MAJOR ARTICLE

Dendritic Cells under Influence of Mouse Cytomegalovirus Have a Physiologic Dual Role: to Initiate and to Restrict T Cell Activation

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The aim of this study is to analyze the dynamics of the mouse cytomegalovirus (MCMV)–dendritic cell (DC) interaction. Immature and mature DCs derived from the mouse stem cell line factor-dependent cell Paterson mixed potential were infected with a recombinant MCMV expressing green fluorescent protein. Infection of immature DCs resulted in DC activation and virus production, both of which may contribute to viral dissemination. The infection of mature DCs was nonproductive and was restricted to immediate-early and early viral protein expression. During early stages of MCMV infection, mature DCs up-regulated major histocompatibility complex (MHC) and costimulatory molecules and activated autologous, but not allogeneic, naive T cells. At later times of MCMV infection, DCs prevented T cell activation by down-regulation of MHC and costimulatory molecules. Thus, DCs under the influence of MCMV have a physiologic dual role: to initiate and to restrict T cell activation. The lack of immunostimulation in allogeneic settings may explain the increased risk of MCMV morbidity after allogeneic transplantation.

Human cytomegalovirus (HCMV) is an important pathogen with a high prevalence in the human population [1]. Primary CMV infection in immunocompetent hosts is controlled by immune functions that prevent overt CMV disease. After the initial acute infection, however, CMV establishes a lifelong persistent infection in the host, an infection that is characterized by alternating latent and productive stages. The persistence of the infection demonstrates that the host immune response fails to completely eliminate the virus. In immunocompromised persons, reactivation of latent CMV is frequent and is still responsible for severe and even fatal disease, despite the development of effective antiviral drugs [1]. Furthermore, CMV may be involved in the pathogenesis of certain malignancies [2, 3], insulin-dependent diabetes [4, 5], and atherosclerosis [6, 7].

HCMV is species-specific, but, because HCMV shares many features with mouse CMV (MCMV) in biology and pathogenesis, MCMV is frequently used as a model to study CMV infection [8, 9]. Both HCMV

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and MCMV use a series of strategies to avoid detection and elimination by the host immune system. For instance, CMV interferes with the major histocompatibility complex (MHC) class I pathway of antigen presentation, by several molecular mechanisms that allow virus-infected cells to escape immune surveillance by cytotoxic T cells [10, 11]. Unlike MHC class I expression, which is ubiquitous, constitutive MHC class II expression is restricted to antigen-presenting cells—macrophages, dendritic cells (DCs), and B cells—which take up and present exogenous antigens to $CD4^+$ T cells. Recently, it has also been shown that CMV down-regulates MHC class II proteins on macrophages and thereby reduces the ability of macrophages to present antigen to $CD4^+$ T cells [12–14]. Although the role of monocytes/macrophages in the pathogenesis of CMV infection is well established, there is a lack of data concerning CMV infection of DCs.

DCs are the most potent antigen-presenting cells and are capable of initiating several T cell–dependent immune responses [15, 16]. Immature DCs, which are distributed in virtually all peripheral tissues, are specialized in antigen uptake and processing. When induced to mature by several immune stimuli, DCs up-regulate MHC class I and II molecules, costimulatory molecules, and accessory molecules, while DCs migrate out of the tissues to secondary lymphoid organs. There, they present processed peptides very efficiently to antigen-specific T cells. This results in the activation of naive and resting antigen-specific T cells and in the differentiation of effector T cells.

The importance of DCs in viral infections stems from their major role in initiation and maintenance of an antiviral immune response [17]. From the view of a "selfish" virus, it might be attractive to counteract the functions of DCs to replicate to high titers, in a host, and to guarantee transmission and survival, in a host population. This may especially apply to viruses, such as herpesviruses and adenoviruses, that manage to establish a persistent infection in their host. Thus, for viruses, DCs represent an ideal target for immune evasion, and interference with DC function has indeed been observed for a number of viruses [18, 19]. Although it is now well established that DCs are targeted by CMV, the phenotypic and functional alterations of DCs that have been reported to be caused by CMV vary considerably between different studies [20–23]. Although Andrews et al. [20] observe productive MCMV-infection of DCs, the study by Raftery et al. [22] indicates that HCMV-infected DCs do not release infectious particles. In 2 separate studies that involve MCMV and HCMV, respectively, maturation of immature DCs after CMV infection was observed [20, 22]. However, another study suggests an inhibition of DC maturation by HCMV [21]. Furthermore, it is not clear whether only MHC are down-regulated [22] or whether both MHC and costimulatory molecules are down-regulated, on mature DCs [20, 21], by CMV infection. Although HCMV and MCMV may use different strategies to interfere with DC function, a possibility that could explain the different outcomes in the studies undertaken so far [20–23], the different alterations described in these studies cannot currently be assigned to either MCMV or HCMV. Remarkably, however, all studies report a complete impairment of DC function, irrespective of whether or not the down-regulation of MHC and costimulatory molecules on the cell surface of DCs was observed [20–22]. This raises the question, how are the high frequencies of CMV-specific T cells [24] that are critically involved in the efficient combat of CMV in healthy humans and mice generated?

To further clarify the role of DCs in CMV infection, we undertook a detailed study of the MCMV-DC interaction. As a model system that reliably and reproducibly recapitulates differentiation into functional DCs, in vitro, the mouse multipotent hematopoietic stem cell line factor-dependent cell Paterson mixed potential (FDCP-mix)—which can be induced to differentiate into mature functional DCs, in a directed and synchronous manner, by physiologic conditions [25]—was used. In the present study, we infected FDCP-mix–derived DCs, at different maturation stages, with a recombinant MCMV expressing green fluorescent protein (GFP). This allowed us to directly monitor the phenotypic and functional changes that were occurring in MCMV-infected DCs.

MATERIALS AND METHODS

Virus and cells. FDCP-mix cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% pretested horse serum (Sigma) and mouse interleukin (IL)–3-conditioned medium [26] at a concentration that stimulated optimal cell proliferation (which corresponds to 150 U of recombinant IL-3/mL). Cells were kept at a density between 6×10^4 and 1×10^6 cells/mL and were regularly checked to verify absence of *Mycoplasma* contamination, by use of a *Mycoplasma* PCR ELISA kit (Roche). M2-10B4 cells and mouse embryonic fibroblasts were cultured in Dulbecco's modified Eagle medium (MEM) supplemented with 10% fetal calf serum. Stocks of the recombinant GFP-MCMV strain were prepared on M2-10B4 cells, and virus titers were determined by standard plaque assay on mouse embryonic fibroblasts [8].

