Murine Model of Interstitial Cytomegalovirus Pneumonia in Syngeneic Bone Marrow Transplantation: Persistence of Protective Pulmonary CD8-T-Cell Infiltrates after Clearance of Acute Infection

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Interstitial pneumonia (IP) is a severe organ manifestation of cytomegalovirus (CMV) disease in the immunocompromised host, in particular in recipients of bone marrow transplantation (BMT). Diagnostic criteria for the definition of CMV-IP include clinical evidence of pneumonia together with CMV detected in bronchoalveolar lavage or lung biopsy. We have used the model of syngeneic BMT and simultaneous infection of BALB/c mice with murine CMV for studying the pathogenesis of CMV-IP by controlled longitudinal analysis. A disseminated cytopathic infection of the lungs with fatal outcome was observed only when reconstituting CD8 T cells were depleted. Neither CD8 nor CD4 T cells mediated an immunopathogenesis of acute CMV-IP. By contrast, after efficient hematolymphopoietic reconstitution, viral replication in the lungs was moderate and focal. The histopathological picture was dominated by preferential infiltration of CD8 T cells confining viral replication to inflammatory foci. Notably, after clearance of acute infection, CD62Llo and CD62Lhi subsets of CD44+ memory CD8 T cells were found to persist in lung tissue. One can thus operationally distinguish an early CMV-positive IP (phase 1) and a late CMV-negative IP (phase 2). According to the definition, phase 2 histopathology would not be diagnosed as a CMV-IP and could instead be misinterpreted as a CMV-induced immunopathology. We document here that phase 1 as well as phase 2 pulmonary CD8 T cells are capable of exerting effector functions and are effective in protecting against productive infection. We propose that antiviral “stand-by” memory-effector T cells persist in the lungs to prevent virus recurrence from latency.

Even though cytomegaloviruses (CMV) typically cause disease with multiple organ involvement, the lungs are a particularly conspicuous organ site of CMV pathogenesis, latency, and reactivation. Specifically, CMV-associated interstitial pneumonia/pneumonitis (CMV-IP) is the most fatal among the clinical manifestations of human CMV (hCMV) disease in the immunocompromised host, such as recipients of bone marrow transplantation (BMT) and solid organ transplants, and recipients of heart-lung transplants in particular (for reviews, see references 10, 17, 18, 43, 45, and 56). While the incidence of hCMV infection is similar after autologous and allogeneic BMT, CMV-IP is more common after allogeneic BMT, specifically in association with graft-versus-host (GvH) disease (26, 27, 50, 55). However, in the less frequent cases of CMV-IP observed after autologous BMT, the disease was just as severe and the fatality rate was outstandingly high (11, 25, 41).

Experimental CMV-IP occurring during lethal infection of immunodepleted BALB/c mice with murine CMV (mCMV) confirmed the role of the lungs as a prominent site of CMV pathogenesis in the immunodeficient host and identified stromal and parenchymal lung cells, such as interstitial fibrocytes, alveolar epithelial cells, and endothelial cells, as target cells of productive mCMV infection in the absence of leukocytic infiltrates (40). Selective exogenous reconstitution of the infected recipients with presensitized antiviral T cells by means of cell transfer demonstrated antiviral control (38, 40) and, notably, also prevention of lung histopathology (39), mediated by CD8 T cells but not by CD4 T cells. The validity of this mCMV model was proven by clinical application of T-cell transfer as a specific preemptive cytoimmunotherapy of hCMV disease after BMT (44), and an originally suspected immunopathology caused by the transferred CD8 T cells was not observed in patients (for a review, see reference 43). Extension of the mCMV infection model by a concurrent experimental BMT, either a syngeneic BMT (19, 32) or a BMT performed across a single major histocompatibility complex (MHC) class I antigen difference (1, 33), documented a preferential recruitment of endogenously reconstituted CD8 T cells into the infected lungs. Specifically, CD8 T cells were recruited much more efficiently than CD4 T cells, and recruitment to infected lungs by far exceeded the recruitment to uninfected lungs (19). A curative antiviral rather than an immunopathological function of CD8 T cells in pulmonary infiltrates was concluded from four facts: (i) T-cell infiltration correlated with a decline in virus replication (1, 19), (ii) depletion of CD8 T cells during reconstitution abolished the antiviral control with fatal outcome (32), (iii) CD8 T cells isolated from pulmonary infiltrates showed an ex vivo MHC-restricted cytolytic activity against infected but not against uninfected target cells (19), and (iv) adoptive transfer of pulmonary infiltrate-derived CD8 T cells controlled virus replication in various organs of indicator recipients, the lungs included (1). While a correlation between reconstitution of CD8 T cells after BMT and control of infection was also observed in patients (42), the mCMV model has provided proof of the protective principle.

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A special role of the lungs in the biology of CMV is also inferred from studies on mCMV latency, reactivation, and recurrence. Thus, in the quite diverse models of neonatal infection (3, 37) and infection of BMT recipients (22–24, 49), the lungs consistently proved to be a prominent site of viral latency, as indicated by a high copy number of latent viral genomes and an associated high risk of transcriptional reactivation and recurrent infection upon secondary immunomodulatory treatment. By controlling virus replication during acute infection, CD8 T cells limited the load of latent viral genomes in the lungs, and, accordingly, reduced the risk of recurrent infection of the lungs (49).

While all this cumulative evidence argued for a protective role of the immune response to mCMV as well as hCMV, Grundy et al. highly influenced the field by proposing an immunopathogenesis of CMV-IP based on their interpretation of clinical data as well as on data from specifically designed murine models (13–15). In essence, the authors highlighted the clinical data on the statistical association between CMV-IP and GVH disease, and this line of argument was supported by murine infection models in which a GVH reaction which was experimentally enforced by administration of fairly high numbers of mature allogeneic T cells caused lung pathology that was preventable by immunosuppression. A second argument was the absence of CMV-IP in athymic nude mice. A third argument was the usually benign course of lung infection by hCMV during earlier stages of AIDS, which the authors ascribed to a lack of CD4 T-cell-driven immunopathology. Finally, Grundy et al. cited cases in which antiviral therapy had apparently failed to resolve established pulmonary symptoms. Since then, the literature on clinical CMV-IP has been filled with case reports that interpret single clinical observations in favor or disfavor of Grundy’s immunopathogenesis hypothesis. Retrabbul came primarily from clinicians who outlined in detail that the immunopathological characteristics ascribed to mCMV pneumonia do not apply to most cases of hCMV pneumonia (4, 29). Specifically, Morris (29) concluded that the murine model is not valid.

