

Dendritic Cell Programming by Cytomegalovirus Stunts Naive T Cell Responses via the PD-L1/PD-1 Pathway¹

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Early during infection, CMV targets dendritic cells (DC) and alters their functions. Herein we show that CMV-infected DC maintain the ability to present both virus-derived and exogenous Ags, but that they actively induce tolerance or anergy in Ag-specific T cells. CMV accomplishes this by selectively maintaining high-level expression of the negative costimulatory molecule programmed death ligand-1 (PD-L1), while commensurately down-regulating positive costimulatory molecules and MHC on the DC surface. Consequently, CD4 and CD8 T cells activated by these infected DC have a stunted phenotype, characterized by poor proliferation, effector function, and recall responses. Blocking PD-L1, but not PD-L2, during direct priming of naive T cells by infected DC significantly restores Ag-specific T cell functions. Using systems where direct and cross-priming of T cells can be distinguished revealed that PD-L1/PD-1 signaling contributes only when naive T cells are primed directly by infected DC, and not upon cross-presentation of viral Ags by uninfected DC. These data suggest that murine CMV programs infected DC during acute infection to inhibit early host adaptive antiviral responses by tipping the balance between negative and positive cosignals. *The Journal of Immunology*, 2008, 180: 4836–4847.

Dendritic cells (DC)³ play a central role in the initiation and regulation of both innate and adaptive immune responses to viral pathogens. DC not only activate naive CD4 and CD8 T cells, but they also dictate the acquisition of T cell effector functions and confer the capacity for T cell survival, homeostasis, and memory formation (reviewed in Refs. 1–3). DC function closely correlates with their maturation state. Immature DCs display strong phagocytic capacity but poor T cell-activating capacity due to their intermediate surface expression of MHC class II (and I) and low levels of costimulatory molecules. It is generally thought that T cell activation by immature DC leads to peripheral tolerance through the induction of T cell anergy, T cell depletion, or generation of regulatory cells. In contrast, mature DC display diminished phagocytic capacity but are proficient in T cell activation through the up-regulation of MHC classes I and II, costimulatory molecules, and cytokine/chemokine production (1–4).

Perhaps not surprisingly given their central role in promoting antiviral immune responses, many viruses have evolved strategies to specifically modulate DC phenotype and/or function. Murine cytomegalovirus (MCMV, a β -herpesvirus) directly infects DC in vivo (5, 6) and encodes several gene products that specifically interfere with various aspects of DC function. Infection of DC by

MCMV leads to the induction of a so-called “paralyzed” phenotype, characterized by the down-regulation of MHC classes I and II (7), costimulatory molecules, and proinflammatory cytokines (8). As a consequence, these infected DC are unable to promote MLR or activate T cells (8, 9). Similar negative effects on T cell activation have been reported for human CMV (HCMV) infection of DC or monocytes (10–15). Inhibition of T cell activation by CMV-infected DC may be due both to inhibition of positive signals (i.e., MHC and costimulation) and to the active targeting of negative cosignaling pathways, which has not yet been examined. Support for this idea does exist in the primate CMV, which encode a homolog of herpesvirus entry mediator, UL144 (16), a ligand for the negative cosignaling receptor B and T lymphocyte attenuator (17), and UL144 is a potent inhibitor of CD4 T cell proliferation (18).

The programmed death ligand (PD-L1/PD-1) pathway has recently been implicated in viral suppression of adaptive T cell responses (19, 20). PD-1 is inducibly expressed upon activation of T, B, and NK T cells and confers a negative signal when engaged simultaneously with the TCR or BCR. PD-L1, one of the two known ligands for PD-1, is constitutively expressed on a variety of hematopoietic and nonhematopoietic cells and can be up-regulated upon activation (19–21). Blockade of the PD-L1/PD-1 pathway has been shown to improve T cell function and proliferation in HIV patients and in mice chronically infected with lymphocytic choriomeningitis virus (22–25). Additionally, respiratory syncytial virus and human rhinovirus infection increase PD-L1 expression on DC, resulting in decreased antiviral T cell responses (26, 27).

Because CMV infection is associated with immunosuppression and paralyzed DC, we have investigated the biological consequence when a naive T cell encounters a MCMV-infected DC, and the relative contribution of the PD-L1/PD-1 pathway in its fate. Our studies reveal a key role for PD-L1/PD-1 interactions in the stunting of naive T cells that directly encounter infected DC both in culture and in vivo, whereas there is a negligible contribution for the PD-L1/PD-1 pathway when MCMV Ag is cross-presented by uninfected APC. This work elucidates a novel molecular mechanism used by MCMV to “program” infected APC and shape the adaptive immune response by shifting the balance of positive and negative cosignals.

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Received for publication July 23, 2007. Accepted for publication January 22, 2008.

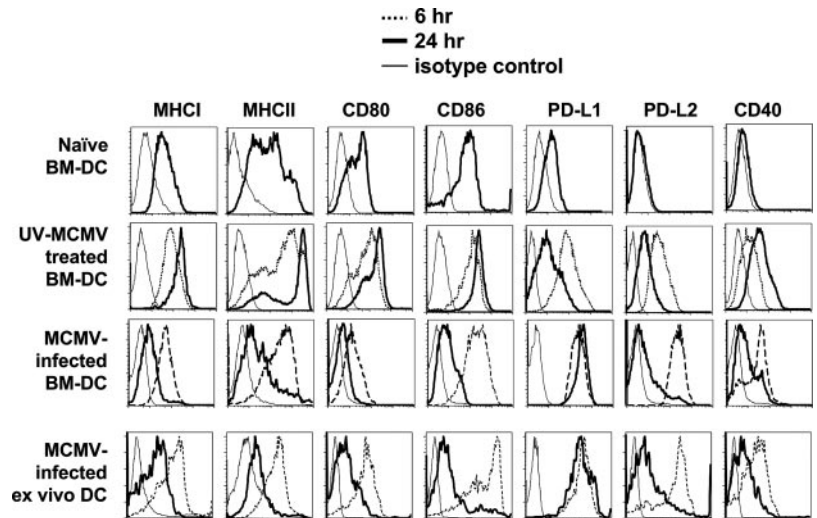
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¹ This work was supported in part by National Institutes of Health Grants R01 AI 34495, CA72669, and P01 AI056299 (to B.R.B.), American Heart Association Grant 0330064N (to C.A.B.), and Leukemia and Lymphoma Society 3248-05 (to E.M.J.).

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³ Abbreviations used in this paper: DC, dendritic cell; HCMV, human CMV; mDC, dendritic cells derived from bone marrow; MCMV, murine CMV; PD-L, programmed death ligand; PI, propidium iodide; 7-AAD, 7-aminoactinomycin D.

FIGURE 1. MCMV selectively down-regulates MHC and costimulatory molecules on DC. DC were isolated from spleens of B6 wild-type mice (ex vivo) or were generated from bone marrow (BM). CD11c⁺ DC were left untreated (naive), exposed to UV-MCMV, or infected with MCMV-GFP (multiplicity of infection of 1). After 6 (dotted lines) and 24 h (bold lines) the expression of MHC classes I and II, costimulatory molecules CD40, CD80, and CD86, and PD-L1 and PD-L2 was determined in the total DC population (naive and UV-treated MCMV-exposed DC) or within the infected (GFP⁺) DC. Data are representative of at least five independent experiments.



Materials and Methods

Mice and materials

C57BL/6, B6.C-H2^{bmi1}/ByJ (K^{bmi1}), B6.SJL.Ptpr^a (B6/CD45.1), and B6.SJL/I-A^{b-/-} mice were purchased from The Jackson Laboratory. C57BL/6Ji-K^{bmi1}(K^{b-/-}) were obtained from Taconic. C57BL/6Ji-K^{bmi1}D^{bmi1} (K^{b-/-}D^{b-/-}) were a gift from Dr. H. Cheroutre (La Jolla Institute for Allergy and Immunology, La Jolla, CA (LIAI)). ActmOVA transgenic mice were a gift from Dr. M. Jenkins (University of Minnesota Medical School, Minneapolis, MN). ActmOVA transgenic mice crossed to a K^{bmi1}, K^{b-/-}, and I-A^{b-/-} background and OT-1 K^{bmi1}/CD45.1 were a generous gift from Dr. S. Schoenberger (LIAI), and OT-2/CD90.1 mice were kindly provided by Dr. K. Sugie (LIAI). Mice were maintained by in-house breeding at LIAI, and they were maintained under specific pathogen-free conditions in accordance with guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Purified and fluorescent/biotin-labeled Abs directed against CD3, CD4, CD8a, CD25, CD11c, CD40, CD44, CD45.1, CD45.2, CD54, CD69, CD80, CD86, CD90.1, CD90.2, PD-1 (J43), PD-L1 (MIH-5/MIH-7), PD-L2 (TY25), K^b, D^b, I-A/E, V α 2, IFN- γ , FoxP3, isotype controls, and reagents to study apoptosis (annexin V, 7-aminoactinomycin D (7-AAD), and propidium iodide (PI)) were obtained from eBioscience and BD Pharmingen. PD-L1 blocking Ab (MIH-7) for in vivo studies was a kind gift from Prof. M. Azuma (Tokyo Medical and Dental University, Tokyo, Japan) (28). CD4, CD8, and CD11c and anti-biotin beads were obtained from Miltenyi Biotec. GM-CSF was from Peprotech, and CFSE was from Invitrogen: Molecular Probes. OVA₃₂₃₋₃₃₉ and MCMV-specific peptides identified by Dr. Ann Hill, M33₄₇₋₅₅, M38₃₈₋₄₅, M44₁₃₀₋₁₃₈, M57₈₁₆₋₈₂₄, M86₁₀₆₂₋₁₀₇₂, M139₄₁₉₋₄₂₆, M141₁₅₋₂₃, and IE3₄₁₆₋₄₂₃ were purchased from A&A Labs.

