Antigen-presenting cells of haematopoietic origin prime cytomegalovirus-specific CD8 T-cells but are not sufficient for driving memory inflation during viral latency

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Expansion of the CD8 T-cell memory pool, also known as 'memory inflation', for certain but not all viral epitopes in latently infected host tissues is a special feature of the immune response to cytomegalovirus. The Ld-presented murine cytomegalovirus (mCMV) immediate–early (IE) 1 peptide is the prototype of an epitope that is associated with memory inflation. Based on the detection of IE1 transcripts in latently infected lungs it was previously proposed that episodes of viral gene expression and antigenic activity due to desilencing of a limited number of viral genes may drive epitope-specific memory inflation. This would imply direct antigen presentation through latently infected host tissue cells rather than cell death-associated cross-presentation of viral antigens derived from productively infected cells through uninfected, professional antigen-presenting cells (profAPCs). To address the role of bone marrow-derived profAPCs in CD8 T-cell priming and memory to mCMV, we have used here a combined sex-mismatched and MHC class-I mismatched dual-marker bone marrow chimera model in which presentation of the IE1 epitope is restricted to donor-derived sry\(^+\)Ld\(^+\) cells of haematopoietic differentiation lineages. Successful CD8 T-cell priming specific for the L\(^d\)- and D\(^d\)-presented inflationary epitopes IE1 and m164, respectively, but selective failure in IE1 epitope-specific memory inflation in these chimeras indicates different modes of antigen presentation involved in CD8 T-cell priming and memory inflation. These data suggest that memory inflation during mCMV latency requires expression of the epitope-presenting MHC class-I molecule by latently infected non-haematopoietic host tissue cells and thus predicts a role for direct antigen presentation in memory inflation.

INTRODUCTION

It is current opinion that priming of CD8 T-cells during acute viral infections is accomplished mainly by uninfected professional antigen-presenting cells (profAPCs) that take up and ‘cross-present’ viral antigens derived from infected cells undergoing infection-associated cell death (den Haan & Bevan, 2001; Heath & Carbone, 2001; Kurts et al., 2010; Shen & Rock, 2006). For the infection with murine cytomegalovirus (mCMV), comparably efficient priming of CD8 T-cells in the presence and absence of ‘viral inhibitors of direct antigen presentation’, known as immunoevasins (for reviews, see Doom & Hill, 2008; Hansen & Bouvier, 2009; Lemmermann et al., 2011a; Reddehase, 2002), provided a reasonable argument for priming by cross-presentation (Böhm et al., 2008; Gold et al., 2002; Munks et al., 2007). More direct evidence for cross-presentation of mCMV epitopes was provided only recently by in vivo priming of CD8 T-cells with MHC class-I (MHC-I)-deficient fibroblasts infected with a spread-defective virus mutant, mCMV-AgL, conditions which plausibly prevent any direct antigen presentation (Snyder et al., 2010). A potent cross-presenting cell type is the CD8\(^+\)CD11c\(^+\) subset of dendritic cells (DCs) (Allan et al., 2003; Belz et al., 2004; Schnorrer et al., 2006), which are bone marrow (BM)-derived haematopoietic cell progeny. Usually, after the primary response to infection and first wave of antigen-driven proliferation, the numbers of viral epitope-specific CD8 T-cells contract and central memory cells are maintained in low numbers (Klenerman & Dunbar, 2008). It is a special feature of the immune response to both human cytomegalovirus (hCMV) (reviewed by Wills et al., 2006) and mCMV (Holtappels et al., 2000; Karrer et al., 2003; Munks et al., 2006a) that
The obvious question of why some epitopes drive memory inflation while others do not still awaits the answer. Notably, the magnitude of the primary immune response to an epitope does not predict memory inflation (Munks et al., 2006a). An intrinsic property of CD8 T-cells of certain T-cell receptor (TCR) specificities and/or affinities or an influence of the size of the naïve T-cell repertoire appear to be an unlikely explanation in view of the finding that the IE1 epitope drives memory inflation only when expressed in the context of mCMV infection but not when expressed in a vaccinia virus recombinant (Karrer et al., 2003). Thus, the underlying mechanism must somehow relate to the specific biology of mCMV. As mCMV, unlike vaccinia virus, establishes a lifelong latent infection, it has been an immediate idea that viral genes encoding inflationary epitopes might possibly be expressed in latently infected cells at least intermittently, resulting in sporadic peptide presentation and consequent memory cell restimulation (Holtappels et al., 2000). Such gene expression episodes, including past episodes, are thought to be most sensitively 'memorized' by memory cells, even if such episodes remain undetectable by conventional molecular methods. For IE1 memory inflation, this concept was supported by the detection of correctly spliced IE1 transcripts in latently infected lungs (Grzimek et al., 2001; Kurz et al., 1999) and by the detection of higher levels of IE1 transcripts in lungs latently infected with mutant virus mCMV-IE1-L176A, in which IE1-specific immune control is selectively prevented by point mutation of the C-terminal amino acid residue of the IE1 peptide (Simon et al., 2006; for a review, see Lemmermann et al., 2011b). As a further corroborative evidence, unrelated transgenic epitopes, if expressed under the control of the mCMV major IE enhancer-promoter, were found to acquire the characteristics of inflationary epitopes (Karrer et al., 2004). From all this it was proposed that memory cells sense and terminate episodes of viral gene expression during latency (reviewed by Reddehase et al., 2008).

