Lymphoid-Related CD11c⁺ CD8α⁺ Dendritic Cells Are Involved in Enhancing Herpes Simplex Virus Type 1 Latency[▽]

Kevin R. Mott,¹ David Underhill,² Steven L. Wechsler,^{3,4,5} and Homayon Ghiasi^{1*}

Center for Neurobiology & Vaccine Development, Ophthalmology Research Laboratories,¹ and Immunobiology Research Institute,² Cedars-Sinai Medical Center, Los Angeles, California 90048; The Eye Institute, University of California Irvine, School of Medicine, Irvine, California 92697³; Department of Microbiology and Molecular Genetics, University of California Irvine, School of Medicine, Irvine, California 92697⁴; and The Center for Virus Research, University of California, Irvine, Irvine, California 92697⁵

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The mechanism(s) by which herpes simplex virus type 1 (HSV-1) latency is established in neurons is not known. In this study, we examined the effect of dendritic cells (DCs) on the level of HSV-1 latency in trigeminal ganglia (TGs) of ocularly infected BALB/c and C57BL/6 mice. We found that immunization of wild-type mice with FMS-like tyrosine kinase 3 ligand (Flt3L) DNA, which increases the number of DCs, increased the amount of latency in infected mice. Conversely, depletion of DCs was associated with reduced latency. Latency was also significantly reduced in Flt3L^{-/-} and CD8^{-/-} mice. Interestingly, immunization of Flt3L^{-/-} but not CD8^{-/-} mice with Flt3L DNA increased latency. Transfer experiments using DCs expanded ex vivo with Flt3L or granulocyte-macrophage colony-stimulating factor suggested that increased latency was associated with the presence of lymphoid-related (CD11c⁺ CD8α⁺) DCs, while reduced latency was associated with myeloid-related (CD11c⁺ CD8α⁻) DCs. Modulation of DC numbers by Flt3L DNA immunization or depletion did not alter acute virus replication in the eye or TG or eye disease in ocularly infected mice. Our results suggest that CD11c⁺ CD8α⁺ DCs directly or indirectly increase the amount of HSV-1 latency in mouse TGs.

A main characteristic of infection with herpes simplex virus type 1 (HSV-1) is the ability of the virus to establish latency in sensory neurons of an infected host (13, 49, 54). At various times throughout the life of the latently infected individual, the virus may reactivate and travel back to the original site of infection, causing recurrent disease. Recurrence of HSV-1 in the eye is the leading cause of corneal blindness by an infectious agent in developed countries (5, 10, 55). HSV-1 induced eye disease ranges in severity from blepharitis, conjunctivitis, and dendritic keratitis to disciform stromal edema and necrotizing stromal keratitis (7, 10). Approximately 500,000 people in the United States have a history of recurrent ocular HSV-1. There are approximately 30,000 episodes of recurrent ocular HSV-1 in the United States per year that result in doctor visits (26). HSV-1-induced eye disease is mainly associated with recurrences, which may correlate with latent virus load in the trigeminal ganglia (TGs) (8, 43). Thus, reducing the level of HSV-1 latency may reduce recurrences, which in turn may alleviate recurrent eye disease, cold sores in the mouth, and genital lesions.

Because of the critical role that dendritic cells (DCs) play in orchestrating the immune response, there is an increasing interest in using signals that are known to activate DCs in order to improve vaccine efficacy. FMS-like tyrosine kinase 3 ligand (Flt3L) is known to be a potent stimulatory factor for DCs in vivo (31, 40). Flt3L is a growth factor that binds to early hematopoietic precursor cells in fetal liver or bone marrow (30,

48). Recently, it has been shown that a single immunization of mice with Flt3L DNA resulted in up to 44% of splenocytes becoming CD11c⁺ and the total number of DCs increasing by 100-fold (34). DC expansion effects lasted for more than 35 days. Similarly, treatment of adult humans with Flt3L resulted in increased numbers of biologically active DCs in peripheral tissues (29, 47). These findings suggest that Flt3L might be a useful adjuvant for use with an anti-HSV-1 vaccine.

