cell transfer. The predicted and observed efficacies of antiviral protection are qualitatively classified from very efficient (+++) to inefficient (). Experimental assessment of antiviral protection was performed by measuring virus replication in spleen, lungs, and liver. For experimental details and the original quantitative data, see [51].

mutated peptide can be enforced by high peptide concentration (Figure 3(a1)), cells infected with virus mutant mCMV-IE1-L176A are not recognized by polyclonal IE1 peptide-specific CTL (Figure 3(a2)). Importantly, the mutated virus also failed in priming of an IE1 epitope-specific CD8 T-cell response *in vivo* (Figure 3(b)).

3.2. Insertion of Foreign Epitope-Encoding Sequences. Ectopic expression of a full-length antigenic protein, for instance ovalbumin (OVA) or the influenza virus nucleoprotein (Flu-NP), is widely employed [104, 105] whereas more recent approaches used an ectopic expression of a foreign antigenic peptide C-terminally attached to an intrinsic viral protein as a fusion protein. Specifically, Karrer and colleagues [106] expressed the lymphocytic choriomeningitis virus-(LCMV-) derived peptide $GP_{(33-41)}$ as well as the influenza virus-derived peptide $NP_{(366-374)}$ in respective BAC-cloned recombinant mCMVs in the context of the IE2 protein under the control of the major immediate-early (MIE) enhancer promoter and showed the induction of a protective CD8 T-cell response against these unrelated viral pathogens. This finding implies successful proteasomal processing of the peptides from the respective fusion proteins. A similar approach was used by Peter Doherty's group in the influenza virus system, namely, the insertion of the OVA-derived peptide SIINFEKL ($OVA_{(257-264)}$) into the neuraminidase stalk [107]. In our lab, a modified approach was taken recently, namely, an "orthotopic peptide swap" mutation (Figure 4) replacing an immunodominant peptide in a nonessential viral carrier protein, specifically peptide AGPPRYSRI in protein gp36.5/m164 [87], position precisely with a foreign antigenic peptide, with SIINFEKL in the specific case [89] (Table 1). The rationale for this strategy was to choose a viral protein as a carrier protein that based on its expression kinetics, abundance, and biochemical features supports the processing of an immunodominant peptide, as well as an integration site that is flanked by sequences which support proteasomal cleavage in principle. Although each modification of a viral protein, regardless of whether sequences are replaced or additional sequences attached, can alter its conformation and can have adverse or more subtle and difficult to predict effects on viral functions, which may apply even to proteins that are nonessential for viral replication, orthotopic peptide swap represents the minimal manipulation needed for the intended immunological phenotype. Nonetheless, we routinely measure viral replicative fitness *in vivo* by virus doubling times in various immunocompromised host organs to exclude replicative attenuation of the mutant [8, 86, 108]. One might argue that orthotopic peptide swap automatically implies a phenotype of reduced antigenicity and immunogenicity due to the replacement of an immunodominant intrinsic epitope. It is, therefore, important to emphasize that the replaced antigenic peptide and the replacing antigenic peptide are chosen so as to differ in their MHC-I restriction. Specifically, in the example of SIINFEKL presented by MHC-I allele K^b in mice of H-2^b haplotype, for instance, in C57BL/6 mice, the replaced peptide was presented by the MHC-I allele D^d and was thus immunodominant only in mice of H-2^d haplotype, for instance in BALB/c mice [89] (Figure 4).

4. Major Scientific Insights Gained with BAC-Cloned Viral Epitope Mutants

During the past few years, our lab has generated a panel of mCMV recombinants with manipulated CD8 T- cell epitopes (Table 1). These recombinants have helped elucidating some aspects of the interplay between mCMV and the immune system of its host. Here, we briefly summarize published findings made in our lab or by our associates.

4.1. Epitope-Specificity of CD8 T Cell-Mediated Antiviral Protection. Earlier work had shown that CD8-positive CTL lines (CTLL) specific for pMHC complexes formed with mCMV-encoded antigenic peptides protect against mCMV multiple-organ infection and disease upon adoptive cell transfer into immunocompromised recipients, a preclinical model for cytoimmunotherapy of CMV disease in the immunocompromised host that is aimed at curing HSCT recipients (reviewed in [35]). Notably, in the murine model, CTLL of different peptide specificities were similarly efficient in antiviral protection independent of the magnitude of the CD8 T-cell response against the cognate pMHC during mCMV infection, that is, independent of their so-called "immunodominance". In fact, even epitopes at the low end of the response hierarchy with barely detectable *in vivo* immunogenicity upon host infection, such as m04-D^d or M84-K^d, mediated protection by the cognate CTLL [109, 110]. Although it has always been the tacit and generally accepted understanding that antiviral protection by CTLL is mediated by epitope-specific pMHC-TCR interaction, this was never formally documented for protection against CMV infection, and thus the virtual irrelevance of the type of viral peptide for protection by CTLL in the adoptive transfer experiments raised the question of whether receptor-ligand pairs other than pMHC-TCR, for instance, RAE1-NKG2D or yet unidentified interactions, might actually trigger an antiviral effector function of the CD8 T cells and CTLL. Most relevant in this context are our observation that CTLL can indeed lyse B7-expressing target cells through interaction with CD28 independent of pMHC-TCR interaction (Holtappels and Reddehase, unpublished data) and the published finding that CD28-B7 is critical for early control of mCMV infection [111]. This issue was finally settled by showing that IE1 epitope-specific CTLL as well as memory CD8 T cells sort purified *ex vivo* with pMHC multimers failed to protect adoptive cell transfer recipients against infection with the loss-of-presentation mutant mCMV-IE1-L176A but protected against infection with the revertant virus mCMV-IE1-A176L [29]. Notably, in this study, focal infiltration of infected host tissues by the transferred IE1 epitope-specific cells required the presentation of the cognate epitope, indicating that virally induced or encoded chemokines alone [112] may attract CD8 T cells but are not sufficient to retain them at the sites of viral replication.