A recently raised argument against CD8 T-cell memory inflation by repetitive restimulation was the absence of CD4 T-cell memory inflation specific for an epitope in protein IE3, a protein that also contains an inflationary CD8 T-cell epitope (Arens et al., 2008). This objection, however, did not consider the possibility that memory inflation may be driven by direct antigen presentation in latently infected non-haematopoietic MHC class-II (MHC-II)-negative host tissue cells, which would explain why memory inflation during mCMV latency appears to be a feature of MHC-I-restricted CD8 T-cells but not of MHC-II-restricted CD4 T-cells. The identification of the type of APC involved in memory inflation, either profAPCs of haematopoietic origin or stromal and parenchymal tissue cells, is therefore an important missing link for a better understanding of memory inflation associated with viral latency.

Here, we provide data supporting the interpretation that memory inflation during mCMV latency requires epitope presentation by latently infected non-haematopoietic host tissue cells.

RESULTS

Characterization of bone marrow chimeras expressing the IE1 epitope-presenting MHC-I molecule selectively on cells of haematopoietic lineages

Clinical BM transplantation (BMT) after haematoablative treatment of recipients is an established therapeutic approach to replace malignant haematopoietic cells of a recipient with healthy haematopoietic cells of a donor. The BMT model was used here in a dual-marker version to distinguish between donor-derived haematopoietic profAPCs and recipients’ cells (Fig. 1). Specifically, male...
BALB/c mice served as donors carrying the heterosomal (Y-chromosomal) gene sry as a genotypic marker and expressing the IE1 epitope-presenting MHC-I molecule L<sup>d</sup> as a phenotypic marker (sry<sup>+</sup>L<sup>d</sup>·<sup>+</sup>), whereas female BALB/c-H-2<sup>dm2</sup> mice lacking L<sup>d</sup> expression due to a genetic deletion (Rubocki et al., 1986) served as recipients (sry<sup>–</sup>L<sup>d</sup>·<sup>–</sup>). Thus, the L<sup>d</sup>-restricted IE1 epitope can be presented in the resulting H-2<sub>d</sub>·<sub>x</sub>dm2 BM chimeras exclusively by donor BM-derived profAPCs of haematopoietic lineages but not by the recipients’ own tissue cells or residual haematopoietic cells. Note that immunological tolerance precludes an L<sup>d</sup>-directed immune response in this model (Alterio de Goss et al., 1998) and that the two mouse strains are co-isogenic, sharing minor histocompatibility antigens, so that donor-derived cells are not destroyed by a host-versus-graft reaction. By using the pan-genomic library of mCMV-ORF expression plasmids established by Munks et al. (2006b), we first characterized the acute and memory CD8 T-cell responses to mCMV in immunocompetent mice of the chosen donor and recipient mouse strains (Fig. 2). At a glance, antigenicity profiles in the acute response are comparable between the two strains, except the expected lack of IE1-epitope recognition in the L<sup>d</sup>-deletion mutant BALB/c-H-2<sup>dm2</sup>. The broader specificity repertoire of the acute response focuses on the known two dominant specificities IE1 and m164 in the memory response in BALB/c (Holtappels et al., 2002b) and, logically, just to the D<sup>d</sup>-restricted m164 epitope in BALB/c-H-2<sup>dm2</sup> mice.