Construction of recombinant GFP-MCMV by BAC mutagenesis. To generate the recombinant GFP-MCMV strain, the GFP gene was inserted into the MCMV BAC plasmid pSM3fr [27], by homologous recombination in *Escherichia coli,* as described elsewhere [28, 29]. In brief, shuttle plasmid pST76KS-GFP [28], which contains the GFP open-reading frame under control of the HCMV immediate-early promoter, flanked on each side by ~3.5 kbp of viral DNA sequences homologous to the

MCMV immediate-early region (*Hin*dIII L fragment [30]), was transformed into the *E. coli* CBTS strain that already contained the MCMV BAC plasmid pSM3fr [29]. Bacterial clones with cointegrates between the BAC plasmid and the shuttle plasmid were selected at 43°C on agar plates containing chloramphenicol (30 μg/mL) plus kanamycin (50 μg/mL). With use of the negative selection marker *sac*B [31], bacteria harboring resolved cointegrates were obtained on agar plates containing chloramphenicol (30 μg/mL) plus 5% sucrose. Correct insertion of the GFP gene was verified by restriction-enzyme analysis of DNA of the MCMV BAC plasmid. The recombinant GFP virus was reconstituted by transfection of the MCMV BAC plasmid into mouse embryonic fibroblasts, by means of the calcium phosphate transfection method, as described previously [28].

Generation of immature and mature DCs. FDCP-mix cells were differentiated into DCs, in culture medium consisting of IMDM supplemented with 20% pretested fetal calf serum (FCS; Sigma), 50 μ*M* 2-mercaptoethanol (Gibco), 500 U/mL mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), and 500 U/mL mouse IL-4, as described previously [25]. Conditioned medium of the 1F5 cell line [25] was used as a source for mouse GM-CSF, and conditioned medium of the cell line X63Ag8-653-IL-4 [32] was used as a source for mouse IL-4. Cells were kept at a density of 5×10^5 cells/mL. Every second day of culture, half of the medium was exchanged, and cytokines were added according to the initial activity. To generate mature DCs, lipopolysaccharide (LPS; 100 ng/mL) was added, at day 12 after induction of DC differentiation, for 2 more days. In vitro–generated FDCP-mix DCs were pulsed with 25 μg/mL keyhole limpet hemocyanin (Sigma) or tetanus toxoid C fragment (1 μg/mL; Calbiochem), 1 or 2 days before the functional assay.

Generation of macrophages. FDCP-mix cells were differentiated into macrophages in IMDM supplemented with 20% pretested FCS (Sigma) and mouse IL-3 (2 U/mL), GM-CSF (250 U/mL), and macrophage colony-stimulating factor (M-CSF; 20 U/mL), as described previously [33].

Infection of DCs or macrophages with GFP-MCMV. DCs or macrophages, in 6-well plates, were subjected to centrifugal enhanced infection (300 *g* for 30 min) [8] with recombinant GFP-MCMV at an MOI of 1, 3, 10, 30, or 100, or with UV-inactivated GFP-MCMV at an MOI of 10. After centrifugation, cells were washed 3 times with PBS and were resuspended in complete medium with GM-CSF and IL-4 or M-CSF.

Determination of virus production. DCs or macrophages were infected with GFP-MCMV at an MOI of 10. The cell-free supernatants were collected from infected DCs at 0, 1, 2, 3, 4, and 6 days after infection and were stored at −80°C until use. Supernatants were serially diluted (10^{-1} to 10^{-6}) and were plated for 1 h on mouse embryonic fibroblasts. An overlay consisting of MEM (Gibco) supplemented with 5% FCS and 0.75% carboxymethyl cellulose (Sigma) was placed on the cells to prevent secondary plaque formation. At day 5 after infection, plaques were counted under the UV fluorescence microscope, and virus titers were calculated.

Flow cytometry. Fluorescein isothiocyanate–conjugated, phycoerythrin-conjugated, allophycocyanin-conjugated, or biotin-conjugated monoclonal antibodies directed against CD86 (no. 09275), CD80 (no. 09602), CD40 (no. 09664 and no. 09665), MHC class II (no. 06044 and no. 06045), MHC class I (no. 06105), CD11b (Mac-1; no. 01719), CD11c (no. 09705), CD14 (no. 09475), and CD34 (no. 09434) (all Pharmingen) were used as cell differentiation markers for FDCP-mix cells and for the analysis of phenotypic changes. The streptavidin-allophycocyanin conjugate was used to visualize primary biotinylated antibodies. Cells were harvested by centrifugation and were resuspended in PBS containing 3% FCS. Fc Block (no. 01241D; Pharmingen) was added, at a dilution of 1:100, for 5 min at room temperature. Subsequently, an antibody directed against the respective cell surface antigen, or an isotype-matched control antibody was added at a dilution of 1:100. After incubation for 45 min at 4°C, in the dark, cells were washed and were resuspended in PBS containing 1% FCS and 1 μg/mL propidium iodide. Fluorescence-activated cell-sorting analysis was done with a FACScalibur machine (Becton Dickinson) equipped with the Cell Quest software (Becton Dickinson), by use of standard procedures.

Fluorescent-activated cell sorting. Cells were harvested 12 h after infection, by centrifugation, and were resuspended in PBS containing 3% FCS, 2 μg/mL propidium iodide, and 25 μg/mL DNase (Roche). The cell suspension was filtered through a nylon mesh (to remove clumps of cells), and living GFP-positive cells were sorted with a MoFlow machine (Cytomation), by use of standard procedures. Uninfected cells were used to set gates for GFP-negative cells.

Immunofluorescence. Cytospin (Shandon) preparations of DCs were prepared, were air dried, and were fixed in methanol for 7 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100, in PBS, for 10 min at room temperature, and nonspecific binding was blocked with 10% FCS, in PBS, for 30 min at room temperature, which was followed by incubation with the primary antibody for 1 h at 37°C. Primary monoclonal antibodies against the MCMV proteins ie1 (Croma101), e1 (20/238/28) [34], and gB (Croma7) were used, nondiluted and supplemented with 3% FCS. After 3 washes in PBS, the cells were incubated with the secondary antibody for 1 h at 37°C. A Cy3-conjugated goat anti-mouse IgG antibody (Becton Dickinson) was used, at a dilution of 1:100, in 3% FCS, in PBS. Subsequently, the cells were washed 3 times in PBS and were mounted in fluorescent mounting medium (DAKO). Microphotographic slides were taken with an Axiophot microscope (Zeiss); identical exposure times were

used for all immunofluorescent pictures. Slides were scanned with a Sprint Scan 35 slide scanner (Minolta), with identical settings for all slides, and slides were compiled to 1 figure with use of Powerpoint software (Microsoft).