Virus and quantification of MCMV genome copy number

MCMV recombinants expressing GFP were derived from the bacterial artificial chromosome-cloned MCMV-GFP genome pSM3f-GFP (9). Reconstitution of bacterial artificial chromosome-derived virus, virus stock preparation, and quantification of stock PFU were conducted as described previously in NIH 3T3 (29).

MCMV genome copy numbers were determined by quantitative PCR. Briefly, DNA from spleen and liver was isolated using the Qiagen DNeasy tissue isolation kit, using twice the recommended amounts of supplied buffer and proteinase K. Quantitative PCR was conducted with the SYBER Green reagent (Roche) according to the manufacturer's instructions using 500 μ g of DNA template and 0.2 μ M of primer(s). Each sample was analyzed in triplicate (60°C annealing, 45 cycles, Stratagene Mx4000), and the level of MCMV IE1 detected was normalized to the murine L32 gene. Dissociation curves revealed that the designed primers were specific. Primers were as follows: MCMV IE1 (+), 5'-AGC TGT TGG TGG TGT CAC TCA A; MCMV IE1 (-), 5'-GGC TGG GAC TCA TCT TCT TCA G; mL32 (-), 5'-GGA TCT GGC CCT TGA ACC TT; mL32 (+), 5' GAA ACT GGC GGA AAC CCA.

DC isolation, generation, and infection

DC were isolated from spleens of naive C57BL/6 mice as described before (30). Bone marrow cells from indicated strains were isolated and cultured

with GM-CSF (20 ng/ml) as previously described (31). After 7 days, DC were sorted based on their expression of CD11c by MACS MicroBeads according to the manufacturer's instruction (Miltenyi Biotec) and checked for MHC classes I and II and CD80/CD86 expression by flow cytometry. DC were left untreated (control), infected with live MCMV-GFP (multiplicity of infection of 1) or exposed to UV-inactivated MCMV-GFP (10⁶ cells/well in 24-well plates). DC were analyzed at various times after infection for the expression of surface molecules by flow cytometric analysis. For in vitro and in vivo T cell activation studies, infected DC were purified by negative selection using biotinylated Abs to CD80 and CD86 followed by Anti-Biotin MicroBeads according to the two-step isolation protocol provided by the manufacturer (Miltenyi Biotec). Sorting of GFP⁺ DC by flow cytometry yielded DC with a comparable phenotype and immunomodulatory capacity as those sorted by microbeads. Purity of sorted cells was >90% and viability was >95% as determined by GFP expression and 7-AAD staining.

Determination of priming and cross-priming by infected DC

DC (10⁵; naive, infected) were incubated with irradiated actmOVA T cells (3000 rad) on a I-A^{b-/-}, K^{b-/-}, or K^{bmi1} background in a 1:3 ratio or with 100 μ g/ml OVA (30) in 96-well U-bottom plates. DC derived from actmOVA mice, which present OVA peptides as self-peptides, were used directly after purification. After 24 h, 10⁵ CFSE-labeled OT-1 or OT-2 were added to the wells with 10 μ g/ml blocking Ab to PD-1, PD-L1, PD-L2, or the appropriate isotype control. After 70 h, OT-1 and OT-2 proliferation and survival was determined by analysis of CFSE dilution together with staining for V α 2⁺, CD4/CD8, annexin V, and 7-AAD. Expansion of OT-1 cells at the end of the culture by the number of cells added at the start of culture. Cytokine production by OT-1 cells was determined after a 5-h stimulation with cognate peptide (5 μ g/ml) in the presence of brefeldin A by intracellular staining for IFN- γ using the Perm/Fix kit as described by the manufacturer (BD Pharmingen). The cytolytic activity on a per cell basis was evaluated by a JAM assay as previously described (32), using [³H]thymidine-labeled EL-4 cells loaded with OVA₂₅₇₋₂₆₄ peptide or irrelevant peptide E1B₁₉₂₋₂₀₀. Specific killing was calculated as: (spontaneous cpm - experimental cpm)/100/spontaneous cpm.

Cytokine production of OT-2 cells was determined by intracellular staining after an additional stimulation with OVA₃₂₃₋₃₃₉ in the presence of T-depleted splenocytes for 24 h, where brefeldin A was added for the final 4 h. The proliferative capacity of OT-2 cells was determined by a standard lymphocyte stimulation assay 6 days after stimulation with the DC. In brief, OT-2 cells were cultured in 96-well U-bottom plates (10⁴ cells/well) with 10⁵ T-depleted irradiated splenocytes (1500 rad) from wild-type mice with medium or increasing doses of OVA or OVA₃₂₃₋₃₃₉ for 60 h, after which the cells were pulsed for 8 h with [³H]thymidine (0.1 μ Ci/well). Proliferation was calculated by dividing the incorporated cpm of the OVA/OVA₃₂₃₋₃₃₉-stimulated cells by the cpm of medium-stimulated cells. Regulatory T cell function was determined by T cell suppressor assay as described previously (33). Briefly, 10⁵ OT-2 cells were cultured with 10⁵ CFSE-labeled naive CD4 T cells and 10⁵ T-depleted splenocytes in the presence of 0.6 μ g/ml CD3 Ab in 96-well U-bottom plates. After 72 h, proliferation of the naive CD4 T cells was determined by CFSE dilution. As controls, 10⁵ purified CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells derived from wild-type mice were added (33).