4.2. Impact of Antigenic Mismatches between Donor and Recipient Virus Variants in Cytoimmunotherapy. Reactivated CMV infection is the most critical viral complication after HSCT. The risk of HSCT-associated CMV-disease is defined by the CMV status of transplantation donor (D) and recipient (R). Of the four possible combinations (D^-R^-,D^+R^-,D^-R^+, and D^+R^+), D^-R^+ bears the highest risk by reactivating latent CMV from the recipients' own tissues [21, 113]. In contrast, a protective effect of preexisting donor immunity leads to a lower risk of CMV disease in the D^+R^+ combination despite a potential additional virus reactivation from the transplanted donor cells. One promising approach for preventing CMV reactivation and disease is the adoptive transfer, clinically also known as donor lymphocyte infusion (DLI), of donor-derived CMV-specific CD8 T cells. Since the donor's CMV variant (CMV-D) and the recipient's CMV variant (CMV-R) can differ in their antigenic peptide repertoire, transferred donor immunity and recipient pMHC presentation may, however, not always fully match, with the consequence of an inefficient antiviral control in the recipients. We addressed this problem experimentally in the BALB/c mouse model by using the dual epitope-loss-mutant mCMV-[IE1 L176A + m164 I175A] (briefly mCMV-mut) and the corresponding revertant virus mCMV-[IE1 A176L + m164 A175I] (briefly mCMV-rev) (Table 1) for infecting immunocompetent donor mice and immunocompromised recipient mice in all four possible combinations of antigenic match and mismatch for a criss-cross adoptive transfer experiment [51] (Figure 5). The IE1-L^d and m164-D^d pMHC complexes have previously been identified as "immunodominant" epitopes in the memory CD8 T-cell repertoire of mCMV-infected BALB/c mice, as defined by the magnitude of the immune response [53]. Thus, if donors are primed with mCMV-mut, CD8 T cells of these two specificities are missing in the memory CD8 T-cell pool for protection against mCMV-rev infection of the recipients. Reciprocally, if donors are primed with mCMV-rev, CD8 T cells of these two specificities are useless for protection against mCMV-mut infection of the recipients. In the first combination (D-mut/R-rev), absence of "immunodominating" epitopes was predicted to favor the expansion of donor CD8 T cells specific for subdominant epitopes, and this compensation may in part restore protective activity. In contrast, in the second combination (D-rev/R-mut), the focus of the donor immune response on the immunodominant epitopes might inhibit the response to subdominant epitopes, a mechanism known as "immunodomination". Thus, from the recipients' perspective, the latter combination ought to be most unfavorable for protection since the donor immune response is directed against "useless epitopes" and even disfavors "useful epitopes". We, therefore, expected a protection hierarchy of D-rev/R-rev > D-mut/R-mut > D-mut/R-rev $\gg$ D-rev/R-mut (Figure 5(a)). The result of this experiment, however, did not really follow the theory (Figure 5(b)). Protection of the recipients turned out to be comparably efficient in all four combinations, with some disadvantage for D-mut/R-rev, suggesting that inhibition of subdominant epitopes by dominant epitopes rather operates on the level of antigen presentation by the infected target cells in the recipients' tissues. Altogether, these data are encouraging from a medical point of view as they show that CD8 T-cell-based immunotherapy of CMV infection is rather robust, tolerating even major antigenicity differences between CMV variants. The data also indicate that antiviral protection does not critically depend on immunodominant epitopes, which applies at least to complex viruses with high coding capacity, as it is the case for CMVs.

4.3. Control of Cytomegalovirus Latency by Immune Sensing of Transcriptional Reactivation Episodes. During mCMV latency in the lungs, viral genomes are not silenced all the time but spontaneous or cytokine-triggered desilencing of genes can lead to transcriptional reactivation episodes that are terminated before viral DNA replication and virion assembly (reviewed in [9]). Theoretically, gene desilencing might occur sporadically at any viral gene locus during latency, but only desilencing at the MIE locus has the potential to proceed in the viral replicative cycle, since IE genes, specifically IE2 of hCMV and IE3 of mCMV, code for essential transcriptional transactivators. Detection of mCMV IE1 and IE2 transcripts in latently infected lungs, indicating MIE locus desilencing [11, 114], along with the finding that recently sensitized CD62L-low effector-memory CD8 T cells specific for the IE1 peptide persist in the lungs and increase in number during latency [115], led to the hypothesis that episodes of transcriptional activity correspond to episodes of antigenic activity driving the expansion of the IE1 epitope-specific cells, which in turn terminate the reactivation episodes by means of their antiviral effector functions. This is, in essence, what the "immune sensing hypothesis of cytomegalovirus reactivation" proposes (see [46, 115], reviewed in [9, 10]). For a limited number of other mCMV epitopes, CD8 T cells also expand or "inflate" during latency [53, 54], and this is usually regarded as an indirect evidence for an expression of the corresponding viral genes during latency. It must be noted, however, that the prototype example IE1 still remains the only example for which the transcripts were actually detected during latency. A gap in the chain of evidence is the missing molecular detection of the IE1 protein and of the presented IE1 peptide, which may relate to the low incidence of transcriptional episodes. We thus argued that genetic deletion of the IE1 epitope from the viral epitope repertoire, which precludes immunological sensing of IE1 transcription episodes, should alter the transcription profile in latently infected host tissue. By using the loss-of-presentation mutant mCMV-IE1-L176A in comparison to corresponding revertant viruses (Figure 2), Simon and colleagues [8] could indeed document an increased number of detectable IE1 transcription episodes and a progression to splicing of the essential transactivator transcript IE3. Thus, the epitope mutant provided the first and still only molecular evidence for antigenic peptide presentation during episodes of transcriptional reactivation.