For BMT, mCMV-naïve female recipients received haematoloblatifve conditioning before BM cells from male mCMV-naïve donors were transferred, followed by intraplantar infection (Fig. 1). Successful repopulation of the recipients’ BM with donor haematopoietic cells was monitored by sry-specific quantitative PCR (qPCR), revealing efficient replacement of recipient-genotype with donor-genotype haematopoietic cells in H-2<sub>d</sub>·<sub>x</sub>dm2 chimeras (Fig. 3a). Chimerism in the recipients was demonstrated for lung cells enriched for MHC-II+ profAPCs and for CD146+ endothelial cells that proved to be primarily of donor genotype (sry<sup>+</sup>) and recipient genotype (sry<sup>–</sup>), respectively (Fig. 3b). Finally, in spleen (Fig. 3c; c1, c2) and lungs (Fig. 3c; c3, c4) essentially all D<sup>d</sup>·<sub>x</sub>CD11c+ cells as well as cells of the cross-presenting CD8+CD11c+ DC subset thereof expressed L<sup>d</sup> and were

**Fig. 2.** Specificity repertoires of acute and memory CD8 T-cell responses. The specificities of CD8 T-cells in immunocompetent BALB/c and BALB/c-H-2<sup>dm2</sup> mice were recorded by using a pan-genomic mCMV-ORF library of expression plasmids for transfection of stimulator cells used in a cytofluorometric intracellular cytokine (IFN-γ) assay. Responder cells were spleen cells isolated on day 7 (acute response) or at 1 year (memory response) after intraplantar infection with 10<sup>5</sup> p.f.u. of mCMV-WT.Smith. For the analysis, electronic gates were set on lymphocytes in the forward versus sideward scatter plot and on cells expressing CD8α. ORFs eliciting prominent peaks of response are indicated. For quantifying CD8 T cells responding to known epitopes (sequences listed in Holtappels et al., 2008a), cell aliquots were incubated with optimized concentrations of synthetic peptides and were run in parallel in the same assay (inset figures). Arrows highlight the absence of IE1-specific CD8 T-cells in the L<sup>d</sup> deletion mutant BALB/c-H-2<sup>dm2</sup>.
thus donor-derived. Note that the percentage of CD8+CD11c+ DCs among all Ld+ cells was just ~9% in the lungs compared with ~40% in the spleen, which suggests a lower cross-presentation potential in the extralymphoid tissue. Taken together, this set of experiments revealed successful repopulation of infected sry–Ld– BMT recipients with sry+Ld+ profAPCs capable of Ld-restricted epitope presentation.

Most of the IE1 peptide in the lungs after BMT is processed in non-haematopoietic cells

Macrophages and DCs are targets of productive mCMV infection and are, in principle, capable of IE1 peptide processing and direct presentation (Andrews et al., 2001; Benedict et al., 2008; Dalod et al., 2003; Hengel et al., 2000; Holtappels et al., 2006). In addition, uninfected DCs can take up and process viral proteins for peptide cross-presentation (see Introduction). Nevertheless, quantification of processed IE1 peptide from lungs during acute infection after BMT revealed that Ld+ haematopoietic lung infiltrate cells account for only low amounts of IE1 peptide in H-2d x dm2 chimeras when compared with Ld+ haematopoietic plus non-haematopoietic lung cells in...
H-2d/δ homo-chimeras (Fig. 4a, b). This relates to the fact that in the immunocompromised host mCMV replicates in a variety of non-haematopoietic stromal and parenchymal cell types (Podlech et al., 1998), including pneumocytes, fibrocytes and endothelial cells of the lungs (Reddehase et al., 1991; Falk et al., 1990). This negative control confirms the conclusion that IE1 peptide isolated from H-2d/δ homo-chimeras is produced by processing in donor-derived haematopoietic cells only.