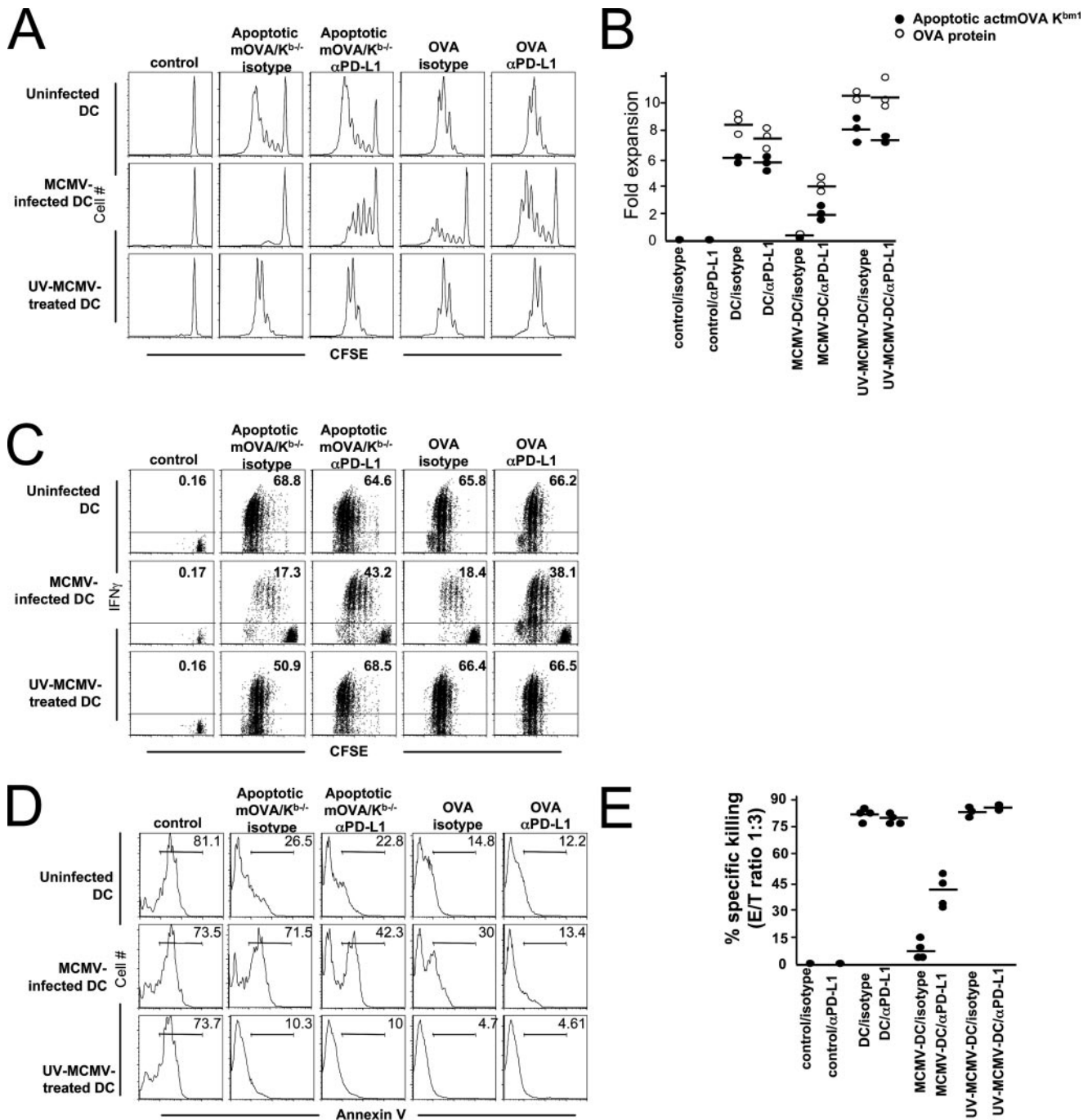


FIGURE 2. MCMV-infected DC stunt CD8 T cells through engagement of PD-L1/PD-1. Uninfected CD11c⁺ mDC, MCMV-infected mDC, and UV-MCMV-exposed mDC were incubated with OVA or irradiated actmOVA/K^{b/-} T cells. Twenty-four hours later, CD45.1⁺ CFSE-labeled OT-1 T cells were added with blocking Ab to PD-L1 or isotype control and incubated for 70 h. *A*, Proliferation of OT-1 T cells (CD8⁺, CD45.1⁺, Vα2⁺, and 7-AAD⁻) as determined by CFSE dilution. *B*, Fold expansion of OT-1 T cells. *C*, IFN-γ production by OT-1 T cells upon OVA_{257–264} peptide stimulation. *D*, Percentage of annexin V⁺-expressing cells in the total OT-1 population. *E*, Cytolytic activity of OT-1 T cells toward OVA_{257–264}-pulsed EL-4 cells. Background killing was <3% killing in all groups. Data in *A*, *C*, and *D* are representative data of at least three independent experiments. Data in *B* and *E* represent mean and individual values ($n = 3–4$) and are representative of at least two independent experiments.

In vivo experiments

Transgenic transfer model (34). K^{bmi}CD45.2 recipients received 10⁶ CFSE-labeled purified OT-1-K^{bmi}CD45.1 cells together with 10⁶ CFSE-labeled nontransgenic purified CD8-K^{bmi}CD45.2 cells that served as an internal control. The next day the mice received 2 × 10⁵ naive or purified MCMV-infected actmOVA DC i.v. PD-L1-blocking Ab (MIH-7 or control, rat IgG2a; 300 μg/mouse) was administered daily by i.p. injection starting at the day of DC transfer (see Fig. 6 for experimental design). Three days after the transfer of DC, spleens were isolated and the OT-1-K^{bmi}CD45.1 responses were analyzed for proliferation by CFSE dilution,

for expansion by determining the ratio of OT-1-K^{bmi}CD45.1/CFSE control cells, and for cytokine production after a 5-h stimulation with cognate peptide in the presence of brefeldin A, followed by intracellular cytokine staining for IFN-γ (Perm/Fix kit). The cytolytic activity was evaluated on purified total CD8 T cells by a JAM assay as described above. CD8 T cells were positively selected using MACS beads, and frequencies of OT-1-K^{bmi}CD45.1 cells were determined in each CD8 T cell preparation and adjusted with nontransgenic CD8 K^{bmi}T cells so that each sample contained the same percentage of transgenic OT-1-K^{bmi}CD45.1 cells (34).

Table I. Effect of PD-1, PD-L1, and PD-L2 blocking on OT-1 T cell expansion, survival, and IFN- γ production^a

	Cells ($\times 10^5$) ^b	Fold Expansion	% IFN- γ ^c	% 7-AAD/Annexin V ⁺
NO DC				
IgG2a	0.7 \pm 0.3	0.1 \pm 0.1	0.1 \pm 0.1	90.3 \pm 18.4
Anti-PD-1	0.6 \pm 0.2	0.1 \pm 0.1	0.2 \pm 0.2	87.1 \pm 9.0
Anti-PD-L1	0.7 \pm 0.3	0.1 \pm 0.1	0.1 \pm 0.1	89.5 \pm 9.5
Anti-PD-L2	0.6 \pm 0.3	0.1 \pm 0.1	0.1 \pm 0.1	82.4 \pm 13.3
Uninfected DC				
IgG2a	28.1 \pm 4.2	7.0 \pm 1.2	69.8 \pm 6.9	24.2 \pm 8.5
Anti-PD-1	32.7 \pm 5.7	8.1 \pm 1.4	73.2 \pm 10.7	22.9 \pm 12.4
Anti-PD-L1	34.1 \pm 6.1	8.4 \pm 1.5	71.5 \pm 5.4	27.1 \pm 8.7
Anti-PD -L2	31.6 \pm 3.3	7.9 \pm 0.8	67.9 \pm 9.3	24.6 \pm 11.3
MCMV-infected DC				
IgG2a	1.7 \pm 0.6	0.4 \pm 0.2	14.7 \pm 4.3	74.4 \pm 11.9
Anti-PD-1	8.3 \pm 2.7*	2.1 \pm 0.5*	44.3 \pm 7.80*	40.7 \pm 7.7*
Anti-PD-L1	8.9 \pm 1.3*	2.2 \pm 0.3*	47.1 \pm 11.4*	43.1 \pm 8.8*
Anti-PD-L2	1.5 \pm 0.4	0.4 \pm 0.1	18.2 \pm 10.1	81.2 \pm 12.1

^a Purified uninfected and MCMV-infected DC were incubated with irradiated actmOVA-K^{b-/-} T cells. After an overnight incubation, CD45.1⁺ CFSE-labeled OT-1 T cells were added to the wells with blocking antibody to PD-1, PD-L1, PD-L2, or isotype control. Proliferation, fold expansion, apoptosis, and IFN- γ production by OT-1 T cells were determined 70 h later.

^b Number of live cells at the end of the 70-h culture ($n = 3/\text{group}$).

^c Determined within the 7-AAD/annexin-V-negative population.

*, $p < 0.05$ compared to isotype-treated culture.

Endogenous transfer model. K^{bm1} recipients received 10⁵ purified MCMV-infected DC from either wild-type, K^{b-/-}, or K^{b-/-}D^{b-/-} mice. In this setting, MCMV did not spread from transferred DC to infect the recipient host cells at a level that would result in a detectable virus-specific CD8 T cell response (data not shown). Direct infection of K^{bm1} mice does result in a K^b-restricted, MCMV-specific CD8 T cell response, although it is diminished in magnitude when compared with infection of wild-type B6 mice. PD-L1-blocking Ab (MIH-7) or rat IgG2a (300 $\mu\text{g}/\text{injection}$) was given i.p. for 3 days starting at the day of DC transfer. Seven days after the transfer of DC, spleens were isolated and the MCMV-specific responses were determined by intracellular staining for IFN- γ in CD45.2⁺CD8 T cells after a 5-h stimulation with MCMV-derived peptides (5 $\mu\text{g}/\text{ml}$) in the presence of brefeldin A. In parallel, the number of MCMV-specific CD8 T cells was determined by standard IFN- γ ELISPOT (eBioscience) using a serial dilution of splenocytes and OVA₂₅₇₋₂₆₄ (10 $\mu\text{g}/\text{ml}$) medium or PMA as Ags. Both assays were performed in medium containing 10% heat-inactivated horse serum to reduce background IFN- γ production. As controls, mice of the indicated strains were infected i.p. with 2×10^4 PFU of MCMV.

Results

MCMV "programs" DC through regulation of positive and negative cosignaling molecules

MCMV has been reported to down-modulate the cell-surface expression of several positive cosignaling molecules in infected macrophage or DC-like cell lines (8, 9, 29, 35), but little of this analysis has been done in primary DC. Consequently, DC were isolated from spleens of naive mice (CD11b⁺/CD11c⁺) and infected with a recombinant MCMV that expresses GFP (MCMV-GFP) (29). As controls, DC were left untreated or were exposed to MCMV that was inactivated by UV light (UV-MCMV). Exposure of DC to UV-MCMV resulted in persistent up-regulation of MHC classes I and II and costimulatory molecules CD40, CD80, and CD86 (Fig. 1). Both PD-L1 and PD-L2 were up-regulated within 6 h of exposure, and then declined over time, returning to levels comparable to control DC at 24 h after exposure (Fig. 1). In contrast, when various costimulatory molecules (CD80, CD86, and CD40), adhesion molecules (CD54, CD11b, and CD11c), or MHC classes I and II were analyzed in MCMV-GFP⁺ DC 24 h after infection, all were present at significantly lower levels than on naive or UV-MCMV DC. This was a result of active down-regulation, as levels of these molecules were significantly higher in the uninfected "bystander" DC in the same MCMV-infected DC cul-

tures at 6 h (Fig. 1 and data not shown). Interestingly, expression of the inhibitory cosignaling ligand PD-L1 was increased upon infection and remained very high over time, in contrast to the transient up-regulation observed in UV-MCMV-exposed DC. This regulation of PD-L1 was specific, as the closely related molecule PD-L2 was only transiently induced and down-modulated with comparable kinetics as observed in UV-MCMV-exposed DC (Fig. 1). DC derived from bone marrow with GM-CSF treatment (mDC) were also infected with MCMV, and comparable "programming" of the infected DC was seen; that is, MHC classes I and II and costimulatory molecules were down-regulated, whereas PD-L1 expression was up-regulated and maintained over time (Fig. 1). This DC programming (i.e., regulation of molecules that confer DC priming capacity) required live virus, as UV-MCMV induced significant DC activation and up-regulation of MHC and costimulatory molecules (Fig. 1).