However, the pathogenesis and histopathology of murine CMV-IP have so far not been thoroughly studied by longitudinal analysis in a setting of BMT that is more closely related to the clinical situation. Based on the finding that hCMV can also result in severe CMV-IP after autologous BMT (11, 25, 41), the present study is focused on syngeneic BMT and on the role of CD8 T cells in mCMV-associated pathogenesis during acute and latent infection of the lungs.

MATERIALS AND METHODS

BMT, infection, and in vivo T-cell subset depletion. Animal experiments were approved by the Ethics Commission, permission no. 177-07/931-17, according to German federal law. Donors and recipients of a syngeneic experimental BMT approved by the Ethics Commission, permission no. 177-07/931-17, according to (MAb) YTS 191.1 and YTS 169.4 (6), respectively (kindly provided by the group subsets were depleted in vivo by i.v. infusion of 1 mg of monoclonal antibodies strain Smith ATCC VR-194/1981 (24). Under the specific conditions chosen for of infected cells present in lung tissue sections and detected either by in situ

(i) Detection of virus titers in organs. Infectious virus was quantified in organs by plaque assay performed on neonatal-passage mouse fetal fibroblast monolayers under conditions of centrifugal enhancement of infectivity as described previously (19, 40). The virus titers represent the number of infectious units per organ and are expressed as PFU* to indicate the enhancement. It is worth noting that 1 PFU* corresponds to ca. 25 viral genomes, whereas a noncentrifugal PFU* represents ca. 500 viral genomes (24).

(ii) ISH of viral DNA in intranuclear inclusion bodies. Detection of viral DNA was performed as described previously (32). In essence, in order to get an optimal sensitivity of detection, a mixture of digoxigenin (digoxigenin-11-dUTP)-tagged plasmids containing HindIII fragments A, I, and K, altogether representing 51.2 kbp of the mCMV Smith genome, was used as the specific probe. Labeling was performed with alkaline phosphatase-conjugated antidigoxigenin antibody and new fuchsin as the substrate, yielding a brilliant red. ISH detects infected cells in the late phase of viral replication, when viral DNA accumulates for packaging and nucleocapsid assembly in an intranuclear inclusion body.

(iii) Two-color IHC. Infected lung cells and lung-infiltrating T cells were simultaneously visualized in 2-μm paraffin-embedded sections of lung tissue by two-color IHC as described in detail previously (19). In essence, infected lung cells were visualized by detection of intranuclear viral IE1 protein with MAb CROMA 101 (kindly provided by S. Jonic, University of Rijeka) using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method with new fuchsine as the substrate (red staining). T cells were visualized by staining of membrane CD3e, using the avidin-biotin-peroxidase complex (ABC) method with biotinylated tetramethyl benzidine hydrochloride as the substrate and development with nickel sulfate hexahydrate (black staining). Viral IE1 protein is present in the nucleus of the infected cell throughout the viral replication cycle. Therefore, IE1-specific IHC detects more infected cells than does ISH. In our experience, the ratio between IHC-positive and ISH-positive cells is ca. 2.3 during florid infection of permissive tissues.

(iv) Quantitation of histological data. To avoid artificial compression of lung tissue, it was crucial to dissect alveolar spaces by instillation of the fixative (phosphate-buffered saline [PBS] [pH 7.4]) containing 4% [vol/vol] formalin) into the trachea. The numbers of infected tissue cells (red) and infiltrating T cells (black) were counted for representative areas of tissue sections (in the lungs, usually 100-mm2 areas) compiled from sections representing the organ macroscopic specimen and taken in distances of different cm from each other (10–20 cm) to avoid a redundant counting of the same cells. Numbers of infected tissue cells were related to the number of lung cells counted in uninfected lung tissue after hematoxylin staining of nuclei. It should be mentioned that infiltrating leukocytes were found not to be infected, with the exception of a very few alveolar macrophages. Therefore, the reported percentage of infected cells in the lungs refers to the stromal-parenchymal compartment. An estimate of the total number of infected cells per lung was made by relating section volumes (2 μm by 100 mm2) to the total lung volume imbedded in paraffin (0.135 cm3). The extrapolation to the total organ volume results in an overestimation because sections may cut the same nucleus more than once depending on the ratio between nucleus (assumed to be a sphere) diameter, D, and thickness of the tissue section, t: N = 4πD3/3t. Extrapolated numbers are corrected for the morphometric formula N = m × n/D, where m is, in nuclei, particularly those of endothelial cells, are ellipsoids rather than spheres, and since the size and form of nuclei differ between cell types in the lungs, the estimate should be seen as a rough approximation to give an impression of the order of magnitude.

Isolation and immunomagnetic enrichment of pulmonary CD8 T cells. Mono- nuclear leukocytes were isolated from lung tissue as described previously (19) by collagenase-DNase digestion of lung parenchyma followed by Ficoll density gradient centrifugation. Analyses were performed with cells pooled from at least 10 mice per group. It is important to emphasize that intravascular leukocytes had been largely removed by perfusion, but the presence of intracapillary leukocytes that stick to endothelial cells can never be excluded. Cells were either used directly for cytofluorometric analyses of the phenotypes present in the pulmonary infiltrate population (see below) or were subjected to positive immunomagnetic separation from the stromal-parenchymal compartment. An estimate of the total number of infected cells in the lungs was made by relating section volumes (2 μm by 100 mm2) to the total lung volume imbedded in paraffin (0.135 cm3). The extrapolation to the total organ volume results in an overestimation because sections may cut the same nucleus more than once depending on the ratio between nucleus (assumed to be a sphere) diameter, D, and thickness of the tissue section, t: N = 4πD3/3t. Extrapolated numbers are corrected for the morphometric formula N = m × n/D, where m is, in nuclei, particularly those of endothelial cells, are ellipsoids rather than spheres, and since the size and form of nuclei differ between cell types in the lungs, the estimate should be seen as a rough approximation to give an impression of the order of magnitude.