Theoretically, a lower amount of processed IE1 peptide during the acute infection in H-2d/δm2 chimeras could reflect also a lower viral productivity and thus a lower antigen load. This alternative explanation was ruled out by comparable viral productivity and clearance kinetics in H-2d/δ, H-2d/δm2 and H-2d/δm2/δm2 chimeras (Fig. 5). This finding suggests that the observed differences in the amounts of IE1 peptide do not result from differences in the overall viral replication but instead reflect the cellular distribution of the IE1 peptide-presenting Ld molecule in the different chimeras. From the low amount of IE1 peptide present in the lungs of H-2d/δm2 chimeras we thus conclude that profAPCs are only a minor source of processed IE1 peptide under the experimental conditions studied herein.

Chimerism does not significantly influence viral genome load and epitope-encoding gene expression during latency

To comply with the hypothesis of memory inflation driven by antigenic activity of latently infected cells, epitope-encoding genes must be expressed during latency. Accordingly, differences between the chimeras in viral genome load and transcriptional activity during latency would affect the provision of antigenic peptides for presentation and should thus have an impact on memory inflation. Specifically, in addition to the presence or absence of the IE1 epitope-presenting Ld molecule on latently infected cell types, differences in IE1 gene expression during latency could influence IE1-specific memory inflation. This possibility needed to be addressed.

Like in our previous work on mCMV latency (reviewed by Reddehase et al., 2008), latency was here defined by the presence of the viral genome in tissues after its PCR-verified clearance from BM and blood, and by absence of infectivity at all typical organ sites of mCMV replication, including the salivary glands, a privileged site of delayed mCMV clearance (for a review, see Campbell et al., 2008). Residual low-level productive infection was most rigorously excluded by centrifugal infection of highly permissive mouse embryo fibroblast cell cultures with organ homogenate, followed by incubation for three cycles of multistep viral growth (72 h) and quantification of IE1 transcripts. The sensitivity of this assay has been shown to be 0.01 non-centrifugal p.f.u. per culture, which corresponds to ~2 × 10^-9 p.f.u. per cell (Kurz et al., 1997). At a time when infectivity was undetectable with this assay in the lungs of both H-2d/δ and H-2d/δm2 chimeras (negative data not shown), latent viral genome load (Fig. 6a) as well as the

![Fig. 4. Most of the naturally processed IE1 peptide in infected lungs of BMT recipients is produced in non-haematopoietic tissue cells. At the indicated times, IE1 peptide was isolated from the lungs and used for pulsing TAP-deficient T2-L^d transfectants for a cytolytic assay with an IE1-specific CTL line. Diagrams show peptide-specific reactivity in HPLC fractions for different volume aliquots (out of a total of 800 μl) tested. The calculated numbers of IE1 peptide molecules per lung are indicated. (a) Positive control quantifying IE1 peptide generated by donor- and recipient-derived cells in the lungs of H-2d/δ homo-chimeras. (b) IE1 peptide processed in donor-derived haematopoietic cell progeny in the lungs of H-2d/δm2 chimeras. (c) Negative control demonstrating the absence of processed IE1 peptide in the lungs of H-2d/δm2 homo-chimeras.]
amount of IE1 transcripts (Fig. 6b) did not differ statistically, albeit there appeared to be a tendency to an even somewhat higher load and transcriptional activity in the H-2d/dm2 chimeras. So, in case memory inflation would be driven by latently infected or cross-presenting donor-derived Ld⁺ profAPCs, there was no reason for a failure in IE1-specific memory inflation in H-2d/dm2 chimeras.