Virally programmed DC stunt CD8 and CD4 T cell responses via PD-L1/PD-1

The selective maintenance of PD-L1 expression on MCMV-infected DC suggested that the PD-L1/PD-1 system might play an important role in prevention or suppression of antiviral T cell responses. To test this hypothesis, uninfected, UV-MCMV-exposed, and highly purified MCMV-infected mDC were used. These three mDC populations were incubated with OVA protein or apoptotic T cells derived from mice expressing OVA under the actin promoter crossed to a K^{b-/-} background to prevent direct presentation of the OVA₂₅₇₋₂₆₄ peptide. The following day, CFSE-labeled OT-1 CD8 T cells were added in combination with blocking Ab to PD-L1 or isotype control, and T cell activation, proliferation, and development of effector function were determined (Fig. 2). Cross-presentation of soluble and cell-associated OVA by uninfected DC induced proliferation and expansion of the CFSE-labeled OT-1 T cells (Fig. 2, A and B). Addition of anti-PD-L1 to these cultures did not significantly increase proliferation or expansion of OT-1 T cells. In contrast, MCMV-infected mDC induced very poor proliferation and expansion of (Fig. 2, A and B), and IFN- γ production by (Fig. 2C), OT-1 T cells regardless of whether soluble or cell-associated OVA was used. Importantly, although no proliferation was observed, the OT-1 T cells were activated as determined by their

cell-surface marker phenotype (CD44^{high}, CD69^{high}; data not shown). Additionally, OT-1 T cells incubated with MCMV-infected mDC showed a significant increase in T cell apoptosis, as determined by staining for annexin V and 7-AAD (Fig. 2D; Table I).

Strikingly, when anti-PD-L1 Ab was added to MCMV-infected mDC and T cell cocultures, significant amounts of proliferation, expansion, and survival of OT-1 cells were restored. The PD-L1/PD-1 and not the PD-L2/PD-1 interaction was responsible for the inhibitory effect on T cell proliferation and survival, as Ab blocking PD-1, but not PD-L2, gave comparable results to PD-L1 blockade (Table I; and data not shown). Again, this DC programming was only observed when infecting with live virus, as mDC exposed to UV-MCMV induced dramatically more T cell proliferation than naive DC, most likely due to the increased expression of MHC and costimulatory molecules in this case (Fig. 1).

Although some of the OT-1 T cells activated by MCMV-infected mDC were capable of producing low levels of IFN- γ upon restimulation with OVA peptide (Fig. 2C), they showed very poor secondary expansion and restricted cytolytic activity on a per-cell basis compared with OT-1 T cells that had encountered Ag in the context of uninfected or UV-MCMV-treated mDC (Fig. 2E). OT-1 T cells primed by MCMV-infected DC maintained this stunted phenotype, independent of the concentrations of OT-1 T cells used, including if cytolytic capacity was normalized on a per-cell basis to the numbers of IFN- γ ⁺ cells. Similar to what was seen for proliferation, addition of anti-PD-L1 to the cultures also partially restored OT-1 cytolytic capacity, secondary expansion, and IFN- γ production on a per-cell basis. MCMV infection has been reported to inhibit the ability of DC to phagocytose dextran (8). To verify that the negative effects of MCMV on mDC-induced OT-1 T cell activation were not simply due to impaired Ag uptake, parallel studies were performed using mDC from mice that constitutively express OVA protein (actmOVA) and present OVA_{257–264} as a self-Ag (36). Importantly, virtually identical results were obtained with MCMV-infected actmOVA-DC as was observed with MCMV-infected wild-type DC (Fig. 3, A and B, and data not shown), ruling out that altered Ag uptake is the major cause of the poor immunostimulatory capacity of the infected mDC.

MCMV-infected mDC inhibit CD4 T cell responses

Given the dramatic contribution that negative PD-L1 signals played in the stunting of naive CD8 T cells by MCMV-infected mDC, we examined whether MCMV could exert a similar effect on CD4 T cell responses. Uninfected, MCMV-infected and UV-MCMV-exposed mDC were incubated with OVA or apoptotic actmOVA T cells (I-A^{b-/-} background) and cultured with naive, OT-2 CD4 T cells. Both uninfected and UV-MCMV-exposed mDC induced OT-2 proliferation to soluble and cell-associated OVA, and addition of PD-L1 blocking Ab did not significantly increase proliferation or expansion (Fig. 4A; Table II). In contrast, MCMV-infected mDC given soluble or cell-associated OVA induced very poor proliferation and expansion of OT-2 cells. Comparable results were found when mDC were generated from actmOVA mice (Fig. 3, C and D). Only very few of the OT-2 T cells that were activated by MCMV-infected mDC had the capacity to produce IFN- γ , and those that did produced less on a per-cell basis (Fig. 4, A and B). Similar to that seen with OT-1 T cells, the OT-2 T cells that did not undergo proliferation still displayed an activated phenotype and increased frequency of apoptosis (Fig. 4C; Table II). Blocking of PD-L1, but not PD-L2, during priming partially restored proliferation, expansion, survival, and cytokine induction by OT-2 cells (Fig. 4; Table II).

The effect of PD-L1 blockade during priming with MCMV-infected mDC also improved OT-2 T cell recall responses. When

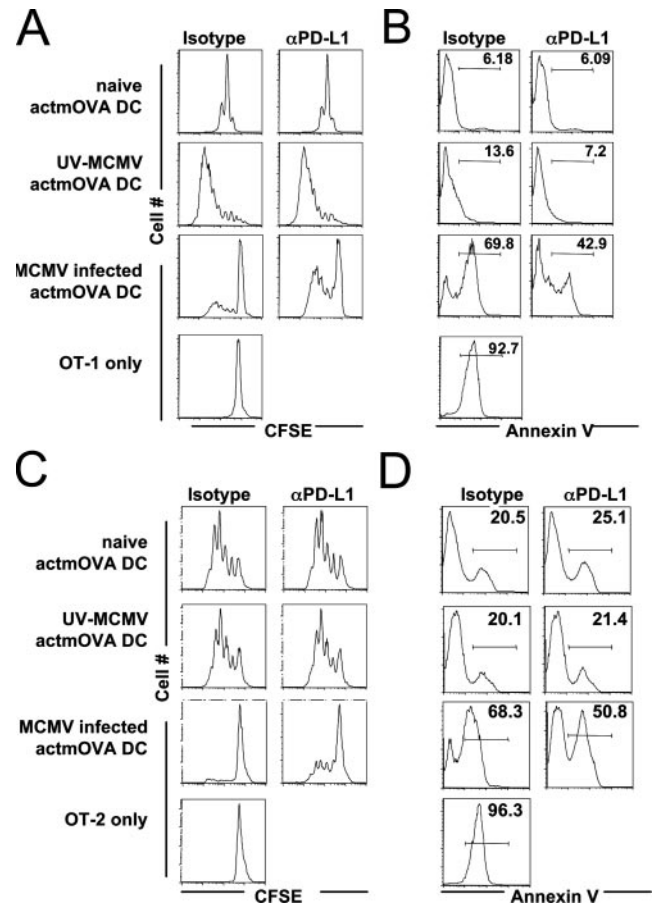


FIGURE 3. PD-L1-mediated regulation of T cell responses by MCMV-infected DC when presenting “self” Ag. DC were generated from bone marrow of actmOVA B6 mice. Purified untreated DC, MCMV-infected, or UV-MCMV-exposed DC were cultured with CD45.1⁺ CFSE-labeled OT-1 (A and B) or CD90.1⁺ CFSE-labeled OT-2 T cells (C and D) in the presence of blocking Ab to PD-L1 or isotype control. A and C, Proliferation of OT-1 and OT-2 T cells was determined 70 h later by analysis of CFSE dilution in combination with 7-AAD staining and Abs to CD4/8, CD45.1/90.1, and V α 2. B and D, Expression of annexin V by total OT-1 and OT-2 T cells upon 70 h of culture under the indicated conditions. Numbers in the upper right corner represent the percentage of annexin V⁺ OT-2 T cells. Data are representative of three independent experiments.