Assay of CD3ε-restricted cytolytic activity. The CD3ε assay measures the total cytolytic potential of an effector cell population by antigen-independent polyclonal signaling via the T-cell receptor (TCR)-CD3 complex. Target cells were 51Cr-labeled P815 mastocytoma cells infected with the T-cell receptor-bound anti-CD3ε MAb. Immunomagnetically purified pulmonary CD8 T cells were tested as effector cells. A standard 4-h 51Cr release assay was performed with graded numbers of effector cells and with 103 target cells per 0.2-ml microtiter plate. The percentage of specific cytolysis was determined after subtraction of spontaneous release (percent release of infected cells). Since nuclei, in particular those of endothelial cells, are ellipsoids rather than spheres, and since the size and form of nuclei differ between cell types in the lungs, the estimate should be seen as a rough approximation to give an impression of the order of magnitude.
anti-CD3ε MAb and that no cytolytic activity was triggered by anti-CD3ε MAb in unprimed T cells isolated from the lungs after BMT with no infection (19).

Three-color cytofluorometric analyses. Cytofluorometric analyses were performed with a FACSort (Becton Dickinson, San Jose, Calif.) by using CellQuest software (Becton Dickinson) for data processing. Overlaps in the emission spectra of fluorescent dyes were compensated for throughout, and thresholds were set in the forward-versus-side scatter (FSC-vs-SSC) plot to exclude particles the size of erythrocytes or smaller and to exclude dead cellscorrecting data acquisition. For calculations, a “lymphocyte gate” was set in the FSC-vs-SSC plot so as to largely exclude macrophages and residual granulocytes from the analysis but include all living T cells. Staining for the expression of TCR εβ (see below) was used to define the lymphocyte gate. This is of particular importance; since the scatter characteristics of activated pulmonary infiltrate T cells differ somewhat from those of resting T cells in lymphoid organs. Basic technical aspects, such as the preparation of cells for cytofluorometric analysis, the composition of buffers, and the blocking of nonspecific binding sites, have been described previously (1). Throughout, fluorescence channel 1 (FL-1) represents the fluorochrome fluorescein (fluorescein isothiocyanate; FITC), FL-2 represents the fluorochrome R-PE, and FL-3 represents either the tandem fluorochrome PE-Cy5, also known as Cy-Chrome, or the tandem fluorochrome PE-Texas Red, also known as RED613 or duochrome.

(i) Determination of CD8/CD4 subset ratios among αβ T cells. Pulmonary infiltrate cells retrieved from the Ficoll interphase were labeled with FITC-conjugated MAb anti-CD8 (clone 53-6.7, rat IgG2a; catalog no. 01044A; Becton Dickinson), PE-conjugated MAb anti-TCR εβ (clone H57-597, hamster IgG; catalog no. 01305A; PharMingen), and PE-Cy5-conjugated MAb anti-CD4 (clone 129.19, rat IgG2a; catalog no. 00908A; PharMingen). The analysis was restricted to αβ T cells by setting an electronic gate on signals with positive FL-2.

(ii) CD44 and CD62L activation phenotyping of CD8 T cells. Pulmonary infiltrate cells retrieved from the Ficoll interphase were labeled with FITC-conjugated MAb anti-CD44 (clone IM7, rat IgG2b; catalog no. 012248; PharMingen) and PE-conjugated MAb anti-CD8α (see above). CD62L was labeled indirectly with biotinylated MAb anti-CD62L (clone MEL-14, rat IgG2a; catalog no. 01262D; PharMingen) and streptavidin-RED613 (catalog no. 19540-010; Life Technologies). The analysis was restricted to CD8 T cells by setting an electronic gate on signals with positive FL-2.

(iii) Detection of intracellular IFN-γ. The functional capacity of pulmonary CD8 T cells to produce gamma interferon (IFN-γ) (35) was tested by antigen-independent intracellular signaling via the TCR/CD3 complex. Pulmonary infiltrate cells retrieved from the Ficoll interphase were seeded in replicate 0.2-ml cultures in 96-well round-bottomed microwell plates at a concentration of 10^5 cells per culture. The composition of the culture medium, which included 10 μg of brefeldin A per ml, was described previously (20). For polyclonal signaling, cells were stimulated with 0.4 μg (per culture, i.e., per 10^6 cells) of MAb anti-CD3ε (clone 145-2C11, hamster IgG; Dianova catalog no. 1530-14; Southern Biotechnology Associates Inc., Birmingham, Ala.). It should be noted that a log2 titration of anti-CD3ε from 0.2 to 1.6 μg gave identical results (data not shown). The cells were harvested after 5 h of incubation and processed for cytofluorometric analysis as described previously (20). Surface staining was performed with FITC-conjugated anti-CD8ε (clone MEL-14, rat IgG2a; catalog no. 01044A; PharMingen) and PE-Cy5-conjugated MAb anti-CD4 (clone 53-6.7, rat IgG2a; catalog no. 01048A; PharMingen). After washing fixation was performed for 20 min at ca. 20°C by addition of 100 μl of PBS containing 2% (vol/vol) paraformaldehyde. Cells were washed to remove the fixative and permeabilized with biotinylated MAb anti-CD62L (clone MEL-14, rat IgG2a; catalog no. 01262D; PharMingen) and streptavidin-RED613 (catalog no. 19540-010; Life Technologies). The analysis was restricted to CD8 T cells by setting an electronic gate on signals with positive FL-2.

The results of the experiments are shown in Fig. 1, all recipients survived long term (Fig. 1A, solid squares). The protective principle within the reconstituting myeloid and lympho- phoid cell lineages was revealed by specific in vivo depletion of T-cell subsets during the process of reconstitution after BMT. The efficacy of the depletions was controlled by cytofluorometric analysis of pulmonary infiltrate cells. In accordance with a previous report (32), depletion reduced the respective T-cell subsets to ≤0.5% of the T cells (not shown here). Selective depletion of CD4-positive cells, which includes CD4 T cells and subsets of bone marrow myeloid cells, had no significant
effect on the survival rate (Fig. 1A, solid circles). By contrast, selective depletion of CD8 T cells inevitably resulted in death from the disease within 3 weeks (Fig. 1A, open circles). Simultaneous depletion of both T-cell subsets did not accelerate the lethal course (Fig. 1A, open squares). It must be emphasized that depletion of CD8 T cells is not associated with lethal disease after BMT in the absence of infection (not shown). In accordance with previous work (32; for a review, see reference 21), this experiment has thus unequivocally identified CD8 T cells as the protective principle preventing CMV-associated mortality after BMT. CD4 T cells were not required for protection, nor could they functionally substitute for the eliminated CD8 T cells in this particular experimental setting. The result is also decisive regarding the controversial hypotheses of “viral pathogenesis” versus “immunopathogenesis” of CMV disease. Recipients survived when CD8 T cells or both CD8 and CD4 T cells were present, and they died when CD8 T cells or both subsets were absent. Thus, clearly, the observed lethal outcome of mCMV infection was not the result of an immunopathology.