4.4. Molecular Quantitation of mCMV-Immunoevasion. CMVs code for glycoproteins that are dedicated to subvert immune recognition of infected cells and are, therefore,

referred to as "immunoevasins", as "viral proteins interfering with antigen presentation (VIPRs)", or as "viral regulators of antigen presentation (vRAPs)" [15, 52, 116, 117]. For immunoevasins affecting the MHC-I pathway of antigen presentation, downmodulation of cell surface MHC-I is a hallmark often used in cytofluorometric analyses as an indicator of immunoevasin function. Since all MHC-I molecules normally reach the cell surface via the constitutive vesicular transport as pMHC complexes loaded with self-peptides in uninfected cells or with self and viral peptides in infected cells, downmodulation of cell surface MHC-I impacts the presentation of antigenic peptides. The term "downmodulation", however, is somewhat imprecise with regard to the underlying mechanism in that it leaves open if MHC-I molecules are actively removed from the cell surface or if their disappearance is indirectly mediated by the immunoevasins and rather reflects constitutive cell surface MHC-I turnover in the absence of resupply by newly generated pMHC complexes. Addressing this question requires to distinguish between MHC-I present at the cell surface in advance of infection and new pMHC complexes travelling to the surface after viral peptide loading onto nascent MHC-I molecules in the ER. For this, an antibody is needed that specifically recognizes a defined pMHC complex, but to date no such antibody is available for a complex between an MHC-I molecule and a CMV peptide. To overcome this technical limitation and to quantitate specific pMHC complexes at the cell surface, we used BAC mutagenesis of mCMV for "orthotopic peptide swap" replacing the intrinsic antigenic sequence in the viral carrier protein gp36.5/m164 with the OVA-derived model peptide SIINFEKL [89] (Figure 4), for which a monoclonal antibody T-AG25.D1-16 exists that recognizes the SIINFEKL-K^b presentation complex [118]. With this tool (see Table 1 for the respective viruses), we could show that after infection of cells in the absence of viral immunoevasins ~10% of all cell surface K^b molecules present endogenously processed SIINFEKL at the cell surface, namely ~10,000 out of ~100,000 in absolute terms, which is an enormous occupancy by a single antigenic peptide. Importantly, this number was reduced to <100 SIINFEKL-K^b complexes after infection with the corresponding virus expressing the immunoevasins. Thus, immunoevasins strongly inhibit the transport of recently formed pMHC complexes to the cell surface. To answer the initial question of the mode of MHC-I cell surface downmodulation, cell surface K^b molecules were exogenously preloaded with synthetic SIINFEKL peptide followed by infection with SIINFEKA viruses that were lacking or expressing immunoevasins (see Table 1 for the respective viruses) but were unable to present endogenously processed SIINFEKL due to the C-terminal anchor residue mutation (same principle as outlined in Figure 2). Notably, after a time of infection when presentation of endogenously processed peptide was already almost completely blocked, specifically at 8 h, cell surface exposure of preloaded SIINFEKL-K^b complexes was still hardly affected. This data led us to the conclusion that immunoevasins inhibit the transport of endogenously loaded pMHC complexes to the cell surface and that the eventual downmodulation of total MHC-I cell surface expression results from turnover in the absence of resupply. Block of pMHC resupply is explained by retention of pMHC complexes in a cis-Golgi compartment by mCMV immunoevasin gp40/m152 [66] and rerouting of pMHC complexes for lysosomal degradation by mCMV immunoevasin gp48/m06 [119].

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References

[1] A. J. Davison, A. Dolan, P. Akter et al., "The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome," *Journal of General Virology*, vol. 84, no. 1, pp. 17–28, 2003.

[2] A. Dolan, C. Cunningham, R. D. Hector et al., "Genetic content of wild-type human cytomegalovirus," *Journal of General Virology*, vol. 85, no. 5, pp. 1301–1312, 2004.

[3] W. D. Rawlinson, H. E. Farrell, and B. G. Barrell, "Analysis of the complete DNA sequence of murine cytomegalovirus," *Journal of Virology*, vol. 70, no. 12, pp. 8833–8849, 1996.

[4] Q. Tang, E. A. Murphy, and G. G. Maul, "Experimental confirmation of global murine cytomegalovirus open reading frames by transcriptional detection and partial characterization of newly described gene products," *Journal of Virology*, vol. 80, no. 14, pp. 6873–6882, 2006.

[5] S. Landolfo, M. Gariglio, G. Gribaudo, and D. Lembo, "The human cytomegalovirus," *Pharmacology and Therapeutics*, vol. 98, no. 3, pp. 269–297, 2003.

[6] E. S. Mocarski, T. Shenk, and R. F. Pass, "Cytomegaloviruses," in *Fields Virology*, D. M. Knipe and P. M. Howley, Eds., pp. 2701–2772, Lippincott Williams & Wilkins, New York, NY, USA, 2007.

[7] B. Roizman and A. E. Sears, "An inquiry into the mechanisms of herpes simplex virus latency," *Annual Review of Microbiology*, vol. 41, pp. 543–571, 1987.