Ag-experienced OT-2 T cells were restimulated with uninfected mDC pulsed with OVA protein or OVA_{323–339} peptide, OT-2 T cells that had previously interacted with MCMV-infected mDC showed poor proliferative responses in both cases (Fig. 4D). The phenotype of these “stunted” OT-2 T cells resembled a tolerant or anergic state, as their responses were restored when IL-2 was added *in trans* to these cultures (Fig. 5A). Addition of PD-1- or PD-L1-blocking reagents to secondary cultures did not restore T cell responses. Consistent with our other results, OT-2 cells cultured with MCMV-infected mDC in the presence of anti-PD-L1 showed markedly increased proliferation upon restimulation with both OVA protein and peptide. Importantly, when stunted OT-2 cells were examined for expression of FoxP3 and their capacity to suppress bystander cell proliferation, none was observed, suggesting that MCMV-infected mDC do not induce FoxP3⁺ regulatory CD4 T cells in this system (Fig. 5B).

Anti-PD-L1 can circumvent MCMV-induced T cell stunting *in vivo*

Having established that PD-L1 signaling stunts naive T cell responses when directly primed by MCMV-infected mDC *in vitro*,

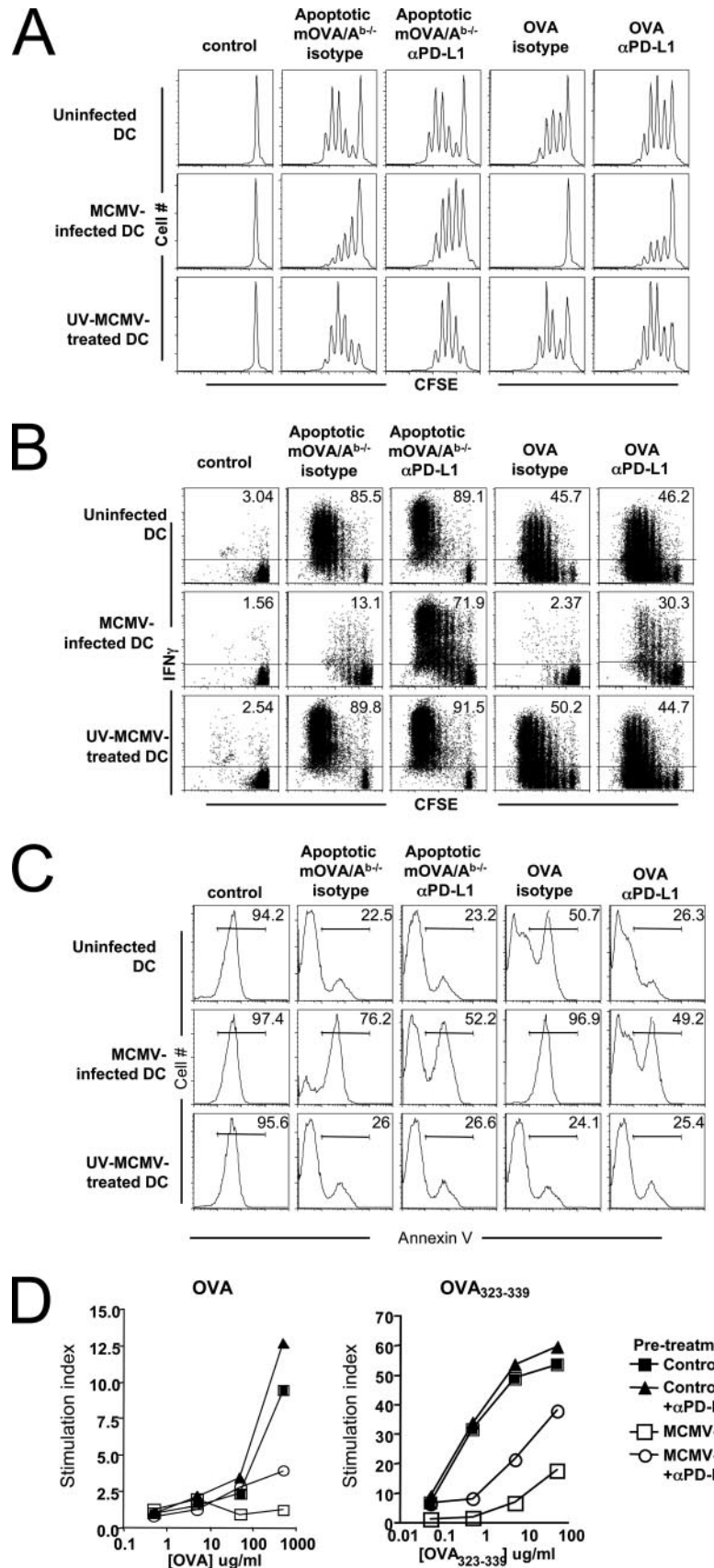


FIGURE 4. MCMV-infected DC stunt CD4 T cells through engagement of PD-L1/PD-1. Uninfected, MCMV-infected and UV-MCMV-exposed DC were the same as in Fig. 2 and were incubated with irradiated actmOVA/I-A^{b-/-} T cells. CD90.1⁺ CFSE-labeled OT-2 T cells were added together with blocking Ab to PD-L1 or isotype control and analyzed after 70 h of culture. *A*, Proliferation of OT-2 T cells as determined by CFSE dilution. *B*, IFN- γ production by OT-2 T cells (CD4⁺, CD90.1⁺, V α 2⁺, and 7-AAD⁻) upon stimulation with OVA₃₂₃₋₃₃₉ peptide. *C*, Percentage of annexin V expressing cells in the total OT-2 population. *D*, Recall responses to OVA protein and OVA₂₃₂₃₋₃₃₉ peptide in the presence of T-depleted splenocytes after a 6-day culture with the different DC. Data in *A-C* are representative data of at least three independent experiments. Data in *D* represent mean \pm SEM ($n = 3-4$) and are representative of at least two independent experiments.

we sought to examine whether PD-L1 could also negatively affect T cell responses primed by infected DC in vivo. Priming of Ag-specific T cells can result from either direct presentation or by

cross-priming. Because APC that cross present viral proteins are not necessarily subject to viral modulation (because they may not be directly infected), an in vivo model was used where

Table II. Effect of PD-1, PD-L1, and PD-L2 blocking on OT-2 T cell expansion, survival, and IFN- γ production^a

	Cells ($\times 10^5$) ^b	Fold Expansion	% IFN- γ ⁺ ^c	% 7-AAD/Annexin V ⁺
NO DC				
IgG2a	0.4 \pm 0.1	0.1 \pm 0.1	2.8 \pm 0.9	95.7 \pm 8.2
Anti-PD-1	0.4 \pm 0.3	0.1 \pm 0.1	2.7 \pm 0.8	96.8 \pm 3.5
Anti-PD-L1	0.3 \pm 0.1	0.1 \pm 0.1	2.6 \pm 0.8	95.3 \pm 4.1
Anti-PD-L2	0.4 \pm 0.2	0.1 \pm 0.1	2.7 \pm 1.1	96.8 \pm 3.3
Uninfected DC				
IgG2a	49.6 \pm 8.3	12.2 \pm 2.1	89.1 \pm 9.2	22.2 \pm 6.5
Anti-PD-1	45.8 \pm 9.6	11.7 \pm 2.4	83.2 \pm 7.5	23.0 \pm 5.7
Anti-PD-L1	54.0 \pm 12.4	13.5 \pm 3.1	91.2 \pm 5.4	23.1 \pm 7.8
Anti-PD-L2	54.5 \pm 8.5	13.6 \pm 2.1	82.2 \pm 11.0	22.6 \pm 3.3
MCMV-infected DC				
IgG2a	2.1 \pm 1.3	0.5 \pm 0.3	16.3 \pm 6.4	86.6 \pm 1.9
Anti-PD-1	20.7 \pm 2.3*	5.2 \pm 0.6*	68.9 \pm 11.7*	51.4 \pm 8.9*
Anti-PD-L1	19.7 \pm 4.5*	4.9 \pm 1.1*	72.1 \pm 10.1*	49.1 \pm 8.6*
Anti-PD-L2	2.6 \pm 1.1	0.7 \pm 0.3	17.9 \pm 12.4	84.2 \pm 7.1

^a Purified uninfected and MCMV-infected DC were incubated with irradiated actmOVA-I-A^{b-/-} T cells. After an overnight incubation, CD45.1⁺ CFSE-labeled OT-2 T cells were added to the wells with blocking antibody to PD-1, PD-L1, PD-L2, or isotype control. Proliferation, fold expansion, and apoptosis production by OT-2 T cells were determined 70 h later. IFN- γ production was determined after an additional 20 incubations with OVA₃₂₃₋₃₃₉ peptide.

^b Number of live cells at the end of the 70-h culture ($n = 3$ per group).

^c Determined within the 7-AAD/annexin V-negative population.