Since CMV disease is characterized by multiple-organ involvement (18, 32), one could argue that CD8 T cells prevent a lethal course of disease by controlling virus replication at vital tissue sites, including liver, adrenal glands, and the bone marrow stroma (9, 32), but contribute to pathogenesis specifically in the lungs. As a first approach to understanding the role of CD8 T cells in the lungs, we quantitated the infection of lung tissue in relation to the presence of T-cell subsets by counting the number of productively infected lung cells. Productive infection was visualized by ISH staining of viral DNA accumulated in intranuclear inclusion bodies during viral DNA packaging and nucleocapsid assembly in the late phase of the viral replication cycle. The analysis was performed at day 19 after BMT and infection, that is, at a prefinal stage of CMV disease (Fig. 1B). The extent of infection of the lungs precisely reflected the overall course of disease. In the two experimental groups with reconstituting CD8 T cells (Fig. 1B, solid symbols), the proportion of infected lung cells was between 0.01 and 0.1%, whereas in both groups that were depleted of CD8 T cells (open symbols), between 1 and 3% of the stromal and parenchymal lung cells, in absolute terms ca. 0.6 × 10⁶ to 1.8 × 10⁶ cells, were found to be in the late phase of infection. In conclusion, reconstitution of CD8 T cells after BMT is essential to prevent massive infection of the lungs.

**Viral histopathology in lungs devoid of T-cell infiltrates.**

The histopathological consequences of viral replication and T-cell control in the lungs were studied by two-color IHC, simultaneously visualizing infected cells by red staining of intranuclear viral IE1 protein and infiltrating T cells by black membrane staining of CD3ε (Fig. 2).

In the experimental group that was selectively depleted of CD4 T cells, infected cells were rare in the lungs and were actually not seen in every tissue section. Notably, infiltrating blastoid CD8 T cells were not distributed randomly in lung tissue but formed inflammatory foci specifically at the sites at which infected lung cells were located (Fig. 2, panel A1 for overview and A2 for details). Alveolar macrophages were also recruited into these foci (Fig. 2A2). As a side aspect, one should note that the alveolar macrophages shown in Fig. 2A2 did not express detectable IE1 protein. From this part of the experiment, we draw the following conclusions: (i) extravasation and recruitment of CD8 T cells to the site of infection do not require help by CD4 T cells, (ii) infiltrating CD8 T cells focus their action towards infected cells, and (iii) in reverse conclusion, CD8 T cells do not damage uninfected parts of tissue, at least not by cell-to-cell delivery of effector functions.

A strikingly different picture was seen when both T-cell subsets were depleted during reconstitution (Fig. 2, panel B1 for overview and B2 for details). Numerous infected cells, distributed randomly throughout the lung parenchyma, were now detected in every tissue section. By cell counting in representative 100-mm² areas of lung tissue, the proportion of IE1-expressing cells was found to range between ca. 2 and 6%. Complete absence of CD3ε staining gives visible proof of the efficacy of T-cell depletion. Alveolar macrophages were frequent but were not found colocalized to infected cells (Fig. 2B1). Altogether, focal infection, as it was seen in the presence of CD8 T cells, was here replaced by disseminated infection. A comparison of the overall tissue architecture (Fig. 2, compare panels A and B) shows a more pronounced widening of alveolar septa and a higher degree of alveolar collapse in the absence of T-cell infiltrates! From this part of the experiment, we draw the following conclusions. (i) Infection of the lungs bursts in the absence of CD8 T cells. In the reverse conclusion, the CD8 T cells shown in Fig. 2A were antivirally active and responsible for limiting the spread of infection in the lungs. (ii) Histopathological characteristics of disseminated interstitial pneumonia develop in absence of T cells, that is, neither CD8 T cells nor CD4 T cells cause the histopathological alterations seen in the acute phase of CMV infection.

In conclusion, the lung pathology found after BMT and acute CMV infection is not a CMV-associated T-cell-mediated immunopathology but represents an authentic viral pneumonia.

**Persistence of T-cell infiltrates after clearance of productive infection.**

Efficient reconstitution of antiviral CD8 T cells after BMT leads to control of CMV infection and prevents a lethal course of disease, but there might be sequelae accounting for late CMV-associated morbidity in survivors of acute CMV disease (5). We therefore performed a longitudinal study of pulmonary infection and T-cell infiltration by two-color IHC, correlating the number of T cells in the lungs with the number of infected cells for an observation period of 10 months after BMT and primary infection, with no experimental depletion of T-cell subsets performed (Fig. 3). In accordance with previous data on the yield of infectious virus (19), the number of infected lung cells reached its peak after 3 weeks and declined sharply thereafter. By 5 weeks, productive infection was resolved. It should be recalled from previous work that the viral genome is not cleared from the lungs but is maintained in a state of latency (22, 24). Infiltration of T cells paralleled the infection during the first 3 weeks, but the infiltrates persisted long term, with only a slow and moderate decline in cell numbers during the observation period (Fig. 3A). By extrapolation, one can predict that infiltrates persist for the life span of the recipients. On the basis of this kinetics, we can now operationally define two phases of CMV-associated histopathology. Phase 1 is defined by the simultaneous presence of infected lung cells and T-cell infiltrates, whereas phase 2 is defined by the presence of T-cell infiltrates in the absence of productive infection. Histopathology representative of these two defined phases is documented in Fig. 3B for the third week (Fig. 3B1, phase 1) and for the third month (Fig. 3B2, phase 2). Figure 3B1 shows a perivascular inflammatory focus with colocalization of infected cells (red) and infiltrating blastoid T cells (black). By contrast, Fig. 3B2 documents a scattered distribution of small, apparently resting T cells (black) in lung parenchyma in the absence of infected cells. The tissue architecture in this late phase is not severely impaired, which is consistent with long-term survival of the recipients and which implies that neither CD8 nor CD4 T cells cause visible tissue damage. In fact, the presence of interstitial T cells is the only significant histopathological sign. In summary, phase 1 histopathology can...
be characterized as a CMV-positive IP with inflammatory foci, whereas phase 2 histopathology can be characterized as a diffuse and very moderate CMV-negative IP with marked T-cell presence in the interstitium and little involvement of alveolar spaces. From a pathologist’s point of view, and with no further information being provided, phase 2 histopathology in clinically asymptomatic BMT recipients would not easily be diagnosed as being associated with CMV. Rather, the presence of T-cell infiltrates in the absence of an infectious agent may tempt one to diagnose an “idiopathic” pneumonia or an immunopathological condition of unknown etiology. That it is in fact an “aged” CMV pneumonia is only revealed by longitudinal analysis of the events.