**profAPCs are not sufficient for driving memory inflation during mCMV latency**

Based on all these conditions, acute and memory CD8 T-cell responses to the two dominant epitopes IE1 and m164 were monitored for a period of 6 months, that is from acute infection to latency (Fig. 7). In accordance with successful physical repopulation of donor-derived Ld⁺ profAPCs (Fig. 3) and with low but detectable IE1 peptide processing (Fig. 4b), IE1-specific CD8 T-cells were indeed primed in the H-2d/dm2 chimeras (Fig. 7a), indicating that repopulation of the recipients with profAPCs was also functional and implying that IE1-specific CD8 T-cells were not missing in the reconstituted repertoire. Moreover, there was no notable quantitative difference to the IE1-specific priming in H-2d/d homo- or H-2d/dm2 chimeras. Importantly, IE1-specific CD8 T-cells were also primed in H-2d/dm2 chimeras established prior to infection (data not shown), which implies that the reconstitution of the virus-specific naïve T-cell repertoire does not require the presentation of viral antigens by thymic DCs.

After the contraction phase, however, IE1-specific memory cells conspicuously failed to expand in lungs and spleen selectively in the H-2d/dm2 chimeras. Importantly, in the very same mice, memory expansion specific for the inflationary m164 epitope presented by MHC-I Dd⁺, which is shared by donor and recipient cells, provided an internal positive control verifying that the failure in IE1-specific memory inflation does not reflect a more general deficiency in BM chimeras but in fact relates specifically to missing IE1-epitope presentation by Ld⁺ tissue cells. It should be mentioned that this important control is unavailable in C57BL/6-based models, where coincidentally all currently known inflationary epitopes share the presenting Kb allele (Munks et al., 2006a).

Finally, in an independent experiment (Fig. 8), selective lack of IE1-specific memory inflation in the lungs of H-2d/dm2 chimeras was verified also by epitope-specific TCR staining with MHC-peptide multimers, whereas m164-specific CD8 T-cells in H-2d/dm2 chimeras as well as both IE1- and m164-specific CD8 T-cells in H-2d/d homo-chimeras were found in significant numbers. Notably, the majority of epitope-specific CD8 T-cells displayed the phenotype CD62L⁻ KLRG1⁺ that is characteristic of sensitized cells under conditions of persistent or repetitive antigen stimulation (Thimme et al., 2005).

**DISCUSSION**

In contrast to the current view of hCMV latency localizing predominantly to haematopoietic cells of the myeloid...
lineage (reviewed by Reeves & Sinclair, 2008), which include profAPCs, there is increasing evidence for mCMV latent genomes localizing primarily to non-haematopoietic tissue cells. In fact, already historic work by the group of D. Spector (Mercer et al., 1988) has provided the first evidence for mCMV latency in MHC-II– sinusoidal lining cells of the spleen. More recently, the liver sinusoidal endothelial cell, which displays the phenotype MHC-II–CD11b–CD11c–CD31+CD146+, was identified as a latently infected cell type expressing IE transcripts, whereas latent viral genomes in the liver did not localize to haematopoietic cell types expressing MHC-II, CD11b or CD11c (Seckert et al., 2009).

Absence of reactivatable, latent mCMV genomes from myeloid lineage stem- and progenitor cells in mouse BM was also suggested from the failure to transmit latent infection by BMT from latently infected donors to naïve recipients (Seckert et al., 2008). Likewise, for the lungs, recent work by Marquardt et al. (2011) has shown that latent mCMV genomes are enriched in non-haematopoietic CD11b– cells, and mCMV reactivation in lung slice explant cultures excluded fractalkine receptor-expressing CX3CR1+ subsets of the myeloid lineage, including CX3CR1+ pulmonary DCs, as cellular sites of virus reactivation. Taken together, these findings questioned the assumption that memory inflation is driven by direct antigen presentation in latently infected profAPCs.