*, $p < 0.05$ compared to isotype-treated culture.

cross-presentation could not occur and Ag presentation was restricted to a specific DC (34). In this model, CFSE-labeled OT-1 CD45.1 cells from K^{bm1} mice were transferred together with CFSE-labeled naive CD45.2 K^{bm1} control CD8 T cells into K^{bm1} CD45.2 recipients. One day later, purified uninfected or MCMV-infected mDC from actmOVA mice (K^{b+/+}) were transferred into the K^{bm1} mice (Fig. 6A). In this case, the trans-

ferred DC are the only cells capable of presenting OVA₂₅₇₋₂₆₄ to the transferred OT-1 cells, as the K^{bm1} recipient mice cannot bind the OVA₂₅₇₋₂₆₄ peptide sufficiently to allow OT-1 activation via cross-presentation.

Transfer of uninfected actmOVA-mDC induced strong proliferation and expansion of the cotransferred OT-1-K^{bm1} CD45.1 T cells, and this was not altered by the coinjection of blocking Abs

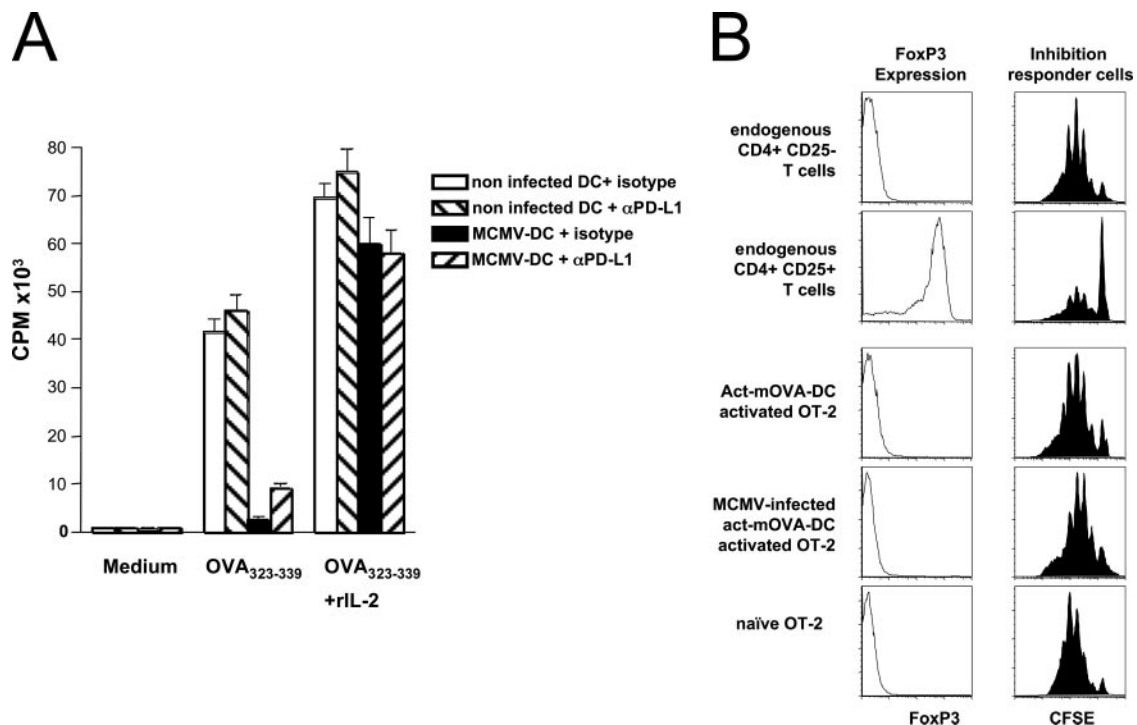


FIGURE 5. Stunted CD4 T cells are rescued by IL-2 and do not acquire regulatory function. *A*, IL-2 restores proliferative capacity in OT-2 T cells activated by MCMV-infected DC. OT-2 T cells were incubated for 3 days with indicated DC and Abs. Subsequently, cells were rested for 3 days and restimulated with OVA and syngeneic T-depleted splenocytes in the presence of absence of recombinant IL-2. *B*, OT-2 T cells activated by MCMV-infected DC do not express FoxP3 or have the capacity to suppress proliferation in CFSE-labeled naive CD4 T cells upon cross-linking of CD3. Data in *A* represent mean \pm SEM ($n = 4$) and are representative of at least three independent experiments. Data in *B* show a representative histogram of two independent experiments ($n = 3$ /group).

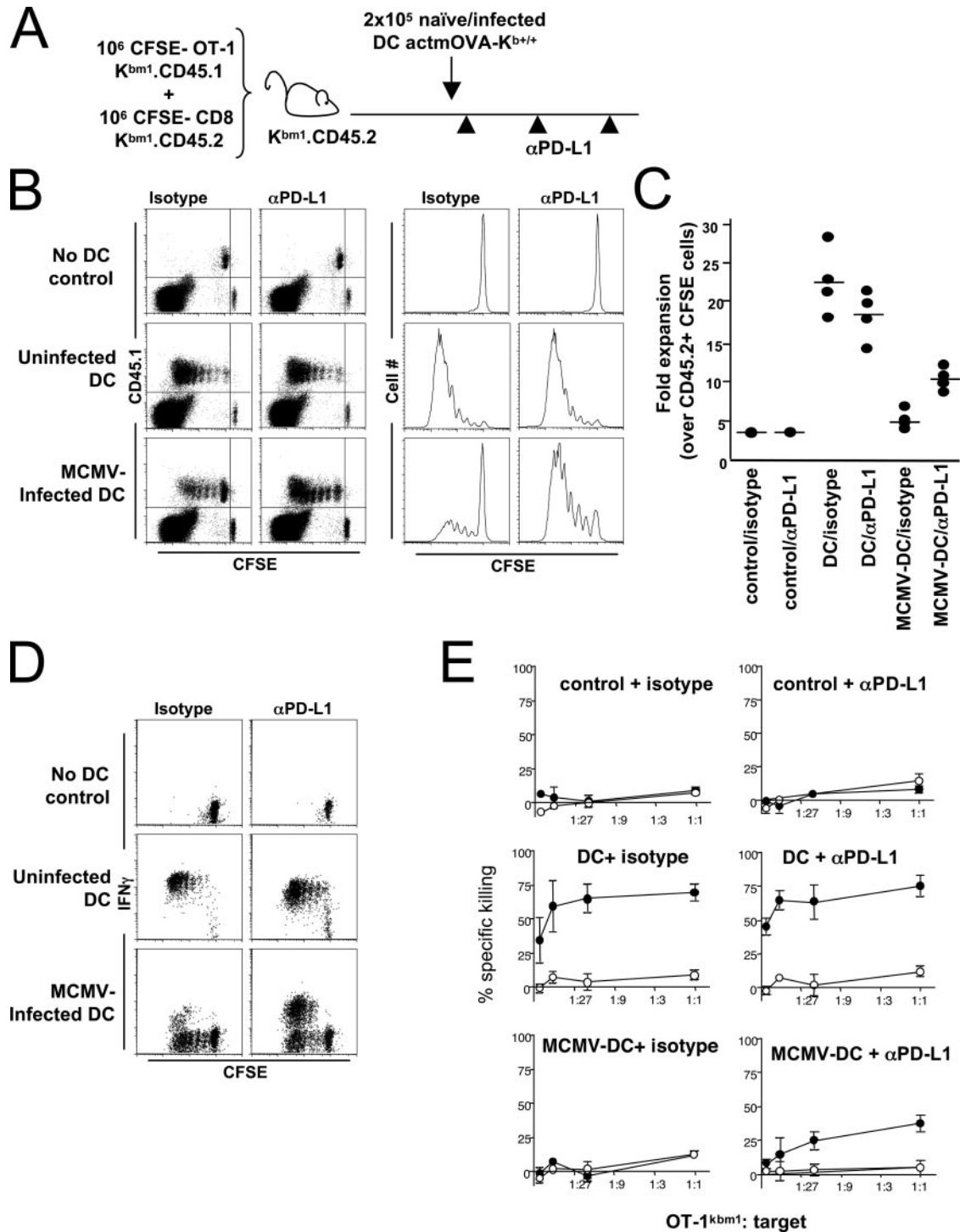


FIGURE 6. Priming by MCMV-infected DC stunts T cells in vivo. C57BL/6- $K^{bm1}/CD45.2^+$ recipient mice received CFSE-labeled purified OT-1- $K^{bm1}/CD45.1^+$ cells mixed in a 1:1 ratio with CFSE-labeled, nontransgenic $K^{bm1}/CD45.2^+$ CD8 T cells. Twenty-four hours later, naive or MCMV-infected actmOVA- $K^{b+/+}$ DC were adoptively transferred. PD-L1-blocking Ab or isotype control was administered daily. Three days after DC transfer, the OT-1- $K^{bm1}/CD45.1^+$ responses were analyzed. *A*, Schema of the transfer and treatments. *B*, Proliferation of OT-1- $K^{bm1}/CD45.1^+$ as determined by CFSE dilution. *Left panels*, CFSE vs CD45.1 staining within the CD8⁺ T cell population. *Right panels*, CFSE histograms of the OT-1- $K^{bm1}/CD45.1^+$ cells. *C*, Expansion of OT-1- $K^{bm1}/CD45.1^+$ T cells in the different groups determined by the ratio of OT-1- $K^{bm1}/CD45.1^+$ T cells compared with the internal control (CFSE-labeled C57BL/6- $K^{bm1}/CD45.2^+$ CD8 T cells). *D*, IFN- γ production by OT-1- $K^{bm1}/CD45.1^+$ T cells after a 5-h stimulation with OVA₂₅₇₋₂₆₄ peptide. Numbers indicate the percentage of IFN- γ -producing cells. *E*, Cytolytic activity on a per-cell basis of OT-1- $K^{bm1}/CD45.1^+$ T cells toward OVA₂₅₇₋₂₆₄-pulsed target cells (●) or control targets (○). Data in *B* and *D* are representative data of at least two independent experiments ($n = 3-4$ in each experiment). Data in *C* and *E* represent mean \pm SEM ($n = 3-4$) and are representative of at least two independent experiments.