Activation phenotypes of phase 1 and 2 pulmonary CD8 T cells. While the preceding experiments have provided conclusive evidence for a protective role of infiltrating CD8 T cells during phase 1, a contribution to late histopathology still has to be considered. As a first approach to understanding the role of CD8 T cells during phase 2, we isolated lymphocytes from phase 1 and 2 pulmonary infiltrates for a comparative cytofluorometric analysis of their phenotypes (Fig. 4). In accordance with our previous work (19), the T-cell population in the phase 1 infiltrates was largely dominated by CD8 T cells. It is instructive to recall from the previous work that the CD8/CD4 T-cell ratio in lung infiltrates is ca. 0.2 after BMT with no mCMV infection, as it is in normal lungs of immunocompetent
FIG. 3. Longitudinal analysis of pulmonary infection and T-cell infiltration after BMT. Histopathology in the lungs was studied by quantitative two-color IHC with infected cells being visualized by red staining of intranuclear viral IE1 protein (red dots) and infiltrating T cells by black staining of membrane CD3ε (black dots). (A) Time course of infection and T-cell infiltration. Each symbol represents data for individual recipients. The lines connect median values. Negative counts are depicted only on the first occasion in the kinetics and remained negative throughout. The left-hand scale and the right-hand scale show the numbers of infected lung cells and of lung-infiltrating T cells, respectively, for representative 100-mm² areas compiled from several lung tissue sections. In a control group receiving BMT but not infection, the tissue architecture was not pathologically altered (19) (not shown), and in phase 2, the average number of T cells per 100 mm² was 880 (arrowhead), in comparison to ca. 200 in lungs of normal mice. (B) Examples of lung histology representative of phase 1 (B1, CMV-positive IP) and of phase 2 (B2, CMV-negative IP). (B1) Perivascular inflammatory focus seen 3 weeks after BMT and infection in tissue that connects two neighboring vessels. T cells located within the inflammatory focus are activated lymphoblasts, as shown by halo-like membrane staining (see also Fig. 2A2). (B2) Random distribution of residual interstitial T cells after clearance of productive infection, as observed after 3 months. Note that most of the T cells (some of which are marked by an arrow) are considerably smaller than those in phase 1 and apparently represent resting cells. Counterstaining was performed with hematoxylin. Bars, 25 μm.
CD4 T cells (CD8/CD4) are indicated.

Percentages of relevant T-cell subsets and the ratios of CD8 to CD4 gated cells as contour plots in a 70% log-density mode (threshold, 2%; smoothing factor, 5). Percentages of relevant T-cell subsets and the ratios of CD8 to CD4 were restricted to ca. 10,000 CD8 T cells by a second gate set on positive FL-2.

FIG. 4. Phenotypes of phase 1 and phase 2 pulmonary infiltrate T cells. Pulmonary infiltrate cells were isolated at 4 weeks (phase 1) and at 10 weeks (early in phase 2) after BMT and mCMV infection. (A and B) Three-color cytfluorometric analysis was performed for the marker combinations FITC (FL-1)-CD8, PE (FL-2)-TCRαβ, and PE-Cy5 (FL-3)-CD4. A gate was set on lymphocytes, and the analysis was restricted to ca. 20,000 αβ T cells by a second gate set on positive FL-2. (C and D) Three-color cytfluorometric analysis was performed for the marker combinations FITC (FL-1)-CD44, PE (FL-2)-CD8, and PE-Cy5 (FL-3)-CD62L. A gate was set on lymphocytes, and the analysis was restricted to ca. 10,000 CD8 T cells by a second gate set on positive FL-2. FL-3 (ordinate) versus FL-1 (abscissa) log fluorescence intensities are shown for gated cells as contour plots in a 70% log-density mode (threshold, 2%; smoothing factor, 5). Percentages of relevant T-cell subsets and the ratios of CD8 to CD4 T cells (CD8:CD4) are indicated.

BALB/c mice (19), whereas this ratio was 2.3 in phase 1 infiltrates of the experiment documented in Fig. 4A. In phase 2, the absolute number of T cells in lung tissue had declined by a factor of ca. 10 (recall Fig. 3A). In addition, the CD8:CD4 ratio had declined to 0.5 (Fig. 4B). Thus, the absolute number of CD8 T cells had actually declined significantly between phase 1 and phase 2, by a factor of ca. 20. The CD44 cell surface glycoprotein (also called Pgp-1 and Ly-24) is required for extravasation of activated T cells into an inflammatory site (8). Accordingly, CD8 T cells isolated from pulmonary infiltrates all expressed CD44, regardless of whether they were isolated during phase 1 or phase 2 (Fig. 4C and D). Like CD44, the CD62L member of the selectin family (also called L-selectin, Ly-22, and MEL-14 antigen) contributes to the recruitment of leukocytes into areas of inflammation by mediating their initial tethering and rolling on endothelial surfaces. However, upon activation of lymphocytes, CD62L is rapidly shed from the cell surface as a result of proteolytic cleavage (12, 54; for a review, see reference 53). Activated cells are therefore supposed to display the phenotype CD44 hi CD62L hi, and naive T cells and quiescent memory T cells are supposed to display the phenotypes CD44 lo CD62L hi and CD44 hi CD62L hi, respectively (28; for a review, see reference 2). In phase 1 pulmonary infiltrates, 90% of the CD8 T cells showed the phenotype of activated cells (Fig. 4C). This finding is in agreement with the antiviral effector function performed by CD8 T cells during acute infection of the lungs. Note that the few CD62L hi CD8 T cells seen in phase 1 were CD44 hi. These cells likely represent naive CD8 T cells, probably residual intracapillary leukocytes. In phase 2 pulmonary infiltrates, the activated population was still prominent (ca. 71%), but a significant population of CD44 lo CD62L hi memory T cells (ca. 22%) had emerged in the infiltrates (Fig. 4D). For completeness, it should be mentioned that a small population (ca. 7%) of CD62L dim CD8 T cells was present in both phases. We do not presently know any role for these cells. In conclusion, the cellular composition of phase 1 and phase 2 pulmonary infiltrate T-cell populations differs in that the proportion of CD8 T cells is reduced in phase 2 while the number of CD8 T cells with memory phenotype is increased. However, the proportion of CD8 T cells with the activation phenotype CD44 hi CD62L hi is still quite considerable in phase 2.