[8] C. O. Simon, R. Holtappels, H.-M. Tervo et al., "CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation," *Journal of Virology*, vol. 80, no. 21, pp. 10436–10456, 2006.

[9] M. J. Reddehase, C. O. Simon, C. K. Seckert, N. Lemmermann, and N. K. A. Grzimek, "Murine model of cytomegalovirus latency and reactivation," *Current Topics in Microbiology and Immunology*, vol. 325, pp. 315–331, 2008.

[10] C. O. Simon, C. K. Seckert, N. K. A. Grzimek, and M. J. Reddehase, "Murine model of cytomegalovirus latency and reactivation: the silencing/desilencing and immune sensing hypothesis," in *Cytomegaloviruses: Molecular Biology and Immunology*, M. J. Reddehase, Ed., pp. 483–500, Caister Academic Press, Norfolk, UK, 2006.

[11] S. K. Kurz and M. J. Reddehase, "Patchwork pattern of transcriptional reactivation in the lungs indicates sequential checkpoints in the transition from murine cytomegalovirus latency to recurrence," *Journal of Virology*, vol. 73, no. 10, pp. 8612–8622, 1999.

[12] B. Polić, H. Hengel, A. Krmpotić et al., "Hierarchical and redundant lymphocyte subset control precludes cytomegalovirus replication during latent infection," *Journal*

of Experimental Medicine, vol. 188, no. 6, pp. 1047–1054, 1998.

[13] M. Ho, "The history of cytomegalovirus and its diseases," *Medical Microbiology and Immunology*, vol. 197, no. 2, pp. 65–73, 2008.

[14] K. Stratton, J. Durch, and R. Lawrence, *Vaccines for the 21st Century: A Tool for Decision Making*, National Academy Press, Washington, DC, USA, 2001.

[15] M. J. Reddehase, "Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance," *Nature Reviews Immunology*, vol. 2, no. 11, pp. 831–844, 2002.

[16] M. G. Smith, "Propagation in tissue cultures of a cytopathogenic virus from human salivary gland virus (SGV) disease," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 92, no. 2, pp. 424–430, 1956.

[17] D. J. Bidanset, R. J. Rybak, C. B. Hartline, and E. R. Kern, "Replication of human cytomegalovirus in severe combined immunodeficient mice implanted with human retinal tissue," *Journal of Infectious Diseases*, vol. 184, no. 2, pp. 192–195, 2001.

[18] F. J. Bravo, R. D. Cardin, and D. I. Bernstein, "A model of human cytomegalovirus infection in severe combined immunodeficient mice," *Antiviral Research*, vol. 76, no. 2, pp. 104–110, 2007.

[19] E. S. Mocarski, M. Bonyhadi, S. Salimi, J. M. McCune, and H. Kaneshima, "Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 1, pp. 104–108, 1993.

[20] M. S. Smith, D. C. Goldman, A. S. Bailey et al., "Granulocyte-colony stimulating factor reactivates human cytomegalovirus in a latently infected humanized mouse model," *Cell Host and Microbe*, vol. 8, no. 3, pp. 284–291, 2010.

[21] M. R. Wills, J. A. Carmichael, and J. G. Sissons, "Adaptive cellular immunity to human cytomegalovirus," in *Cytomegaloviruses: Molecular Biology and Immunology*, M. J. Reddehase, Ed., pp. 341–365, Caister Academic Press, Norfolk, UK, 2006.

[22] S. J. Kaptein, C. Vink, C. A. Bruggeman, and F. R. Stassen, "The rat model of CMV Infection and vascular disease," in *Cytomegaloviruses: Molecular Biology and Immunology*, M. J. Reddehase, Ed., pp. 501–524, Caister Academic Press, Norfolk, UK, 2006.

[23] M. R. Schleiss and J. C. Lacayo, "The Guinea-pig model of congenital cytomegalovirus infection," in *Cytomegaloviruses: Molecular Biology and Immunology*, M. J. Reddehase, Ed., pp. 525–550, Caister Academic Press, Norfolk, UK, 2006.

[24] D. N. Streblow, J. Dumortier, A. V. Moses, S. L. Orloff, and J. A. Nelson, "Mechanisms of cytomegalovirus-accelerated vascular disease: induction of paracrine factors that promote angiogenesis and wound healing," *Current Topics in Microbiology and Immunology*, vol. 325, pp. 397–415, 2008.

[25] S. G. Hansen, C. J. Powers, R. Richards et al., "Evasion of CD8+ T cells is critical for superinfection by cytomegalovirus," *Science*, vol. 328, no. 5974, pp. 102–106, 2010.

[26] C. Powers and K. Früh, "Rhesus CMV: an emerging animal model for human CMV," *Medical Microbiology and Immunology*, vol. 197, no. 2, pp. 109–115, 2008.

[27] R. Holtappels, M. W. Munks, J. Podlech, and M. J. Reddehase, "CD8 T-cell-based immunotherapy of cytomegalovirus disease in the mouse model of the immunocompromised bone marrow transplantation recipient," in *Cytomegaloviruses: Molecular Biology and Immunology*, M. J. Reddehase, Ed., pp. 383–418, Caister Academic Press, Norfolk, UK, 2006.

[28] A. Krmpotic, I. Bubic, B. Polic, P. Lucin, and S. Jonjic, "Pathogenesis of murine cytomegalovirus infection," *Microbes and Infection*, vol. 5, no. 13, pp. 1263–1277, 2003.