to PD-L1 (Fig. 6, *B* and *C*). In contrast, transfer of MCMV-infected actmOVA-mDC resulted in dramatically less proliferation and expansion of the OT-1- K^{bm1} CD45.1 T cells, which was par-

tially restored if anti-PD-L1 was administered (Fig. 6, *B* and *C*). Comparable to the in vitro data, anti-PD-L1 did not further increase the already robust IFN- γ production or cytolytic activity of

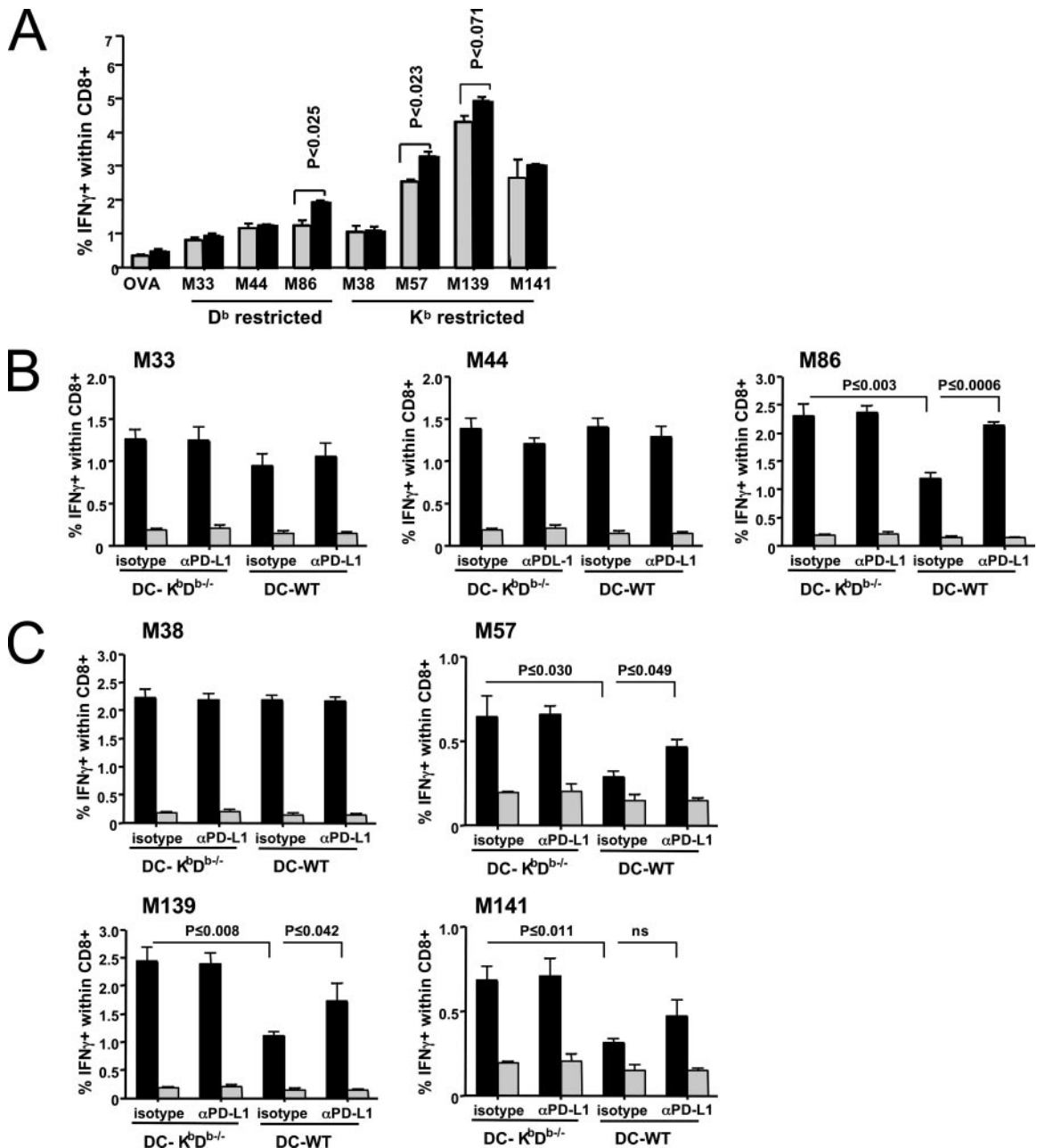


FIGURE 7. Direct priming by MCMV-infected DC inhibits endogenous T cell responses in vivo. *A*, B6 wild-type mice were directly infected with MCMV (5×10^4 PFU) and treated for 3 days with anti-PD-L1 (black bars) or isotype (gray bars). Seven days postinfection the frequency of IFN- γ -producing MCMV-specific CD8 T cell responses was determined after in vitro restimulation with the indicated peptides. For *B* and *C*, DC from either wild-type or K^bD^b -/- mice were infected in vitro for 24 h, and purified infected DC were transferred into K^{bm1} recipients. Mice were treated with anti-PD-L1 or isotype control for the first 3 days following transfer, and 4 days later frequencies of MCMV-specific CD8 T cell responses were determined by IFN- γ production upon stimulation with indicated D^b-restricted (*B*) and K^b-restricted peptides (*C*). Gray bars represent responses to irrelevant peptide. Data are presented as mean \pm SEM ($n = 5$).

OT-1 T cells that were primed by transferred, uninfected actmOVA mDC (Fig. 6*B–E*). In contrast, when OT-1 T cells were cotransferred with MCMV-infected actmOVA mDC, they displayed a stunted phenotype, with very poor IFN- γ production and cytolytic activity on a per-cell basis. Administration of PD-L1-blocking Abs in this scenario partially restored the stunted OT-1 phenotype, increasing proliferation, IFN- γ production, and cytolytic capacity on a per-cell basis.

PD-L1/PD-1 plays a role in shaping the MCMV-specific CD8 T cell response

To evaluate whether the PD-L1/PD-1 system also has an effect on shaping the endogenous, MCMV-specific CD8 T cell response, B6

mice were infected directly with MCMV and treated with anti-PD-L1 or control Ab. Infection of B6 mice results in the induction of MCMV-specific CD8⁺ T cells directed against a wide range of K^b- and D^b-restricted MCMV-derived peptides (37). Treatment with anti-PD-L1 resulted in a modest (but significant) increase in some, but not all, of the epitope-specific CD8 T cell responses examined (Fig. 7*A*).

We postulated that one potential reason for the modest effect of anti-PD-L1 on the endogenous CD8 T cell response to MCMV could be that a significant amount of cross-presentation of viral Ags might contribute to the shaping of this repertoire, as has been recently proposed (38, 39), especially at times of acute infection when viral load is high. To test this hypothesis, we established a

mDC-transfer model where we could distinguish the relative contribution of direct priming from that of cross-priming. Wild type and $K^b^{-/-}D^b^{-/-}$ DC were infected with MCMV, sorted for purity, and transferred into K^{bm1} recipients. In this case, only the wild-type mDC can present viral epitopes and prime directly, while the CD8 T cell responses originating upon transfer of $K^b^{-/-}D^b^{-/-}$ mDC will result only from cross-presentation by host APC upon phagocytosis of infected donor cells. In both transfer scenarios, the donor DC are rapidly rejected based on their absent or mismatched MHC, allowing for the comparison of how cross-presentation alone or the combination of direct presentation and cross-presentation shapes the MCMV-specific CD8 T cell response.