Mixed lymphocyte reaction. Mature DCs were infected with MCMV at an MOI of 10, were mock infected, or were treated with UV-inactivated MCMV. Twenty-four or 48 h after infection, the different DC samples were irradiated with 20 Gy and were seeded, in triplicates, into 96-well flat-bottom plates (Greiner), in serially diluted concentrations. Autologous (from $B_6D_2F_1$ mice) T cells or allogeneic (from BALB/c mice) T cells, from thymus or spleen, were added at 2×10^5 cells/well. As a positive control for T cell proliferation, triplicates of T cells alone were incubated with 4 μg/well phytohemagglutinin-M (Sigma) and 40 U/well human recombinant IL-2 (Roche). Triplicates with T cells or with DCs alone were included as negative controls. On day 5, cells were pulsed with 1 μCi/well tritiated thymidine (Amersham) for 18 h and were harvested onto UniFilterPlate-96 (Canberra Packard). Tritiated thymidine incorporation was measured with a Top-Counter (Packard). Data are expressed as mean counts per minute of triplicate wells.

RESULTS

MCMV infects immature and mature DCs. To determine at which stage DCs are targeted by MCMV, we analyzed the susceptibility of DCs to MCMV infection, at different maturation stages. Immature DCs were generated from undifferentiated FDCP-mix cells, in vitro, by culturing the cells in GM-CSF and IL-4, for 12 days, as previously described [25]. For further maturation, LPS was added on day 12. The generated cells showed the characteristic features of immature and mature DCs (figure 1), efficiently presented foreign antigen to autologous T cells, and induced proliferation of T cells (data not shown). Immature DCs were large nonadherent cells with veiled processes, and they displayed a $CD34^-$, $CD11c^+$, $CD14^-$, $CD80^+$, $CD86^{low/-}$, $CD40^{low/-}$, or MHC class $II^{low/-}$phenotype, as assessed by flow cytometry (figure 1*A*). On further maturation, the DCs became adherent and acquired high levels of CD86, CD40, and MHC class II expression, in addition to CD80 and CD11c expression (figure 1*B*).

To monitor the MCMV infection in DCs, immature or mature DCs were inoculated at different MOIs, with an MCMV recombinant strain that expresses GFP under control of the HCMV immediate-early promoter (GFP-MCMV), and were analyzed for GFP expression 8 h after infection. Depending on the MOI, up to 60% of the $CD80^+$ DCs were infected with MCMV (figure 2*A*). Infection rates were similar for immature and mature DCs (figure 2*B*).

MCMV replication is tightly regulated in a multistep process that is subdividable into the immediate-early, early, and late

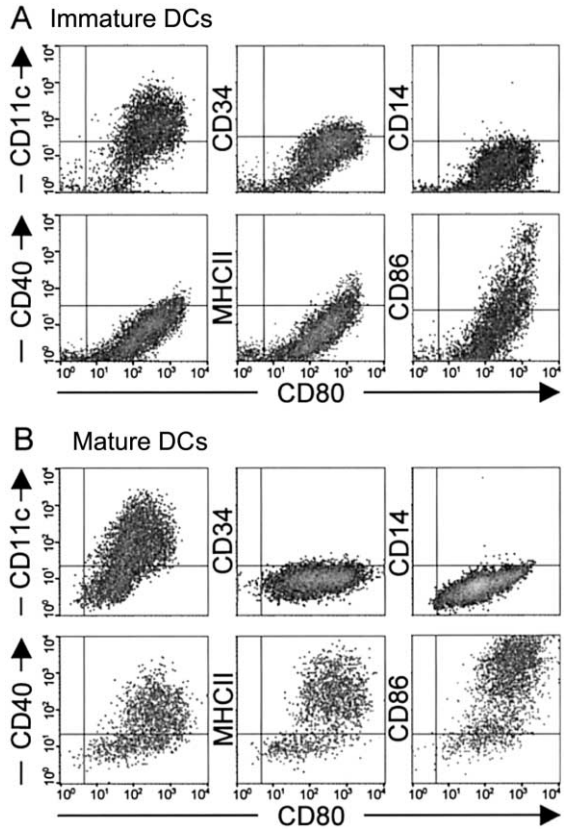

Figure 1. Characterization of immature *(A)* and mature *(B)* factor dependent cell progenitor–mix-derived dendritic cells (DCs). Immature DCs were analyzed 12 days after induction of differentiation and mature DCs were analyzed 14 days after induction of differentiation, for expression of DC markers CD11c, CD40, CD80, CD86, and major histocompatibility complex class II (MHC II), and for absence of stem cell marker CD34 and of macrophage marker CD14. Data shown are representative for immature and mature DCs used throughout this study.

phases of viral gene expression [35]. We used a panel of anti-MCMV antibodies to assess, by immunofluorescence staining, the expression of representative viral proteins from each kinetic class. Immature and mature DCs were inoculated with GFP-MCMV at an MOI of 10, and were analyzed for expression of immediate-early, early, and late viral proteins, at selected times after infection (figure 3). In MCMV-infected cultures of immature DCs, immediate-early, early, and late gene products were readily seen, whereas, in MCMV-infected cultures of mature DCs, only immediate-early and early proteins were detected (figure 3). These data show that both immature DCs and mature DCs are permissive to MCMV infection and that immature DCs, but not mature DCs, are likely to support the full viral replicative cycle.

MCMV infection is productive in immature DCs but not in mature DCs. To determine whether infected DCs produce infectious virus particles, we infected immature and mature DCs with MCMV at an MOI of 10, and we analyzed the pres-

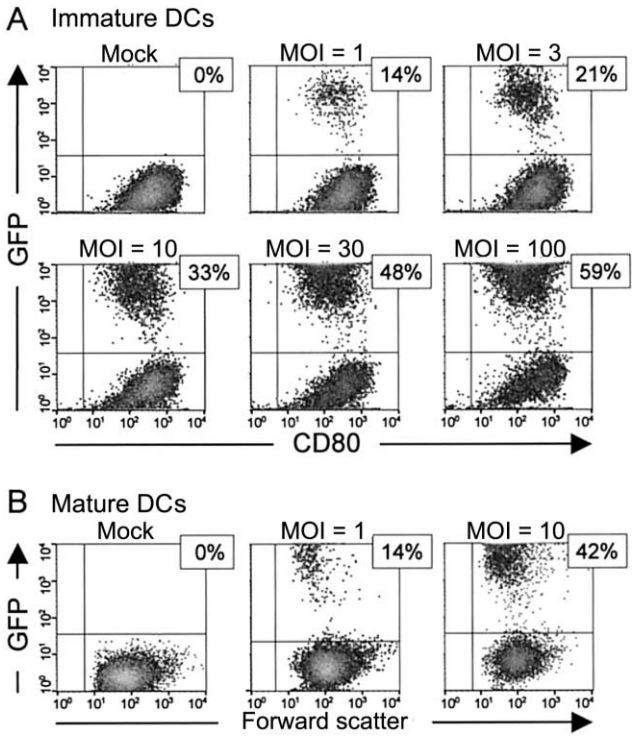

Figure 2. Mouse cytomegalovirus (MCMV) infection of immature *(A)* and mature *(B)* dendritic cells (DCs). Immature and mature DCs were either mock-infected or were inoculated with recombinant MCMV expressing green fluorescent protein (GFP) at indicated MOI. Living cells were analyzed by fluorescence-activated cell-sorting analysis for intracellular GFP expression and for CD80 surface expression, 8 h after infection. Values shown for 1 representative experiment indicate portion (%) of GFP-expressing cells. Experiment was repeated 2 times with virtually identical results. Mock, mock-infected.