Functional properties of phase 1 and 2 CD8 T cells. The finding that a considerable number of phase 2 pulmonary infiltrate CD8 T cells still displayed the phenotype of activated T cells suggested a functional activity. Since the proportion of CD8 T cells specific for any single antigenic peptide of mCMV is proposed to be very low, as it was concluded previously for the immunodominant IE1 peptide (19), the antigen-independent method of eliciting effector function by polyclonal signaling via the CD3-TCR complex was chosen to measure the functional competence of whole CD8 populations. Specifically, the cytolytic potential of immunomagnetically purified phase 1 and 2 pulmonary infiltrate CD8 T cells was tested by CD3ε redirected lysis. In accordance with previous work (19), phase 1 CD8 T cells were cytolytically active (Fig. 5). Notably, even though with somewhat lower cytolytic potential, purified phase 2 CD8 T cells were also functional in this assay (Fig. 5). Accordingly, analysis of perforin gene expression by reverse tran...
scriptase-PCR revealed a similar amount of perforin transcripts for phase 1 and 2 pulmonary infiltrate CD8 T cells in this particular experiment (not shown). It should be mentioned, however, that cytolytic activity during phase 2 is variable and that detection of low activities in the infiltrates requires enrichment for CD8 T cells. From previous work (19) it should be recalled that cytolytic activity is undetectable throughout the kinetics in pulmonary infiltrates after BMT performed in the absence of infection. In conclusion, the experiment has demonstrated that phase 2 CD8 T cells, at least some of them, possess the capacity to exert cytolytic effector function upon engaging their CD3-TCR complex. This quality is dependent upon prior sensitization during a preceding infection of the lungs.

The expression of IFN-γ is another relevant effector quality of CD8 T cells. The capacity to synthesize IFN-γ upon polyclonal signaling via the CD3-TCR complex was tested for phase 1 and 2 pulmonary infiltrate cells by stimulation with MAb anti-CD3ε (Fig. 6A). For comparison, pulmonary T cells isolated at the same time post-BMT from the lungs of uninfected BMT recipients were analyzed accordingly (Fig. 6B). Three-color cytofluorometric analysis was used to combine cell phenotyping for expression of CD8 and CD62L with measurement of accumulated intracellular IFN-γ.

In phase 1 after infection (Fig. 6A, left panel), ca. 36% of the gated CD8 T cells were capable of expressing IFN-γ, and these cells displayed the CD62Llo phenotype. The few CD62Lhi cells (ca. 20%) were mostly refractory to stimulation by anti-CD3ε. In accordance with their CD44lo phenotype (recall Fig. 4), this finding is indicative of naive cells. It should be noted that IFN-γ expression discriminates between two qualitatively different subsets of CD81 CD62Llo T cells. We concluded this from the finding that intensification of the triggering by higher doses of MAb anti-CD3ε did not increase the number of responding cells (not shown). After BMT with no infection (Fig. 6B, left panel), many more CD8 T cells in phase 1 (ca. 72%) displayed the CD62Lhi phenotype of resting cells and accordingly, most of these cells (ca. 63% of the CD8 T cells) were also refractory to stimulation by anti-CD3ε. CD8 T cells producing IFN-γ were less frequent than after infection (ca. 11% versus ca. 36%), but were also of the CD62Llo phenotype.

In phase 2 after infection (Fig. 6A, right panel), the percentage of CD62Lhi CD8 T cells was elevated (ca. 40% versus ca. 20% in phase 1) and the percentage of CD62Llo IFN-γ producers was still substantial, ca. 26%. Remarkably, unlike in phase 1, many of the CD62Lhi cells were now susceptible to stimulation by anti-CD3ε, as is indicated by the shift to a CD62Llo phenotype. This feature is consistent with the known loss of CD62L upon activation of quiescent memory CD8 T cells to memory effector cells (28). After BMT with no infection (Fig. 6B, right panel), many cells among the prevalent CD62Lhi population also showed a memory-type downregulato
tion of CD62L in response to stimulation with anti-CD3ε, but only a few CD62Llo cells (ca. 6% of the CD8 T cells) were able to respond with production of IFN-γ.

In conclusion, infection alters the pulmonary CD8 T-cell population quantitatively and qualitatively. It increases the absolute and relative number of CD8 T cells that can respond to a CD3-TCR-mediated stimulus with the production of IFN-γ.

**Phase 1 and phase 2 pulmonary infiltrate CD8 T cells protect against productive infection.** The finding that “stand-by” effector cells persist in the lungs for many months after clearance of the productive infection raised the question of their functional role, immunopathology or antiviral surveillance. Evidence in favor of antiviral surveillance was provided by adoptive transfer of immunomagnetically purified phase 1 and 2 pulmonary infiltrate CD8 T cells into immunocompromised and lethally infected indicator recipients. Pulmonary CD8 T cells derived from both phases controlled the acute infection in a dose-dependent manner not only at the site of origination, namely in the lungs, but also in the spleen (Fig. 7) and in other organs of the indicator recipients (not shown). Notably, the efficacy per cell was even somewhat higher in phase 2.

In conclusion, phase 2 pulmonary infiltrate CD8 T cells can exert an antiviral effector function in vivo.