If, based on these arguments, we presume an absence of direct antigen presentation in profAPCs during mCMV latency, we can think of two alternative modes of antigen presentation for restimulating memory CD8 T-cells: (i) direct antigen presentation by latently infected tissue cells or (ii) cross-presentation of viral antigens, derived from

**Fig. 6.** Quantification of viral genome load and IE1 transcripts during latency in the lungs. The analysis was performed for seven individual mice of each type of chimera, H-2dxd and H-2dxdm2, after resolution of productive infection (see Fig. 5) at 15 weeks after BMT. (a) Viral DNA load. The post-caval lung lobe of each of the seven mice was divided into two tissue pieces. Symbols represent the qPCR results for these two pieces. (b) Steady-state levels of IE1 transcripts. The superior, middle and inferior lobes of the right lung of each of the seven mice were divided each into two pieces. Symbols represent median values of triplicate RT-qPCR measurements for the overall six pieces analysed per mouse. Log-normal distributions for the load data (n=14) and the transcript data (n=42) were confirmed by the Kolmogorov–Smirnov test. The geometric mean values and their 95% confidence intervals are indicated. P-values (two-tailed) for the differences between the mean values were calculated on the basis of the log-transformed data by using Student's unpaired t-test.

**Fig. 7.** Selective failure in IE1 epitope-specific memory inflation in H-2dxdm2 chimeras. Frequencies of CD8 T-cells specific for the inflationary epitopes IE1 and m164 were determined by ELISPOT assay in lungs and spleen during a period of 6 months after BMT and infection. (a) Time-course of the response in H-2dxdm2 chimeras capable of presenting the IE1 epitope only on donor BM-derived haematopoietic cell progeny. (b) Time-course of the response in H-2dxd homo-chimeras capable of presenting the IE1 epitope also on non-haematopoietic tissue cells of recipient origin. Black bars, IE1-specific CD8 T-cells. Grey bars, m164-specific CD8 T-cells. Error bars represent the 95% confidence intervals.
latently infected tissue cells, by uninfected profAPCs. If the first alternative applies, IE1-specific memory inflation should not occur in H-2d×dm2 chimeras because the IE1 peptide is not presented by Ld– cells. If the second alternative applies, however, IE1-specific memory inflation should take place because the IE1 protein can be processed for peptide presentation in donor-derived Ld+ profAPCs, provided that the latently infected Ld– tissue cells undergo cell death and release the IE1 protein. The data presented here show a selective failure of IE1-specific memory inflation in H-2d×dm2 chimeras and hence clearly argue against cross-presentation of antigen derived from latently infected tissue cells. Importantly, this failure also argues against a relevant role for direct antigen presentation by a putatively existing minor population of latently infected myeloid lineage profAPCs, for instance by donor-derived tissue resident macrophages reported to be low in expression of CX3CR1 and CD11b (Jung et al., 2000; Vermaelen & Pauwels, 2004).

Against our interpretation of the data one may argue that, when compared with H-2d×d homo-chimeras, the overall lower amount of IE1 peptide generated during acute infection in H-2d×dm2 chimeras, rather than the type of APC, may later on make the difference in memory inflation. The different amounts of IE1 peptide generated during acute infection, however, were not paralleled by differences in IE1 gene expression during latency. In this

Fig. 8. Activation phenotype of epitope-specific memory CD8 T-cells in latently infected lungs. After resolution of virus replication in the lungs of H-2d×d and H-2d×dm2 chimeras at 15 weeks after BMT and infection (see Fig. 5), pulmonary lymphocytes were isolated from a pool of seven lungs per group for a four-colour cytofluorometric analysis (FL, fluorescence). Data are displayed as colour-coded density plots (with red and blue representing highest and lowest density, respectively) using logarithmic threshold calculation. Top panels, in the sideward scatter (SSC, linear scale of channels) versus CD8a expression (log FL-1, FITC) plots, an electronic gate was set on CD8a+ cells. Centre panels, percentages of IE1 and m164 epitope-specific cells among the gated CD8 T-cells were determined by staining with the respective, TCR-specific MHC-peptide dextramers (log FL-4, allophycocyanin). Bottom panels, cells within the CD8a+TCR+ gates were analysed for expression of the activation markers CD62L (log FL-5, PE-Cy7) and KLRG1 (log FL-2, PE). Percentages of main interest are indicated. *, Reliable analysis was precluded by too low absolute cell number.
context it is important to consider that the amount of peptide processed in productively infected cells is certainly unrelated to the amount of peptide processed in latently infected cells. Actually, the amount of peptide processed during latency does not suffice for detection, because the frequency of latently infected cells in tissues is too low. Likewise, the IE1 protein proved to be below the limit of biochemical and immunohistological detection (Kurz et al., 1999). In light of this latency-inherent technical dilemma, the internal control provided by an inflammmatory Dd-restricted epitope gains particular importance. As memory inflation specific for the Dd-restricted m164 epitope occurred in both types of chimeras, the qualitative difference seen for the IE1 epitope is unlikely to result from a difference in the overall amounts of antigenic proteins expressed.