Transfer of infected $K^b^{-/-}D^b^{-/-}$ mDC induced MCMV-specific K^b - and D^b -restricted CD8 T cell responses resulting exclusively from cross-priming (Fig. 7, B and C). Treatment of these mice with anti-PD-L1 did not alter the magnitude of these CD8 responses to any of the tested K^b - and D^b -restricted epitopes, again suggesting that in the context of cross-presentation of viral Ags by uninfected DC, the PD-L1/PD-1 system plays a negligible role in shaping the CD8 T cell response. In contrast, upon transfer of MCMV-infected wild-type mDC (e.g., cells that can directly present viral Ags), CD8 T cell responses to the D^b -restricted epitope M86 and K^b -restricted epitopes M57, M139, and M141 were significantly reduced in magnitude when compared with transferring infected $K^b^{-/-}D^b^{-/-}$ mDC (Fig. 7, B and C). When PD-L1-blocking Ab was administered in this case, all four of these stunted CD8 responses were increased, in some cases to near normal levels (Fig. 7, B and C). Importantly, the same D^b - and K^b -restricted epitopes that responded to anti-PD-L1 blockade in mice that were directly infected with MCMV were affected in this transfer model (Fig. 7A). This observation supports the hypothesis that although only a low percentage of DC are directly infected by MCMV under normal experimental conditions (5, 6), these APC have the potential to shape the adaptive immune response to some degree by stunting antiviral T cell responses via PD-1/PD-L1 interactions.

Discussion

Herein we show that MCMV-infected DC have the capacity to directly activate Ag-specific naive T cells and stunt their proliferation, survival, and acquisition of effector function. The programming of the MCMV-infected DC to selectively maintain PD-L1 on the cell surface greatly contributes to this stunted antiviral T cell response. Although one could propose that the maintenance of PD-L1 represents fortuitous neglect in the DC programming strategy of CMV, the observation that PD-L2 is actively down-regulated from the cell surface suggests to us that this is not likely a passive mechanism.

The PD-L1/PD-1 system is part of the balance between the stimulatory and inhibitory signals needed for effective immune responses and maintenance of self-tolerance. In contrast to PD-L2 that is induced on DC and macrophages upon activation (40), PD-L1 is expressed constitutively on many cell types of hematopoietic and nonhematopoietic origin, leading to the hypothesis that PD-L1 may regulate self-reactive T cells or B cells (41). Involvement of PD-1 in regulating T cell tolerance and autoimmunity was first indicated by the autoimmune phenotype of *Pcd11^{-/-}* mice (PD-1^{-/-}) that develop spontaneous late-onset lupus-like disease (42, 43). Later studies found inhibitory roles for PD-L1, PD-L2, and PD-1 in the initial phase of activation and clonal expansion of self-reactive T cells and progression in autoimmune models for diabetes and experimental autoimmune encephalomyelitis (44–46).

The current models view the activation of naive T cells as a sum of positive and negative signals dictated by the relative expression of ligands and receptors on T cells and APC. In this setting, a net

“negative” signal will be tolerogenic whereas a net “positive” signal will lead to T cell activation/priming. Consequently, Ag presentation by fully activated DC would provide sufficient positive signals to override negative signals provided by PD-L1 and PD-L2. In contrast, TCR engagement in the absence of sufficient positive signals (e.g., Ag presentation by immature DC or tissue-associated MHC self-Ags) would result in a dominant negative signal through the PD-L1/PD-1 pathway, leading to T cell tolerance, anergy, or deletion (47, 48). This model is supported by studies showing that PD-1 is important for the induction of peripheral CD8 T cell tolerance by resting DC in vivo, as PD-1^{-/-} CD8 T cells are resistant to tolerance (49).

Our data suggest that MCMV actively exploits the cosignaling balance to induce suppressive DC and inhibit the induction of antiviral T cell responses by down-regulating MHC and costimulatory molecules and maintenance of PD-L1. Importantly, our results clearly show that even though MHC and costimulatory molecule expressions are reduced in MCMV-infected DC, in some cases by several orders of magnitude, upon blockade of PD-L1 these levels are still sufficient to prime an effective CD8 and CD4 T cell response, further supporting the “additive” model of costimulation. The importance of the PD-L1/PD-1 pathway is also highlighted by recent observations that human rhinovirus and respiratory syncytial virus use this pathway to prevent the induction of antiviral immune responses (26, 27). These viruses also tip the costimulatory balance in DC, but do so by enhancing PD-L1 expression while maintaining normal levels of MHC and costimulatory molecules, indicating that the system is quite plastic and not easily saturable.

The mechanisms by which the PD-L1/PD-1 pathway exerts its inhibitory effects could be dual. Early studies reported that PD-1 signaling resulted in cell cycle arrest, whereas later studies indicate that PD-1 signaling promotes death, either through the direct engagement of a death pathway or indirectly by down-regulating survival signals and growth factors (25, 40, 50). Our studies indicate that many of the T cells activated by MCMV-infected DC undergo apoptosis and/or die, and those that survive show poor proliferation and acquisition of effector function. As blocking PD-L1 or PD-1 improves both survival and proliferation, it is likely that both mechanisms are involved in the context of MCMV-programmed DC. Intriguingly, some have proposed that reverse signaling through PD-L1 on DC by PD-1 reduces the expression of CD40, CD80, and CD86 and increases IL-10 production, and this mechanism could also potentially contribute in our studies (51–53).

That the effect of anti-PD-L1 on the MCMV-specific CD8 T cell response is epitope-specific, both upon direct infection of wild-type mice and when transferring infected DC, is quite intriguing. In the case of direct infection, virion-associated and some immediate-early/early proteins could potentially be expressed, processed, and presented by MHC before MCMV could exert its maximum effect on MHC and costimulatory molecule expression, perhaps contributing to the observed epitope-specific differences of anti-PD-L1. However, a comparable pattern for the effect of anti-PD-L1 on the CD8 T cell response was observed whether mice were directly infected or whether DC were first infected for 24 h in culture and then adoptively transferred into mice, with the latter scenario giving ample time for MCMV immunomodulatory genes to act (shown in Fig. 1). This observation argues against a “timing-dependent” explanation for the epitope-specific effects of anti-PD-L1. Alternatively, it is possible that some epitopes may be imperious to MCMV immunomodulation. However, to date, only one epitope, a peptide derived from the M164 early-late gene product presented by D^d in BALB/c mice (54), is known to be presented at

high levels throughout the MCMV replication cycle in cultured fibroblasts. All of the peptides used in this study are “hidden” from recognition by epitope-specific CD8 T cell clones in infected fibroblasts (55), and we think that it is unlikely that they all will escape immune modulation in vivo.

A more plausible explanation might be that peptides with high affinity and/or availability continue to be presented at sufficient levels in MCMV-infected DC to allow for TCR signaling. In contrast, low abundance or low-affinity peptides would occupy only very few MHC molecules on the DC surface, leading to T cell ignorance. Potential support for this model comes from our observation that the CD8 T cell response to the M86-derived peptide was the only D^b-restricted response that was affected by anti-PD-L1 treatment. M86 encodes a major capsid protein of MCMV (56), is expressed at high levels in infected cells, and the M86-derived peptide binds D^b with the highest affinity of the known MCMV peptides measured to date (>10-fold) (55). Also consistent, CD8 T cell responses specific for the K^b-restricted epitopes M57, M139, and M141 are all increased by anti-PD-L1, while the response to the subdominant, M38-derived epitope, which has the lowest known affinity for these four K^b-binding peptides, was completely unaffected by the blocking Ab (55).

We have shown that a key component of MCMV DC programming leading to CD8 and CD4 T cell stunting is the maintenance of PD-L1 expression on the cell surface while commensurately down-regulating expression of MHC and positive costimulatory molecules. Modulating PD-L1/PD-1 in various experimental contexts revealed that these virally programmed DC retain their ability to prime naive, Ag-specific T cells, but lead to the induction of tolerance and/or their deletion. HCMV employs similar mechanisms to down-regulate MHC and costimulatory molecule expression in infected DC, stunting allogeneic T cell proliferation in vitro (11–15). Although we are not aware of any studies directly addressing the role that negative cosignaling molecules play in HCMV-induced T cell stunting, we think it is quite likely that primate CMV will also target the PD-1 system. Human and rhesus CMV do target the B and T lymphocyte attenuator-dependent (17) negative cosignaling pathway by encoding the *ul144* orf, a homolog of the B and T lymphocyte attenuator-ligand herpesvirus entry mediator (16, 18). Importantly, our data indicate a role for PD-L1/PD-1 in regulating the initial priming of naive T cells by MCMV-infected APC, distinct from the role that PD-1 signaling plays in T cell “exhaustion” described for several persistent/chronic viral infections in humans and mice (20), including HCMV (25). Regardless, it is clear from this study that maintaining the PD-L1/PD-1 interaction is a critical component of the overall program used by MCMV to suppress the antiviral T cell response, and our study highlights this negative cosignaling system as a key player in shaping the T cell response to a β -herpesvirus during the acute phase of infection.

Acknowledgments

We thank S. P. Schoenberger for scientific advice, and M. Wolkers and R. Arens for critical reading of the manuscript. This is publication 900 from LIAI.

Disclosures

The authors have no financial conflicts of interest.

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