[29] V. Böhm, J. Podlech, D. Thomas et al., "Epitope-specific in vivo protection against cytomegalovirus disease by CD8 T cells in the murine model of preemptive immunotherapy," *Medical Microbiology and Immunology*, vol. 197, no. 2, pp. 135–144, 2008.

[30] M. J. Reddehase, S. Jonjic, F. Weiland, W. Mutter, and U. H. Koszinowski, "Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors," *Journal of Virology*, vol. 62, no. 3, pp. 1061–1065, 1988.

[31] M. J. Reddehase, W. Mutter, K. Münch, H. J. Bühring, and U. H. Koszinowski, "CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity," *Journal of Virology*, vol. 61, no. 10, pp. 3102–3108, 1987.

[32] M. J. Reddehase, W. Mutter, and U. H. Koszinowski, "In vivo application of recombinant interleukin 2 in the immunotherapy of established cytomegalovirus infection," *Journal of Experimental Medicine*, vol. 165, no. 3, pp. 650–656, 1987.

[33] M. J. Reddehase, F. Weiland, and K. Munch, "Interstitial murine cytomegalovirus pneumonia after irradiation: characterization of cells that limit viral replication during established infection of the lungs," *Journal of Virology*, vol. 55, no. 2, pp. 264–273, 1985.

[34] H.-P. Steffens, S. Kurz, R. Holtappels, and M. J. Reddehase, "Preemptive CD8 T-cell immunotherapy of acute cytomegalovirus infection prevents lethal disease, limits the burden of latent viral genomes, and reduces the risk of virus recurrence," *Journal of Virology*, vol. 72, no. 3, pp. 1797–1804, 1998.

[35] R. Holtappels, V. Böhm, J. Podlech, and M. J. Reddehase, "CD8 T-cell-based immunotherapy of cytomegalovirus infection: "Proof of concept" provided by the murine model," *Medical Microbiology and Immunology*, vol. 197, no. 2, pp. 125–134, 2008.

[36] M. Cobbold, N. Khan, B. Pourgheysari et al., "Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers," *Journal of Experimental Medicine*, vol. 202, no. 3, pp. 379–386, 2005.

[37] H. Einsele and K. Hamprecht, "Immunotherapy of cytomegalovirus infection after stem-cell transplantation: a new option?" *The Lancet*, vol. 362, no. 9393, pp. 1343–1344, 2003.

[38] K. S. Peggs, S. Verfuerth, A. Pizzey et al., "Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines," *The Lancet*, vol. 362, no. 9393, pp. 1375–1377, 2003.

[39] S. R. Riddell, K. S. Watanabe, J. M. Goodrich, C. R. Li, M. E. Agha, and P. D. Greenberg, "Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones," *Science*, vol. 257, no. 5067, pp. 238–241, 1992.

[40] E. A. Walter, P. D. Greenberg, M. J. Gilbert et al., "Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor," *The New England Journal of Medicine*, vol. 333, no. 16, pp. 1038–1044, 1995.

[41] A. R. Townsend, J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael, "The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. 1986," *Cell*, vol. 44, no. 6, pp. 959–968, 1986.

[42] K. L. Rock and A. L. Goldberg, "Degradation of cell proteins and the generation of MHC class I-presented peptides," *Annual Review of Immunology*, vol. 17, pp. 739–779, 1999.

[43] J. G. Donaldson and D. B. Williams, "Intracellular assembly and trafficking of MHC class I molecules," *Traffic*, vol. 10, no. 12, pp. 1745–1752, 2009.

[44] J. Yewdell, L. C. Antón, I. Bacik, U. Schubert, H. L. Snyder, and J. R. Bennink, "Generating MHC class I ligands from viral gene products," *Immunological Reviews*, vol. 172, pp. 97–108, 1999.

[45] J. W. Yewdell and J. R. Bennink, "Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses," *Annual Review of Immunology*, vol. 17, pp. 51–88, 1999.

[46] M. J. Reddehase and U. H. Koszinowski, "Significance of herpesvirus immediate early gene expression in cellular immunity to cytomegalovirus infection," *Nature*, vol. 312, no. 5992, pp. 369–371, 1984.

[47] M. J. Reddehase, J. B. Rothbard, and U. H. Koszinowski, "A pentapeptide as minimal antigenic determinant for MHC class I-restricted T lymphocytes," *Nature*, vol. 337, no. 6208, pp. 651–653, 1989.

[48] M. W. Munks, M. C. Gold, A. L. Zajac et al., "Genome-wide analysis reveals a highly diverse CD8 T cell response to murine cytomegalovirus," *The Journal of Immunology*, vol. 176, no. 6, pp. 3760–3766, 2006.

[49] J. W. Yewdell and M. Del Val, "Immunodominance in TCD8+ responses to viruses: cell biology, cellular immunology, and mathematical models," *Immunity*, vol. 21, no. 2, pp. 149–153, 2004.

[50] A. W. Sylwester, B. L. Mitchell, J. B. Edgar et al., "Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects," *Journal of Experimental Medicine*, vol. 202, no. 5, pp. 673–685, 2005.

[51] R. Holtappels, C. O. Simon, M. W. Munks et al., "Subdominant CD8 T-cell epitopes account for protection against cytomegalovirus independent of immunodomination," *Journal of Virology*, vol. 82, no. 12, pp. 5781–5796, 2008.

[52] N. A. Lemmermann, V. Böhm, R. Holtappels, and M. J. Reddehase, "In vivo impact of cytomegalovirus evasion of CD8 T-cell immunity: facts and thoughts based on murine models," *Virus Research*. In press.

[53] R. Holtappels, D. Thomas, J. Podlech, and M. J. Reddehase, "Two antigenic peptides from genes m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-2d haplotype," *Journal of Virology*, vol. 76, no. 1, pp. 151–164, 2002.