Previous work by Snyder et al. (2008) has indicated that memory expansion involves continuous replenishment of the memory pool by replacement of short-lived memory cells with progeny of restimulated central memory cells primed already during acute infection as well as by recruitment of naïve cells. As shown here for the specific case of the IE1 epitope, both of these sources appear to be inaccessible or insufficient in H-2d/dm2 BM chimeras. While a missing restimulation of central memory cells is best explained by a lack of IE1-epitope presentation on latently infected tissue cells, missing Ld expression on the thymic epithelium might result in a deficiency in the generation of naïve IE1-specific thymic emigrants. Interestingly, however, the quantitative contribution of naïve thymic emigrants to memory inflation needs to be relativised in view of the observation that memory inflation takes place also after thymectomy (noted by Wiesel et al., 2009). Although we cannot formally exclude differences in the primary CD8 T-cell repertoire due to the different genotype of the thymic epithelial cells in H-2d/dm2 and H-2d/d chimeras, this potential caveat appears to be minor in view of quantitatively similar priming of IE1-specific CD8 T-cells (Fig. 7). In any case, our data indicate a critical role of non-haematopoietic host tissue cells for memory inflation to occur.

This conclusion is also compatible with recent work by Hutchinson et al. (2011) who presented evidence in support of the view that inflammatory epitopes correspond to antigenic peptides that are efficiently generated by the constitutive proteasome present in all cell types, whereas non-inflammatory epitopes depend on the immunopro teaseome constitutively expressed in proAPCs or conditionally expressed in other cell types upon induction by IFN-γ. Accordingly, in the absence of a pro-inflammatory cytokine milieu and provided that the presenting MHC-I molecule and the viral antigenic protein are expressed, the constitutive proteasome present in latently infected non-haematopoietic host tissue cells will preferentially generate inflammatory epitopes and thereby drive memory inflation. Thus, for memory inflation to occur, at least three conditions need to be fulfilled: (i) expression of the epitope-encoding viral gene, (ii) efficient proteasomal processing for generating the antigenic peptide even from a low amount of protein, preferentially requiring only the constitutive proteasome, and (iii) expression of the MHC-I molecule presenting the antigenic peptide to memory cells.

Collectively, these data strongly support our original hypothesis of memory inflation being driven by repetitive antigen stimulation mediated by episodes of desilencing of certain viral genes in latently infected cells (Holtappels et al., 2000).

METHODS

Experimental BMT and mCMV infection. BMT and infection were performed as described previously with no depletion of BM-resident T-cells (Podlech et al., 2002). In essence, 8-week-old female mice received haematopoietic total-body γ-irradiation with a single dose of 6.5 Gy. BMT was performed 6 h later by infusion of 5 × 106 femoral and tibial male donor-derived BM cells into the tail vein of the recipients. Intraplantar infection of the recipients with 104 p.f.u. of wild-type (WT) virus mCMV-WT.Smith (strain Smith ATCC VR-1399) was performed 2 h after BMT. Animal experiments were approved according to German federal law under permission number 177-07/G09-1-004.