**DISCUSSION**

Since the time when Grundy et al. (14) postulated an immunopathogenesis triggered by CMV infection, the pathogenesis of CMV-IP has been a matter of ongoing debate. While a number of pros and cons have meanwhile been collected from clinical data and murine models (for reviews, see references 4 and 29), a systematic study of lung histopathology after CMV infection in the specific context of BMT was clearly missing. Murine models of GvH disease and mCMV infection have been used to advocate the immunopathogenesis hypothesis (14, 15, 46), although these models were designed unrelated to the particular conditions imposed by the kinetics of hematopoietic and lymphopoietic reconstitution after BMT. A model of allogeneic BMT in the rat confirmed the postulated correlation between GvH immunogenetics of the transplantation and CMV-IP but did not confirm immunological symptoms of a GvH disease (48) as it was proposed by the immunopathogenesis hypothesis. Likewise, we have evidence for murine CMV-IP caused in the absence of GvH disease by extensive virus replication resulting from a failure in antiviral CD8 T-cell control after BMT performed across an MHC class I difference (31, 33). In the present report, a murine model of syngeneic BMT was used to study the time course of CMV-associated histopathology of the lungs under conditions of endogenous reconstitution of an antiviral immune response. We have operationally defined two phases in the kinetics that will be discussed consecutively: phase 1 is characterized by pulmonary infiltrates and acute infection of the lungs, while phase 2 is characterized by the persistence of interstitial T cells during viral latency after clearance of productive infection.

**Phase 1 scenario: CD8 T cells prevent disseminated viral pneumonia.** The acute phase of pulmonary mCMV infection after syngeneic BMT was characterized by a vigorous and preferential recruitment of reconstituted CD8 T cells that confined the infection by the formation of inflammatory foci. This process was independent of CD4 T cells. The infection of the lungs was moderate, with only ca. 0.04% (ca. 25,000 cells) of the lung stromal and parenchymal cells expressing viral IE1 protein at the peak of infection. At the same time, the number of CD8 T cells in the infiltrates was ca. 5 million, that is, ca. 200 CD8 T cells were present to control 1 infected cell. The actual ratio visible in the inflammatory foci was much lower, which is explained by the fact that infection was already controlled in many foci. The focal character of the inflammation as well as the correlation between CD8 T-cell infiltration and decline of the infection implied an antiviral function of the CD8 T cells. Activation of the CD8 T cells was indicated by blastoid morphology in the immunohistology, by a CD62Llo phenotype, and by a significant proportion of cells that produced IFN-γ upon CD3-TCR triggering.

Proof of the protective principle was provided by two complementary approaches: (i) CD8 T cells isolated from phase 1 infiltrates controlled the infection in cell transfer recipients and (ii) selective elimination of reconstituting CD8 T cells led to an enhancement of lung infection by a factor of 100, that is, ca. 4% of the lung cells, or, in absolute terms, ca. 2.5 million lung cells, were then infected. A disseminated IP with widening of alveolar septa and partial collapse of alveoli was seen also after combined depletion of CD4 and CD8 T cells, that is, in the absence of any T-cell infiltration. It is hence obvious that
phase 1 CMV-IP does not represent a T-cell-driven immunopathology but is caused primarily by cytolytic infection, with a likely contribution of cytokines and chemokines secreted by infected cells as well as by alveolar macrophages and, possibly, by natural killer cells. Clinical observations in BMT recipients have implicated TH2-type cytokines and lack of T-cell-mediated cytotoxicity in the pathogenesis of CMV-IP (47). In a clinical setting, it is difficult to evaluate the contributions of these two parameters to the pathogenesis. We would propose that the failure in the CD8 T-cell control was decisive for the development of CMV-IP in these patients and that the TH2-dominated cytokine profile is an epiphenomenon associated with the CD8 T-cell deficiency. This interpretation is also in accordance with clinical cases of early CMV-IP occurring prior to engraftment and in the absence of GVH disease following T-cell depleted allogeneic BMT (7, 16, 30). In previous models of CMV-IP, reviewed and interpreted by J. Grundy (13), an apparent lack of correlation between virus titers and histopathology associated with T-cell infiltration was used as an argument in favor of the immunopathogenesis hypothesis. Clearly, in our model as well, the lung histopathology is visually dominated by the infiltrating cells, particularly at later stages of phase 1 when the number of infected cells had already declined due to the effect of antiviral CD8 T cells. It is also evident that antiviral treatment at that stage would not resolve the histopathological characteristics. Nonetheless, these infiltrates are protective in that they prevent extensive viral destruction of the tissue, a conclusion that is in accordance with the progressive CMV-IP observed in athymic nu/nu mice (46). The antiviral function of CD8 T cells also explains the benign course of viral function of CD8 T cells also explains the benign course of pulmonary hCMV infection during earlier stages of AIDS (for a review, see reference 45). While J. Grundy proposed a lack of CD4-driven immunopathology as a result of CD4 T-cell depletion in the patients, we infer from our data obtained by selective depletion of the CD4 subset (recall Fig. 1 and 2) that residual CD8 T cells are likely to suffice for controlling the infection except in later stages of disease, when the numbers of CD8 T cells have also declined.

In conclusion, timely reconstitution of CD8 T cells is critical for the termination of acute infection and thus for the prevention of disseminated viral pneumonia.

**Phase 2 scenario:** persistence of pulmonary T cells after clearance of acute infection. A striking feature is the persistence of functionally competent pulmonary T cells after clearance of productive mCMV infection, which is reminiscent of persistent lymphocytosis observed in survivors of clinical CMV-IP (5). This CMV-negative IP can be viewed as an “aged” CMV-IP in survivors in whom the antiviral response has largely prevented viral histopathology. In fact, the presence of high numbers of interstitial T cells per se indicated a pathological condition, whereas the alveolar architecture was apparently intact. With no further information, an association with CMV is impossible to recognize. The T cells in phase 2 differed from those in the inflammatory foci of phase 1 by the following features: (i) they were no longer organized in foci but were randomly distributed in the lung parenchyma, (ii) they were no longer lymphoblasts but were resting cells by morphological criteria, (iii) the proportion of CD8 T cells had declined, even though it was still elevated compared with that in normal lungs or time-matched lungs after BMT with no infection (19), and (iv) many of the CD8 T cells represented memory cells, as was indicated by their CD62L嗨 phenotype in conjunction with responsiveness to triggering via their CD3-TCR complex. The latter property discriminates memory cells from naive cells, which are also CD62L嗨 but are refractory to triggering by MAb anti-CD3e. Notably, however, a high proportion of phase 2 CD8 T cells had maintained or possibly reacquired the CD62L嗨 phenotype, and a significant proportion of the CD8 T cells were able to respond by producing IFN-γ.