ence of infectious MCMV in the supernatants, up to 5 days after infection, with use of MCMV-permissive fibroblasts as indicator cells. Supernatants from infected DCs, collected 2 h after infection, were included as a control to verify that virus particles were not derived from the inoculate initially used to infect the DCs. As a positive control, supernatants of infected FDCP-mix–derived macrophages were used. MCMV-infected immature DCs produced high virus titers, a production that is similar to that of MCMV-infected macrophages, whereas no infectious virus could be detected in supernatants of MCMV-infected mature DCs (figure 4). These data indicate that MCMV productively replicates at high levels in immature DCs but not in mature DCs.

MCMV alters the cell surface expression of functionally important molecules on immature and mature DCs. DCs play an essential role in the initiation of the adaptive immune response against viruses [17]. For priming of naive T cells, efficient expression of MHC and costimulatory molecules on the cell surface of DCs is required. Although T cells specific for CMV antigens are generated in high frequencies in healthy persons, several recent studies suggest that CMV interferes with DC function by down-regulation of expression of some (MHC only) or all (MHC and costimulatory molecules) cell surface immune molecules [20–22].

To analyze in more detail how MCMV infection of DCs affects the expression of these cell surface immune molecules, over time, after infection, MCMV-infected immature and mature DCs were assessed by flow cytometry for the expression of cell surface proteins CD80, CD86, CD40, MHC class I, and MHC class II, at 4, 8, 24, and 48 h after infection. As a control, expression of the myeloid marker Mac1 was also tested. Whereas Mac1 remained unchanged by the infection of MCMV (figure 5), several alterations in the immune phenotype of immature and mature DCs were observed at different times after infection.

During the early phase of viral gene expression (between 4 h and 24 h after infection), immature DCs infected with MCMV, as well as mature DCs infected with MCMV, (as determined by expression of GFP) showed an increased expression of cell surface immune molecules, compared with mock-infected DCs, that is, DCs that were never exposed to virus (figure 5*A* and 5*B*, upper 2 rows, and data not shown). Uninfected (GFP-negative) cells of MCMV-infected cultures also slightly up-regulated expression of CD86, CD40, and MHC class I and II molecules early after infection, albeit not to the extent of infected DCs. CD80 expression, which is already very high in immature and mature DCs, was maintained at a high level in infected (GFP-positive) and uninfected (GFP-negative) DCs of infected cultures, a level similar to that of DCs of mock-infected control cultures. These data indicate that MCMV infection of DCs results in activation of DCs during the early phase after infection.

At 48 h after infection, however, expression of CD80, CD86, CD40, and MHC class I and II molecules was considerably down-regulated, in mature MCMV-infected (GFP-positive) cells, but not in mock-infected control DCs, and only slightly in uninfected (GFP-negative) DCs, of MCMV-infected cultures (figure 5*B*, lower row). Down-regulation of the costimulatory molecule CD80 was most rapid and pronounced in infected (GFP-positive) DCs, and it resulted in complete absence of cell surface expression 48 h after infection. In infected immature DC cultures, expression of CD80 was considerably down-regulated in infected (GFP-positive) DCs 48 h after infection, and expression of CD86, CD40, and MHC class I and MHC class II molecules was reduced to levels of mock-infected immature control DCs (figure 5*A*, lower row). Taken together, these data indicate that cell surface proteins involved in antigen presentation to naive T cells are specifically down-regulated in MCMV-infected DCs after the initial activation.

MCMV-infected DCs efficiently prime autologous naive T cells against MCMV, early after infection. Next we examined