Preparation of lung cell suspensions. Lung cells were isolated by using a modification of the method described in Holtappels et al. (1998). The lungs were perfused via the right ventricle with perfusion solution [Gey’s balanced salt solution (GBSS) + 0.2 U collagenase A ml−1 (catalogue no. 1010358601; Roche)]. The lungs were excised, trachea, bronchi and pulmonary lymph nodes were removed and the five lung lobes were minced with scissors. Digestion of tissue was performed in 15 ml GBSS + 0.2 U collagenase A ml−1 + 1000 U DNase I (catalogue no. 11284952001; Roche) for 2 h at 37 °C in a rotating water bath. Tissue clumps were resolved by straining the digested tissue through a steel mesh and thereafter through a 100 μm nylon mesh. After lysis of red blood cells, the cell suspension was strained through a 40 μm nylon mesh.

Separation and phenotypic analysis of lung and spleen cell suspensions. The following MicroBeads and reagents were used (immuno)magnetic cell separation and cytofluorometric analysis: rat anti-mouse CD146 MicroBeads (clone ME-9F1, rat IgG2a, 20 μl per 107 cells; catalogue no. 130-092-007; Miltenyi Biotech), rat anti-mouse MHC-II MicroBeads (rat IgG2b, 10 μl per 107 cells; catalogue no. 130-052-401; Miltenyi Biotech), mouse anti-mouse H-2Dd FITC-conjugated (clone 34-2-12, mouse IgG2a, 1 μg per 107 cells; catalogue no. 553579; BD Biosciences), mouse anti-mouse H-2Ld PE-conjugated (clone 30-5-7S, mouse IgG2a, 1 μg per 107 cells; catalogue no. CL9011PE; Cederlane), rat anti-mouse CD8α PE-Cy5-conjugated (clone 53-6-7, 0.3 μg per 106 cells, rat IgG2a; catalogue no. 553034; BD Biosciences), hamster anti-mouse CD11c FITC-conjugated (clone N418, 0.5 μg per 106 cells, hamster IgG; catalogue no. 25-0114; eBioscience), rat anti-mouse CD8α FITC-conjugated (clone 53-6-7, 0.5 μg per 106 cells, rat IgG2a; catalogue no. 553031; BD Biosciences), hamster anti-mouse KLRG1 PE-conjugated (clone 2F1, 0.2 μg per 106 cells, hamster IgG; catalogue no. 12-5893-82; eBioscience), rat anti-mouse CD62L PE-Cy7-conjugated (clone ME-L-14, 0.3 μg per 106 cells, rat IgG2a; catalogue no. 731715; Beckman Coulter), MHC-peptide dextramer H-2Ld-IE1(YYPFMPTNL) allopolyocyanin-conjugated (10 μl per 106 cells; code no. JG3532-APC; Immudex), and MHC-peptide dextramer H-2Dd-m164 (AGPPRYSRI) allopolyocyanin-conjugated (10 μl per 106 cells; code no. JB3533-APC; Immudex).
(i) Immunomagnetic cell sorting. For quantification of donor-derived sry+ cells, cell populations positive for MHC-II or CD146 were enriched from lung cell suspensions by magnetic cell sorting (Seckert et al., 2009).

(ii) Four-colour cytofluorometric analysis. Cells were labelled with the indicated directly fluorochrome-conjugated mAbs and MHC-peptide multimers (dextramers) for cytofluorometric analysis performed with a Beckman Coulter FC500 and CXP software, version 2.2 (Beckman Coulter).

Quantification of genomes and transcripts. BM cells were isolated by flushing one tibia (individual mouse testing) with 1 ml PBS. DNA from BM cells, lung tissue pieces or immunomagnetically enriched lung cell subsets was extracted with a DNeasy Blood & Tissue kit (Qiagen) (Seckert et al., 2009). Quantification of the viral gene M555gB, the cellular gene pthp and the Y-chromosomal gene sry was performed by SYBR-Green qPCR (Seckert et al., 2008). Standard curves for quantification were established by using graded numbers of linearized plasmid pDrive_gBPTHrP_Tdy (Simon et al., 2005) as template. RT-qPCR for quantifying spliced IE1 transcripts has been described in detail previously (Lemmermann et al. 2010).

Quantification of naturally processed IE1-peptide in infected lungs. Naturally processed peptides were acid-extracted and Quantification of naturally processed IE1-peptide in infected lung cell suspensions by magnetic cell sorting (Seckert et al., 2009).

REFERENCES