A remarkable set of experiments that are related to these findings have previously been reported by Tanaka et al. (51, 52). After clearance of acute mCMV infection in the lungs, a fulminant and rapidly fatal pneumonia was elicited by in vivo application of MAb anti-CD3e. Since virus recurrence did not occur in the short period until death, only 24 to 48 h after injection of the MAbs, it was obvious that the pneumonia was not caused by cytolytic virus replication. On the other hand, the pneumonia was CMV associated in that the effect was not observed with naive mice. The cause of pneumonia then was a “cytokine storm,” which included IFN-γ and tumor necrosis factor alpha (51), which in turn induced nitric oxide formation via expression of inducible nitric oxide synthetase (52). In addition, we propose also direct damage to lung tissue by elicited cytolytic effector function, such as perforin release, from memory-effector CD8 T cells. From the facts that virus replication was clearly not involved in this acute disease and that the pneumonia was prevented by immunosuppression, the authors believed they had provided direct evidence for an immunopathogenesis of CMV-IP. In order to explain the lack of disease in naive mice, they proposed a role for CMV infection in modulating the responsiveness of pulmonary T cells to stimulation via the CD3e molecule of the CD3-TCR complex.

We have here documented that the proposed modulation of T-cell responsiveness does indeed occur as a result of CMV infection. What has actually happened in the experiments performed by Tanaka and colleagues? In essence, they have observed the in vivo consequences of polyclonal T-cell stimulation via the CD3-TCR complex, that is, the in vivo correlate of the in vitro CD3e-directed lysis assay and the CD3e-mediated induction of IFN-γ synthesis. A quantitative comparison between phase 2 pulmonary infiltrates of infected and uninfected BMT recipients makes it obvious why pneumonia is induced preferentially in the infected group: the extrapolated number of CD8 T cells at 3 months after BMT and infection was ca. 0.5 million per lung, of which 26.4% (recall Fig. 6) were able to respond with IFN-γ synthesis, that is, ca. 10^8 CD8 T cells had the potential to respond to in vivo polyclonal stimulation. For BMT with no infection, the extrapolated number of CD8 T cells was ca. 40,000 per lung, of which only 5.4% were able to respond with IFN-γ synthesis, that is, only ca. 2000 CD8 T cells had the potential to respond to in vivo polyclonal stimulation. In summary, mCMV infection in our model of syngeneic BMT has amplified the number of functionally competent CD8 T cells residing in the lungs by a factor of 50! In addition, cytokine production by CD4 T cells will also be stimulated. That a simultaneous polyclonal in vivo triggering of the effector functions of so many T cells by MAb anti-CD3e will result in an acute cytokine syndrome and associated pneumonia is evident. Was this now the proof of an immunopathogenesis of late CMV-IP?

**Stand-by effector cells: guardians of CMV latency?** The experiments by Tanaka et al. (51, 52) as well as the data presented here have identified a powerful CMV-amplified response potential of the pulmonary CD8 T-cell population, and this indeed entails a risk of immunopathogenesis. However, disease will develop only if the effector functions are called up by polyclonal stimulation. At the moment, we see no disease correlate for such polyclonal in vivo triggering via the CD3-TCR complex, but there may be risk factors that could elicit immunopathogenesis mediated by the tissue-resident T cells. After allogeneic BMT, recruitment of antiviral CD8 T cells into the lungs by CMV-associated chemokines might indeed...
also attract GvH-reactive donor T cells, as was implied by the immunopathogenesis hypothesis (14). However, there is a major problem with this idea: while the statistical correlation between clinical GvH disease and human CMV-IP is undoubted, owing to the Minnesota Bone Marrow Transplantation Program study published in 1986 (27), the authors of this study have specifically emphasized that GvH disease preceded symptomatic hCMV infection by more than a month. One may therefore rather propose that GvH disease causes lung damage, which predisposes the lungs to fulminant CMV infection resulting in CMV-IP.

What else can be the physiological role of the persisting pulmonary T cells? An evident hint for answering this question was given by the antiviral efficacy of phase 2 CD8 T cells in adoptive transfer recipients. We propose that these cells represent tissue-resident “stand-by” effector cells ready for eliminating latently infected lung cells before virus reactivation, as soon as reactivation of viral gene expression has led to the presentation of antigenic peptides. There is clear evidence in support of an immunosurveillance function of T cells in maintaining CMV latency in general and specifically also in the lungs. We have shown previously in the murine model that the lungs carry a particularly high load of latent mCMV genome that entails a high statistical risk of intermittent transcriptional reactivation (3, 37, 49). However, infectious virus remained undetectable (24) unless an immunoablative treatment was performed (22–24, 49). These experiments have also demonstrated that recurrent virus can indeed originate from lung tissue and does not have to be imported from a distant site (23). Notably, as shown by Polic et al. (34), selective depletion of lymphocyte subsets revealed a redundant and hierarchical control of mCMV latency, with CD8 T cells being the principal subset.

In a way, the stand-by effector cells are reminiscent of any ordinary memory T cell, with the notable exception that they stay long term at the extralymphoid tissue site and that a significant subpopulation display the CD62Llo phenotype of acutely sensitized memory-effector cells, as opposed to the CD62Lhi phenotype of quiescent memory T cells in lymphoid tissues. Taken together, both features are likely to reflect a significant subpopulation that display the CD62Llo phenotype of memory T cells in lymphoid tissues. Frequent engagement of CD8 T cells in antiviral responses that predominate in lung tissues and does not have to be imported from a distant site (23) could make them from a distance site (23). Notably, as shown by Polic et al. (34), selective depletion of lymphocyte subsets revealed a redundant and hierarchical control of mCMV latency, with CD8 T cells being the principal subset.

In conclusion, the murine model presented here has given experimental evidence against an immunopathogenesis of primary CMV-IP. In the acute phase, protective antiviral CD8 T cells confer viral replication to inflammatory foci and eventually terminate productive infection of the lungs. Disseminated CMV-IP is caused by extensive cytolitic infection in the absence of CD8 T cells and with no pathogenic involvement of CD4 T cells. After resolution of productive infection, stand-by memory-effector cells remain in the lungs as guardians of viral latency. Their elimination by secondary immunoablative events (23, 34) can result in virus reactivation and late CMV disease. In addition, the persistence of tissue-resident memory-effector cells in the lungs entails a risk of immunopathogenesis after unspecified polyclonal triggering of the T-cell effector functions under disease conditions that still need to be defined.

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