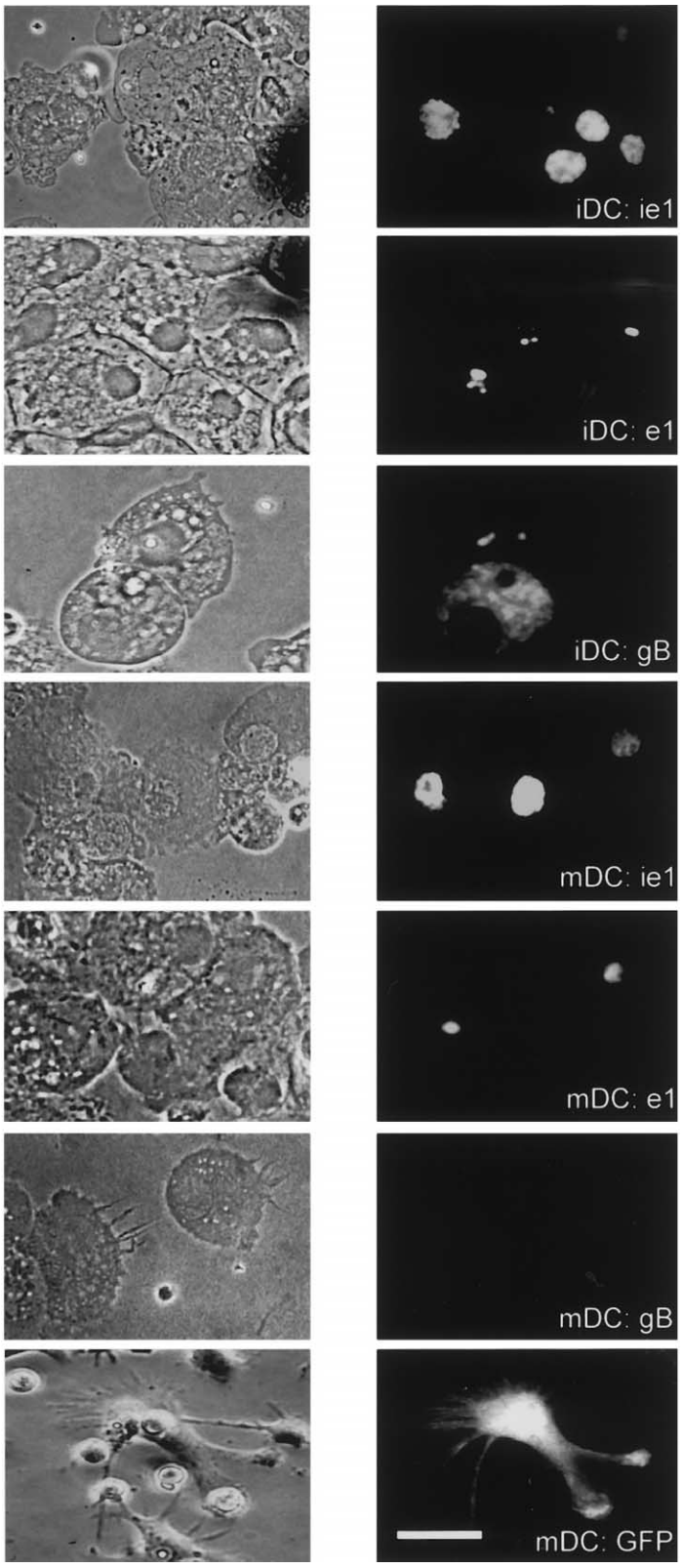

Figure 3. Expression of viral antigens in mouse cytomegalovirus (MCMV)–infected dendritic cells (DCs). Immature DCs (iDC) and mature DCs (mDC) were infected with recombinant MCMV expressing green fluorescent protein (GFP) and were analyzed for viral gene expression, 8 h after infection (immediate-early proteins), 24 h after infection (early proteins), and 48 h after infection (late proteins). Expression of immediate-early proteins was detected by either fluorescence microscopy, for GFP, or immunofluorescence staining with monoclonal antibody directed against MCMV immediate-early protein ie1. For detection of early and late proteins, monoclonal antibodies directed against the MCMV early and late proteins e1 and gB, respectively, were used. Shown is fluorescence microscopy *(right)* and corresponding phase-contrast microscopy *(left)* of representative experiment. Experiment was repeated 3 times with virtually identical results. Bar = 16 μm.

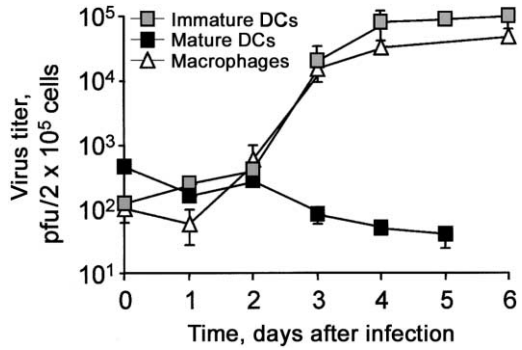

Figure 4. Growth kinetics of mouse cytomegalovirus (MCMV) in immature and mature dendritic cells (DCs). Immature and mature DCs and factor dependent cell Paterson mixed potential–derived macrophages were infected with recombinant MCMV expressing green fluorescent protein (GFP), at day 12 (immature DCs and macrophages) or day 14 (mature DCs) after induction of differentiation. At indicated times after infection, supernatants of cultures were collected, and titers were determined by plaque assay on mouse embryonic fibroblasts. Mean values ± SE from triplicate cultures of 1 representative experiment are shown. Experiment was repeated 3 times with virtually identical results. pfu, plaque-forming units.

the functional consequences of the phenotypic changes that occurred early after MCMV infection of mature DCs. To test whether the initial activation of DCs by MCMV infection leads to the stimulation of T cell responses, we used autologous mixed lymphocyte reaction. T cell proliferation was strongly induced if autologous thymocytes or splenocytes were added to the infected mature DCs at an early time after infection, when the infected DCs had up-regulated MHC class I and II as well as the costimulatory molecules CD80, CD86, and CD40 (figure 6*A*, and data not shown). Intracellular signaling is also initiated by contact of cells with CMV virions without an active infection [36, 37]. To test whether virion that is binding to its cellular receptor on DCs is responsible for the T cell–activating capacity of infected DCs, we compared DCs treated with UV-inactivated MCMV with DCs infected with MCMV, in mixed lymphocyte reaction. In contrast to infected DC cultures that strongly stimulated autologous T cell proliferation, the treatment of DCs with UV-inactivated MCMV resulted in only a lesser activation of T cells, which was similar to treatment of DCs with a foreign antigen (figure 6*A*). This result suggests that the efficient T cell stimulation by infected DCs requires viral gene expression. To further confirm that the infected cells are critical for the increased T cell proliferation, infected (GFP-positive) DCs and uninfected (GFP-negative) DCs were sorted from infected cultures early after infection and were tested for their T cell–stimulatory capacity, in autologous mixed lymphocyte reaction. As shown in figure 7, stimulation of thymocyte proliferation by MCMV-infected (GFP-positive) DCs was considerably higher than that by uninfected (GFP-negative) cells. Taken together, our data indicate that, in the infected cultures, naive T cells were efficiently primed by the up-regulated MHC class I and II molecules presenting viral antigens and by efficient costimulation in infected DCs. Thus, MCVM-infected DCs are potent and specific activators of naive T cells, early after infection.

MCMV interferes with T cell priming by DCs, by means of MHC-dependent and -independent mechanisms. Given the down-regulation of MHC and costimulatory molecules, on mature MCMV-infected DCs, at later stages of infection, we asked whether this would lead to an interference with the capacity to activate T cells in autologous and allogeneic mixed lymphocyte reaction. Mock-infected DCs pulsed with keyhole limpet hemocyanin, and DCs treated with UV-inactivated MCMV efficiently presented the foreign antigen to autologous and allogeneic T cells and stimulated T cell proliferation (figure 6*B* and 6*D*). DCs treated with UV-inactivated MCMV induced levels of T cell proliferation similar to, and in some experiments even higher than, those induced by DCs pulsed with keyhole limpet hemocyanin, a finding that indicates that MCMV structural proteins are highly immunogenic. As expected from the diminished expression of MHC and costimulatory molecules, the proliferative T cell response was strongly reduced in response to stimulation with MCMV-infected DCs, 48 h after infection, in both autologous and allogeneic mixed lymphocyte reactions (figure 6*B* and 6*D*). This indicates that the down-regulation of both MHC and costimulatory molecules by MCMV blocks the T cell–stimulatory capacity of the infected DCs and thereby interferes with an antiviral immune response.

In contrast to our data from the autologous situation, MCMV-infected DCs were unable to stimulate proliferation of allogeneic T cells at early times after infection (figure 6*C*), although they had up-regulated MHC and costimulatory molecules on the cell surface at this time (figure 5*B*, upper row). These data suggest that MCMV affects proliferation of allogeneic T cells by an additional mechanism that seems to be independent of the MHC–T cell receptor interaction. Obviously, this mechanism is operative in the allogeneic mixed lymphocyte reaction, but it is not required in the autologous setting.