[54] M. W. Munks, K. S. Cho, A. K. Pinto, S. Sierro, P. Klenerman, and A. B. Hill, "Four distinct patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection," *The Journal of Immunology*, vol. 177, no. 1, pp. 450–458, 2006.

[55] E. K. Day, A. J. Carmichael, I. J. M. Ten Berge, E. C. P. Waller, J. G. P. Sissons, and M. R. Wills, "Rapid CD8+ T cell repertoire focusing and selection of high-affinity clones into memory following primary infection with a persistent human virus: human cytomegalovirus," *The Journal of Immunology*, vol. 179, no. 5, pp. 3203–3213, 2007.

[56] B. Fleckenstein, I. Muller, and J. Collins, "Cloning of the complete human cytomegalovirus genome in cosmids," *Gene*, vol. 18, no. 1, pp. 39–46, 1982.

[57] R. R. Spaete and E. S. Mocarski, "Insertion and deletion mutagenesis of the human cytomegalovirus genome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 20, pp. 7213–7217, 1987.

[58] G. Kemble, G. Duke, R. Winter, and R. Spaete, "Defined large-scale alteration of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids," *Journal of Virology*, vol. 70, no. 3, pp. 2044–2048, 1996.

[59] D. Wolff, G. Jahn, and B. Plachter, "Generation and effective enrichment of selectable human cytomegalovirus mutants using site-directed insertion of the neo gene," *Gene*, vol. 130, no. 2, pp. 167–173, 1993.

[60] H. Shizuya, B. Birren, U.-J. Kim et al., "Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 18, pp. 8794–8797, 1992.

[61] M. Messerle, I. Crnkovic, W. Hammerschmidt, H. Ziegler, and U. H. Koszinowski, "Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 26, pp. 14759–14763, 1997.

[62] M. Wagner, S. Jonjić, U. H. Koszinowski, and M. Messerle, "Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution," *Journal of Virology*, vol. 73, no. 8, pp. 7056–7060, 1999.

[63] A. Krmpotíc, D. H. Busch, I. Bubíc et al., "MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells in vivo," *Nature Immunology*, vol. 3, no. 6, pp. 529–535, 2002.

[64] M. Lodoen, K. Ogasawara, J. A. Hamerman et al., "NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules," *Journal of Experimental Medicine*, vol. 197, no. 10, pp. 1245–1253, 2003.

[65] L. Zhi, J. Mans, M. J. Paskow et al., "Direct interaction of the mouse cytomegalovirus m152/gp40 immunoevasin with RAE-1 isoforms," *Biochemistry*, vol. 49, no. 11, pp. 2443–2453, 2010.

[66] H. Ziegler, R. Thäle, P. Lucin et al., "A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments," *Immunity*, vol. 6, no. 1, pp. 57–66, 1997.

[67] E.-M. Borst, G. Hahn, U. H. Koszinowski, and M. Messerle, "Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in Escherichia coli: a new approach for construction of HCMV mutants," *Journal of Virology*, vol. 73, no. 10, pp. 8320–8329, 1999.

[68] Y. Saeki, T. Ichikawa, A. Saeki et al., "Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in Escherichia coli: rescue of replication-competent virus progeny and packaging of amplicon vectors," *Human Gene Therapy*, vol. 9, no. 18, pp. 2787–2794, 1998.

[69] H.-J. Delecluse, T. Hilsendegen, D. Pich, R. Zeidler, and W. Hammerschmidt, "Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 14, pp. 8245–8250, 1998.

[70] H. Adler, M. Messerle, M. Wagner, and U. H. Koszinowski, "Cloning and mutagenesis of the murine gammaherpesvirus

68 genome as an infectious bacterial artificial chromosome," *Journal of Virology*, vol. 74, no. 15, pp. 6964–6974, 2000.

[71] W. Dunn, C. Chou, H. Li et al., "Functional profiling of a human cytomegalovirus genome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 2, pp. 14223–14228, 2003.

[72] D. Yu, M. C. Silva, and T. Shenk, "Functional map of human cytomegalovirus AD169 defined by global mutational analysis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 21, pp. 12396–12401, 2003.

[73] W. Brune, M. Wagner, and M. Messerle, "Manipulating cytomegalovirus genomes by BAC mutagenesis: strategies and applications," in *Cytomegaloviruses: Molecular Biology and Immunology*, M. J. Reddehase, Ed., pp. 383–418, Caister Academic Press, Norfolk, UK, 2006.

[74] E.-C. Lee, D. Yu, J. Martinez de Velasco et al., "A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA," *Genomics*, vol. 73, no. 1, pp. 56–65, 2001.

[75] J. P. P. Muyrers, Y. Zhang, G. Testa, and A. F. Stewart, "Rapid modification of bacterial artificial chromosomes by ET-recombination," *Nucleic Acids Research*, vol. 27, no. 6, pp. 1555–1557, 1999.

[76] D. Yu, G. A. Smith, L. W. Enquist, and T. Shenk, "Construction of a self-excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene," *Journal of Virology*, vol. 76, no. 5, pp. 2316–2328, 2002.

[77] Y. Zhang, F. Buchholz, J. P. P. Muyrers, and A. Francis Stewart, "A new logic for DNA engineering using recombination in Escherichia coli," *Nature Genetics*, vol. 20, no. 2, pp. 123–128, 1998.

[78] E.-M. Borst and M. Messerle, "Analysis of human cytomegalovirus oriLyt sequence requirements in the context of the viral genome," *Journal of Virology*, vol. 79, no. 6, pp. 3615–3626, 2005.