DISCUSSION

DCs play a central role in induction of protective immunity to viral infections. However, they can also be exploited by viruses to evade the host immune response and to induce immune suppression. A tightly controlled balance between these processes must exist to prevent the elimination of the host and, consequently, the species-specific virus. Here, we have provided a detailed study of MCMV infection of DCs that includes analysis of susceptibility to virus and of the effects of CMV

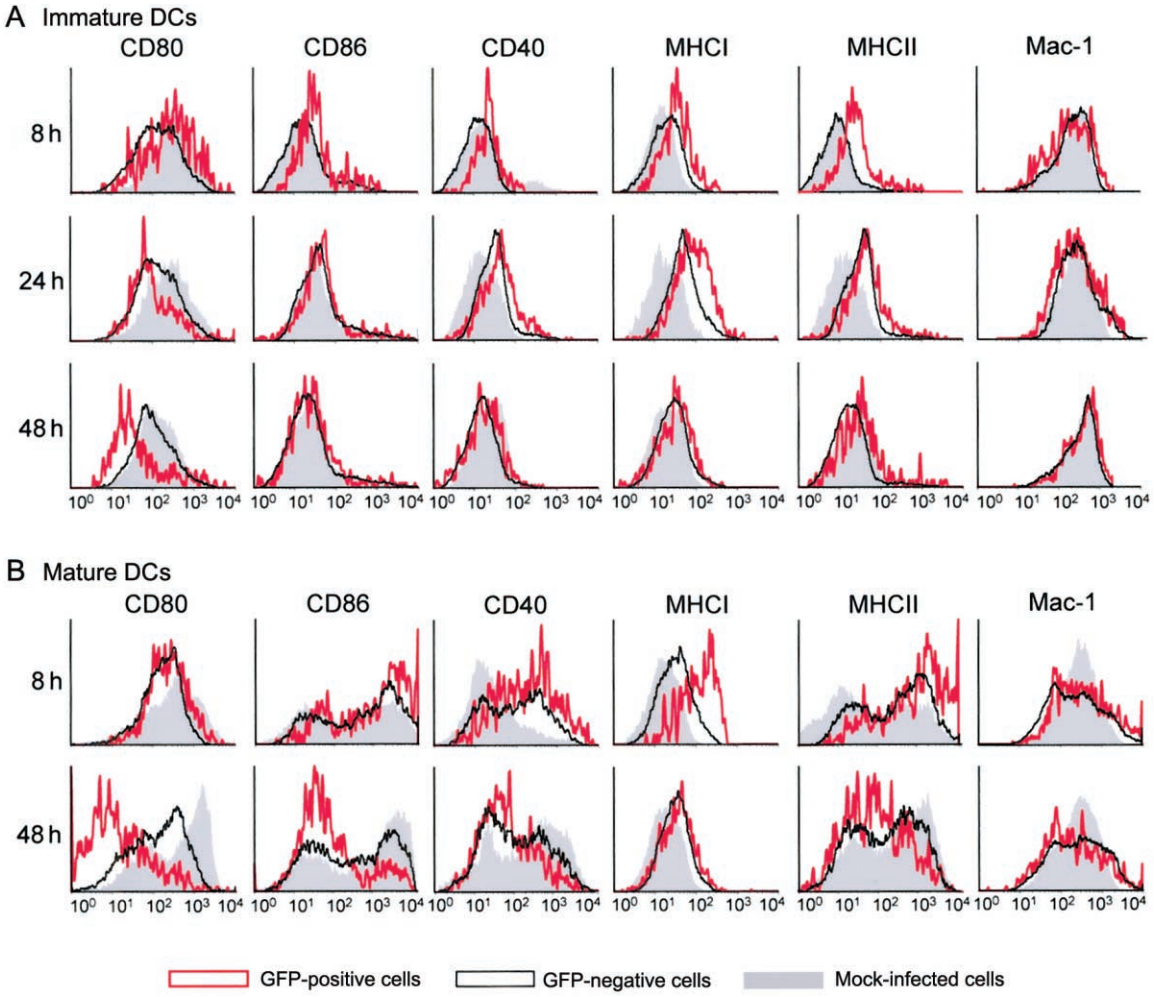

Figure 5. Phenotypic changes of immature *(A)* and mature *(B)* dendritic cells (DCs) after mouse cytomegalovirus (MCMV) infection. Immature and mature DCs were analyzed by flow cytometry 8, 24, and 48 h after infection, with recombinant MCMV expressing green fluorescent protein (GFP). Mock-infected cultures were used as controls. Surface expression of indicated markers on MCMV-infected (GFP-positive) DCs is represented by red lines, on uninfected (GFP-negative) DCs of infected cultures by black lines, and on mock-infected (never exposed to virus) control DCs by shaded areas. Results shown are representative for 3 independent differentiation and infection experiments. MHC, major histocompatibility complex.

infection on the immune function of DCs. Both immature DCs and mature DCs are targets for MCMV infection. MCMV replicates in immature DCs but not in mature DCs. Although MCMV-infected mature DCs efficiently prime naive T cells early after infection, MCMV infection of DCs subsequently prevents T cell activation, by down-regulation of MHC and costimulatory molecules. Our findings not only confirm that DCs are permissive for MCMV but further document that DCs have a dual role during MCMV infections: to initiate a specific immune response and to cause immune suppression. This process is governed by 2 principles: the kinetics of viral gene expression and the kinetics of DC maturation.

In line with previous reports [20–22, 38], we show that both immature and mature DCs can be infected, with a similar efficiency, by CMV. Whereas mature MCMV-infected DCs remain viable and do not release infectious virions, MCMV-infected immature DCs show a difference in reactions that is characterized by the production of viral progeny and by the transient up-regulation of MHC class I and II, CD80, CD86, and CD40 cell surface molecules, the latter indicating the activation of DCs. Because immature DCs reside in the periphery and migrate to lymphoid organs on activation, infected immature DCs may serve as vehicles for transport and spread of virus and thus may contribute to viral pathogenesis. A mononuclear phagocytic cell type that may represent a DC precursor was indeed described as being important for the dissemination of MCMV infection [39]. Similarly, viral dissemination by immature DCs plays a role for other viruses, such as human immunodeficiency virus (HIV) [40] and herpes simplex virus type 1 [41]. Interestingly, although MCMV can infect mature DCs to the same extent as it can infect immature DCs, the infection is highly restricted and nonproductive in mature DCs.