[79] M. Wagner, A. Gutermann, J. Podlech, M. J. Reddehase, and U. H. Koszinowski, "Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus," *Journal of Experimental Medicine*, vol. 196, no. 6, pp. 805–816, 2002.

[80] S. Warming, N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland, "Simple and highly efficient BAC recombineering using galK selection," *Nucleic Acids Research*, vol. 33, no. 4, pp. 1–12, 2005.

[81] B. K. Tischer, J. Von Einem, B. Kaufer, and N. Osterrieder, "Two-step Red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli," *BioTechniques*, vol. 40, no. 2, pp. 191–197, 2006.

[82] E. M. Borst, C. Benkartek, and M. Messerle, "Use of bacterial artificial chromosomes in generating targeted mutations in human and mouse cytomegaloviruses," *Current Protocols in Immunology*, vol. 10, 2007.

[83] E. M. Borst, G. Pósfai, F. Pogoda, and M. Messerle, "Mutagenesis of herpesvirus BACs by allele replacement," *Methods in Molecular Biology*, vol. 256, pp. 269–279, 2004.

[84] M. O'Connor, M. Peifer, and W. Bender, "Construction of large DNA segments in Escherichia coli," *Science*, vol. 244, no. 4910, pp. 1307–1312, 1989.

[85] G. Pósfai, M. D. Koob, H. A. Kirkpatrick, and F. R. Blattner, "Versatile insertion plasmids for targeted genome manipulations in bacteria: isolation, deletion, and rescue of the pathogenicity island LEE of the Escherichia coli O157:H7 genome," *Journal of Bacteriology*, vol. 179, no. 13, pp. 4426–4428, 1997.

[86] K. A. Kropp, C. O. Simon, A. Fink et al., "Synergism between the components of the bipartite major immediate-early transcriptional enhancer of murine cytomegalovirus does not accelerate virus replication in cell culture and host tissues," *Journal of General Virology*, vol. 90, no. 10, pp. 2395–2401, 2009.

[87] T. Däubner, A. Fink, A. Seitz et al., "A novel transmembrane domain mediating retention of a highly motile herpesvirus glycoprotein in the endoplasmic reticulum," *Journal of General Virology*, vol. 91, no. 6, pp. 1524–1534, 2010.

[88] P. A. Lyons, J. E. Allan, C. Carrello, G. R. Shellam, and A. A. Scalzo, "Effect of natural sequence variation at the H-2L(d)-restricted CD8+ T cell epitope of the murine cytomegalovirus ie1-encoded pp89 on T cell recognition," *Journal of General Virology*, vol. 77, no. 10, pp. 2615–2623, 1996.

[89] N. A.W. Lemmermann, K. Gergely, V. Böhm, P. Deegen, T. Däubner, and M. J. Reddehase, "Immune evasion proteins of murine cytomegalovirus preferentially affect cell surface display of recently generated peptide presentation complexes," *Journal of Virology*, vol. 84, no. 3, pp. 1221–1236, 2010.

[90] Y. Deng, J. W. Yewdell, L. C. Eisenlohr, and J. R. Bennink, "MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL," *The Journal of Immunology*, vol. 158, no. 4, pp. 1507–1515, 1997.

[91] A. T. Stock, C. M. Jones, W. R. Heath, and F. R. Carbone, "CTL response compensation for the loss of an immunodominant class I-restricted HSV-1 determinant," *Immunology and Cell Biology*, vol. 84, no. 6, pp. 543–550, 2006.

[92] S. J. Turner, K. Kedzierska, H. Komodromou et al., "Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations," *Nature Immunology*, vol. 6, no. 4, pp. 382–389, 2005.

[93] P. J. Bjorkman, M. A. Saper, and B. Samroui, "The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens," *Nature*, vol. 329, no. 6139, pp. 512–518, 1987.

[94] K. Falk, O. Rotzschke, S. Stevanovic, G. Jung, and H.-G. Rammensee, "Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules," *Nature*, vol. 351, no. 6324, pp. 290–296, 1991.

[95] H.-G. Rammensee, J. Bachmann, N. P. N. Emmerich, O. A. Bachor, and S. Stevanovic, "SYFPEITHI: database for MHC ligands and peptide motifs," *Immunogenetics*, vol. 50, no. 3-4, pp. 213–219, 1999.

[96] H.-G. Rammensee and S. Stevanovic, "MHC ligands and peptide motifs," in *Molecular Biology Intelligence Unit*, Springer, New York, NY, USA, 1997.

[97] H.-G. Rammensee, T. Friede, and S. Stevanovic, "MHC ligands and peptide motifs: first listing," *Immunogenetics*, vol. 41, no. 4, pp. 178–228, 1995.

[98] M. J. Reddehase and U. H. Koszinowski, "Redistribution of critical major histocompatibility complex and T cell receptor-binding functions of residues in an antigenic sequence after biterminal substitution," *European Journal of Immunology*, vol. 21, no. 7, pp. 1697–1701, 1991.

[99] D. Rognan, M. J. Reddehase, U. H. Koszinowski, and G. Folkers, "Molecular modeling of an antigenic complex

between a viral peptide and a class I major histocompatibility glycoprotein," *Proteins*, vol. 13, no. 1, pp. 70–85, 1992.

[100] K. A. Hogquist, M. A. Gavin, and M. J. Bevan, "Positive selection of CD8+ T cells induced by major histocompatibility complex binding peptides in fetal thymic organ culture," *Journal of Experimental Medicine*, vol. 177, no. 5, pp. 1469–1473, 1993.