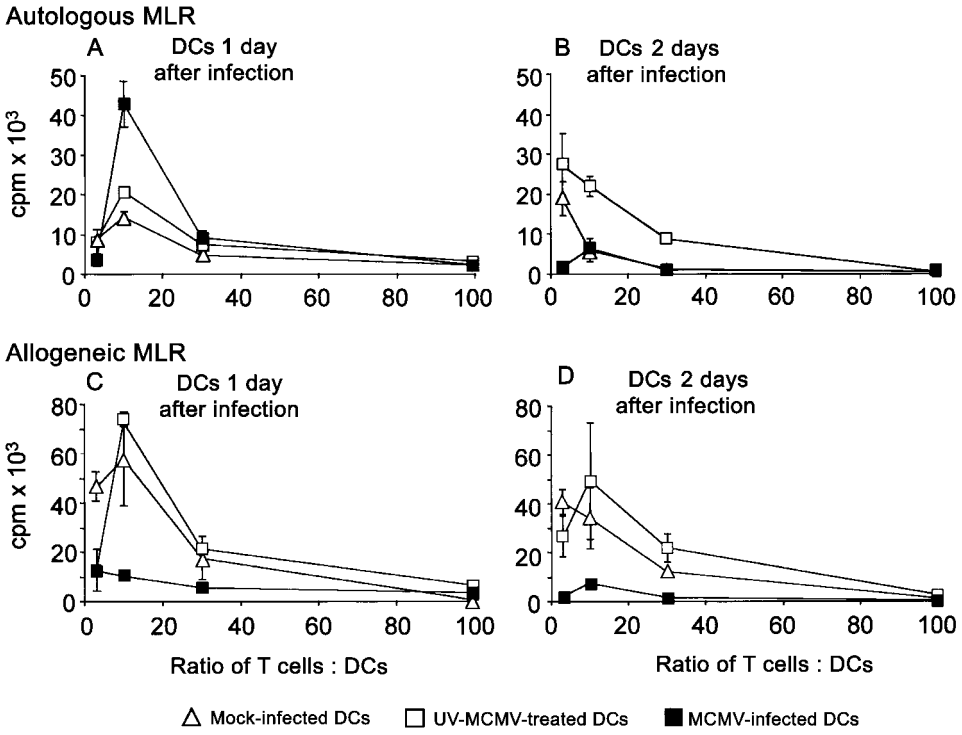

Figure 6. T cell–stimulatory capacity of mouse cytomegalovirus (MCMV)–infected dendritic cells (DCs) and of DCs treated with UV-inactivated MCMV in autologous or allogeneic mixed lymphocyte reaction (MLR). Mature DCs were infected with recombinant MCMV expressing green fluorescent protein (GFP), were mock-infected, or were treated with UV-inactivated MCMV. Twenty-four h after infection *(A* and *C)* or 48 h after infection *(B* and *D),* autologous *(A* and *B)* or allogeneic *(C* and *D)* T cells were added to DCs to start mixed lymphocyte reaction. Mean values ± SE from triplicate cultures of representative experiment are shown. In absence of an additional foreign antigen or when tetanus toxoid, instead of keyhole limpet hemocyanin, was used as foreign antigen, T cell proliferation was also strongly induced by MCMV-infected DCs, early after infection, and was impaired at later stages of infection (data not shown). Experiment was repeated 4 times with virtually identical results.

Our data seemingly differ from another study of MCMV that reported that replication of MCMV occurs also in mature DCs, albeit diminished and delayed [20]. Because some of the LPS-treated D1 DCs used by Andrews et al. [20] retained an immature DC phenotype, it cannot be excluded that virus was produced from remaining undifferentiated D1 DCs. Nonlytic infection was observed for immature and mature monocyte-derived HCMV-infected DCs [22]. At present, it is not clear whether HCMV differs from MCMV with respect to productive infection or whether the lytic versus nonlytic infection depends on the DC type analyzed in the different studies. Further work is needed to address these questions. Resistance of mature DCs to productive infection also has been shown for other viruses [42–44], but the exact mechanism for this phenomenon remains to be elucidated.

To further understand the relevance, regarding DC function, of MCMV infection of mature DCs, we examined the regulation of cell surface proteins involved in the induction of immune responses. During the first hours after infection, expression of the key functional molecules CD80, CD86, CD40, and MHC class I and II was maintained at a high level or was even further up-regulated, but, subsequently, expression of all of these surface markers decreased considerably in infected DCs. Expression of Mac1 antigen was not altered, thus ruling out global effects on the expression of surface molecules. The effects of MCMV infection on the DC phenotype observed by us and, previously, by Andrews et al. [20] suggest that the infected DCs would efficiently activate T cells initially but would then lose this ability. This was indeed the case when we tested DCs in autologous mixed lymphocyte reaction.

Here, we have shown that, during early stages of infection, MCMV-infected DCs can efficiently prime autologous naive T cells. Although CMV has evolved several strategies to prevent its elimination, the acute CMV infection is well controlled by the immune system in an immunocompetent host. A major effector mechanism to contain the CMV infection is the $CD8^+$ T cell response [45, 46]. DCs belong to professional antigen-presenting cells, which are indispensable for the induction of antiviral cytotoxic T cells [17]. DCs are involved, in several ways, to expand $CD8^+$ cells directed against CMV-infected cells. Besides antigen uptake and presentation to $CD4^+$ helper and $CD8^+$ cytotoxic T cells, DCs can also directly stimulate naive and memory $CD8^+$ killer cells, after infection [47, 48]. Both cross-priming (presenting antigens from other infected cells)