[101] M. Nielsen, C. Lundegaard, O. Lund, and C. Keşmir, "The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage," *Immunogenetics*, vol. 57, no. 1-2, pp. 33–41, 2005.

[102] J. Hakenberg, A. K. Nussbaum, H. Schild et al., "MAPPP: MHC class I antigenic peptide processing prediction," *Appl Bioinformatics*, vol. 2, no. 3, pp. 155–158, 2003.

[103] C. Knuehl, P. Spee, T. Ruppert et al., "The murine cytomegalovirus pp89 immunodominant H-2Ld epitope is generated and translocated into the endoplasmic reticulum as an 11-mer precursor peptide," *The Journal of Immunology*, vol. 167, no. 3, pp. 1515–1521, 2001.

[104] J. R. Bennink and J. W. Yewdell, "Recombinant vaccinia viruses as vectors for studying T lymphocyte specificity and function," *Current Topics in Microbiology and Immunology*, vol. 163, pp. 153–184, 1990.

[105] B. J. McCabe, K. R. Irvine, M. I. Nishimura et al., "Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T lymphocyte responses," *Cancer Research*, vol. 55, no. 8, pp. 1741–1747, 1995.

[106] U. Karrer, M. Wagner, S. Sierro et al., "Expansion of protective CD8+ T-cell responses driven by recombinant cytomegaloviruses," *Journal of Virology*, vol. 78, no. 5, pp. 2255–2264, 2004.

[107] M. R. Jenkins, R. Webby, P. C. Doherty, and S. J. Turner, "Addition of a prominent epitope affects influenza A virus-specific CD8+ T cell immunodominance hierarchies when antigen is limiting," *The Journal of Immunology*, vol. 177, no. 5, pp. 2917–2925, 2006.

[108] V. Wilhelmi, C. O. Simon, J. Podlech et al., "Transactivation of cellular genes involved in nucleotide metabolism by the regulatory IE1 protein of murine cytomegalovirus is not critical for viral replicative fitness in quiescent cells and host tissues," *Journal of Virology*, vol. 82, no. 20, pp. 9900–9916, 2008.

[109] R. Holtappels, J. Podlech, N. K. A. Grzimek, D. Thomas, M.-F. Pahl-Seibert, and M. J. Reddehase, "Experimental preemptive immunotherapy of murine cytomegalovirus disease with CD8 T-cell lines specific for ppM83 and pM84, the two homologs of human cytomegalovirus tegument protein ppUL83 (pp65)," *Journal of Virology*, vol. 75, no. 14, pp. 6584–6600, 2001.

[110] R. Holtappels, D. Thomas, J. Podlech, G. Geginat, H.-P. Steffens, and M. J. Reddehase, "The putative natural killer decoy early gene m04 (gp34) of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral CD8 T cells," *Journal of Virology*, vol. 74, no. 4, pp. 1871–1884, 2000.

[111] C. H. Cook, L. Chen, J. Wen et al., "CD28/B7-mediated co-stimulation is critical for early control of murine cytomegalovirus infection," *Viral Immunology*, vol. 22, no. 2, pp. 91–103, 2009.

[112] K. L. Hokeness, E. S. Deweerd, M. W. Munks, C. A. Lewis, R. P. Gladue, and T. P. Salazar-Mather, "CXCR3-dependent recruitment of antigen-specific T lymphocytes to the liver during murine cytomegalovirus infection," *Journal of Virology*, vol. 81, no. 3, pp. 1241–1250, 2007.

[113] V. C. Emery, "Relative importance of cytomegalovirus load as a risk factor for cytomegalovirus disease in the immunocompromised host," in *CMV-Related Immunopathology. Monographs in Virology*, M. Scholz, H. F. Rabenau, H. W. Doerr, and J. Cinatl, Eds., pp. 288–301, Karger, Basel, Switzerland, 1998.

[114] N. K. A. Grzimek, D. Dreis, S. Schmalz, and M. J. Reddehase, "Random, asynchronous, and asymmetric transcriptional activity of enhancer-flanking major immediate-early genes ie1/3 and ie2 during murine cytomegalovirus latency in the lungs," *Journal of Virology*, vol. 75, no. 6, pp. 2692–2705, 2001.

[115] R. Holtappels, M.-F. Pahl-Seibert, D. Thomas, and M. J. Reddehase, "Enrichment of immediate-early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary CD62Llo memory-effector cell pool during latent murine cytomegalovirus infection of the lungs," *Journal of Virology*, vol. 74, no. 24, pp. 11495–11503, 2000.

[116] R. Holtappels, D. Gillert-Marien, D. Thomas et al., "Cytomegalovirus encodes a positive regulator of antigen presentation," *Journal of Virology*, vol. 80, no. 15, pp. 7613–7624, 2006.

[117] J. W. Yewdell and A. B. Hill, "Viral interference with antigen presentation," *Nature Immunology*, vol. 3, no. 11, pp. 1019–1025, 2002.

[118] A. Porgador, J. W. Yewdell, Y. Deng, J. R. Bennink, and R. N. Germain, "Localization, quantitation, and in situ detection of specific peptide- MHC class I complexes using a monoclonal antibody," *Immunity*, vol. 6, no. 6, pp. 715–726, 1997.

[119] U. Reusch, W. Muranyi, P. Lucin, H.-G. Burgert, H. Hengel, and U. H. Koszinowski, "A cytomegalovirus glycoprotein reroutes MHC class I complexes to lysosomes for degradation," *EMBO Journal*, vol. 18, no. 4, pp. 1081–1091, 1999.