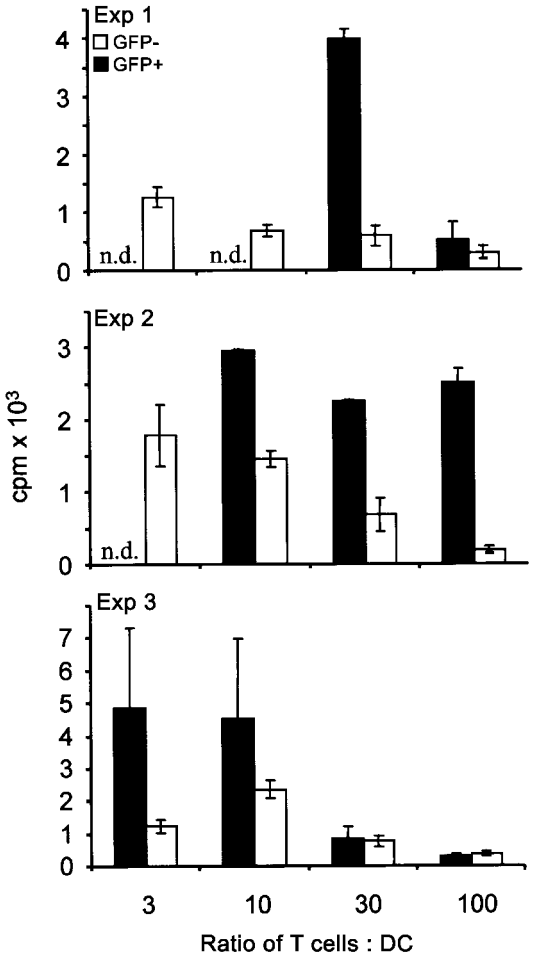

Figure 7. T cell–stimulatory capacity of sorted mouse cytomegalovirus (MCMV)–infected dendritic cells (DCs), in autologous mixed lymphocyte reaction. Mature DCs were infected with recombinant MCMV expressing green fluorescent protein (GFP) and were sorted for MCMV-infected (GFP+) and uninfected (GFP−) cells, 12 h after infection. Subsequently, autologous thymocytes were added to sorted DCs to start mixed lymphocyte reaction. In absence of additional foreign antigen, T cell proliferation was also induced by MCMV-infected (GFP-positive) DCs, but not by uninfected (GFP-negative) DCs (data not shown). Mean values ± SE from triplicate cultures of 3 independent experiments (Exp) are shown.

and direct priming (presenting endogenous antigens) were shown to contribute significantly to the generation of specific $CD8^+$ T cells, in vaccinia virus infection [49]. Thus, in addition to cross-presentation of CMV antigens by uninfected DCs [50, 51], CMV-infected DCs provide a fast and efficient way for the host to prime anti-CMV $CD8^+$ T cells, which is required to control CMV infection.

Remarkably, however, infected DCs were incapable of stimulating T cell proliferation in allogeneic mixed lymphocyte reaction, even at 24 h after infection, when autologous T cells were highly activated by infected DCs. Whereas autologous mixed lymphocyte reaction is a model to study priming of naive T cells to a foreign antigen, the allogeneic mixed lymphocyte reaction is a model to study alloresponsiveness to histocompatibility antigens as it occurs in transplantation. In both settings, the interaction with the MHC and costimulatory molecules is considered to stimulate T cells to proliferate. Recent data, however, indicate that DCs may stimulate allogeneic mixed lymphocyte reactions by transfer of MHC class II molecules to DCs of the responder type, with subsequent stimulation of syngeneic T cells [52]. Because we cannot exclude the presence of syngeneic thymic or splenic DCs in our T cell preparations, it is possible that, as suggested for HIV [53], CMV renders the DCs incapable of transferring antigenic signals to other DCs. Alternatively, the interference of CMV with the ability of DCs to release IL-2 [20] and IL-12 [20, 21] may result in the complete impairment of the capacity of DCs to activate allogeneic T cells, despite the presence of MHC class I, MHC class II, and costimulatory cell surface molecules, as reported by us and by others [20]. Mature DCs express cytokines, such as IL-2 and IL-12 [54], which are released early in allogeneic reaction but not in autologous mixed lymphocyte reaction [55]. Thus, secretion of IL-2 or IL-12 by DCs may be crucial for the initial activation of allogeneic T cells. On the basis of our data presented herein, we conclude that MCMV-induced effects on the immune function of DCs differ in the autologous and allogeneic situations. In the autologous situation, the immune function of DCs after MCMV infection strictly follows the expression pattern of MHC and costimulatory molecules, which results in initial activation of T cells and subsequent T cell suppression. In the allogeneic situation, however, CMV infection leads to a complete absence of T cell activation, which involves 2 different mechanisms, 1 independent of antigen presentation by the MHC of infected DCs and the other responsible for down-regulation of MHC and costimulatory molecules. Thus, the artificial allogeneic situation strongly pushes the balance towards unresponsiveness to viral infection. Histoincompatibility between donor and recipient is associated with a considerably decreased anti-CMV immune reconstitution [56] and is a known risk of CMV disease after bone marrow transplantation [1, 57]. The selective inability of CMV-infected DCs to prime naive T cells in that setting may contribute to reduced antiviral control and, consequently, to CMV disease after allogeneic bone marrow transplantation.

CMV deploys a variety of immune evasion mechanisms to persist and to replicate, even in a fully primed host. Most of the mechanisms analyzed thus far reduce the number of target structures for cytotoxic T cells, by down-regulation of MHC class I molecules [58]. However, viruses can also interfere with the host immune defense by attacking and altering the function of those cells that play a critical role in the adaptive immune response. On the basis of previous studies [20–22] and our findings here, we propose that infected mature DCs contribute, in several ways, to the evasion of T cell attack and to immune

suppression. The down-regulation of MHC class I and II molecules, together with costimulatory molecules on CMV-infected DCs, counteracts the initial activation of naive T cells and renders the infected DCs unable to induce a further T cell response, such as the priming of naive $CD4^+$helper cells and naive and memory $CD8^+$ cytotoxic cells. Because down-regulation occurred primarily in infected DCs and because functional paralysis was induced only by the infected DCs of infected cultures, the effects of CMV on cell surface expression of MHC and costimulatory molecules are most likely directly triggered by viral proteins inside the infected cells. However, uninfected DCs of infected cultures show reduced expression of MHC and costimulatory molecules late in infection, albeit to a much lesser extent. Similarly, uninfected DCs of infected cultures slightly up-regulated MHC and costimulatory molecules early after infection. This suggests that the effects of CMV on MHC and costimulatory-molecule expression may be mediated in part by soluble factors. Recent reports that demonstrate MCMV-induced down-regulation of MHC class II molecules on macrophages, by IL-10 [13] and HCMV-induced modulation of CD83 expression by transforming growth factor-β [59], support this notion. Thus, cytokine-mediated mechanisms for MHC and costimulatory molecule modulation, in uninfected DCs, may help the host to control CMV infection and may help CMV to further interfere with generation of $CD4^+$helper T lymphocytes and, thus, to escape $CD4^+$ T lymphocyte immunosurveillance.

Binding of the HCMV virion to its cellular receptor, via the envelope glycoprotein B, induces alteration in the expression of a series of cellular genes, including the interferon-inducible genes, early during HCMV infection [36, 37]. We have shown here that treatment of DCs with UV-inactivated MCMV results in activation of T cells, similar to that induced by treatment of DCs with the foreign antigen keyhole limpet hemocyanin, but does not result in the altered T cell responses caused by replication-competent CMV. These results suggest that the effects of CMV-infected DCs on T cell stimulation require viral gene expression and that these effects were not induced by the virus-receptor interaction.

How MCMV modulates expression of MHC and costimulatory molecules on DCs requires further investigation. Identification of the MCMV genes that mediate this control will be a critical step in the resolution of the regulation of antigen presentation. An understanding of the mechanisms may help in the design of strategies to prevent the immunosuppressive and immunopathologic conditions associated with CMV infection.

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