The studies described in this report were undertaken to test the hypothesis that increasing the number of DCs in mice will decrease replication of HSV-1 during primary infection and reduce viral latency. We report here that neither stimulation nor depletion of DCs had any effect on virus replication in the eye or TG during primary ocular HSV-1 infection. Surprisingly, in contrast to what might have been expected, we found that immunization of mice with Flt3L DNA enhanced the number of DCs, which significantly increased the amount of latency in TGs of surviving mice, while depletion of DCs reduced the amount of latent virus in TGs. Finally, the increased HSV-1 latency in mouse TGs was associated with the presence of CD11c⁺ CD8α⁺ cells.

MATERIALS AND METHODS

Virus, cells, and mice. The triple-plaque-purified HSV-1 strain was grown in rabbit skin (RS) cell monolayers in minimal essential medium containing 5% fetal calf serum. McKrae, a stromal disease-causing, neurovirulent HSV-1 strain was the ocular challenge virus. Male and female hemizygous BALB/c-DTR [C.FVB-Tg (Itgax-DTR/GFP)57Lan/J] mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice carry a transgene encoding a simian diphtheria toxin (DT) receptor (DTR)-green fluorescent protein (GFP) fusion protein under the control of the murine *CD11c* promoter, which makes them sensitive to DC depletion with DT (23). BALB/c-DTR mice were bred at Cedars-Sinai Medical Center and housed in sterile microisolator units. Inbred BALB/c, C57BL/6, C57BL/6-CD4^{-/-}, C57BL/6-CD8^{-/-}, and C57BL/6-IFN-γ^{-/-} mice

* Corresponding author. Mailing address: Center for Neurobiology and Vaccine Development, D2024, Cedars-Sinai Burns and Allen Research Institute, 8700 Beverly Blvd., Los Angeles, CA 90048. Phone: (310) 423-0593. Fax: (310) 423-0302. E-mail: ghiasih@CSHS.org.

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were purchased from The Jackson Laboratory. C57BL/6-Flt3L^{-/-} mice and their C57BL/6 controls were obtained from Taconic (Germantown, NY). Age- and sex-matched mice used for experiments were between 6 and 8 weeks of age, and all animals were maintained under standard germfree housing conditions at the Cedars-Sinai Medical Center vivarium with the approval of the Institutional Animal Care and Use Committee.

Depletion of DCs. BALB/c-DTR mice were depleted of their DCs using 100 ng of DT in 100 μ l of phosphate-buffered saline (PBS) intraperitoneally 24 h before ocular infection and 2 and 5 days after ocular infection. As negative control, some mice were similarly injected with PBS alone and are referred to here as sham-depleted mice. Efficiency of DC depletion in corneas and spleens was monitored by fluorescence-activated cell sorter (FACS) analysis before ocular infection and 5 days after ocular infection. After the first depletion, more than 75% of DCs were depleted.

Depletion of macrophages. BALB/c-DTR mice were depleted of their macrophages using liposome encapsulation of dichloromethylene diphosphonate as we described previously (16). Depletion was done on days -5, -2, +1, +3, and +6 relative to ocular infection. Sham-depleted mice were similarly treated with PBS liposome. Efficiency of depletion was monitored by FACS analysis before and after ocular infection. After the third depletion, more than 80% of macrophages were depleted.

Immunization. Mice were immunized with Flt3L DNA as described previously (45). In a pilot experiment, mice were immunized intramuscularly using a 27-gauge needle into each quadriceps at 4 h, 3 days, or 10 days after ocular infection with 10 μ g of cesium chloride-purified DNA in a total volume of 100 μ l. On day 3, 5, 7, 10, and 20 postinfection (p.i.), the presence of GFP-positive DCs and CD8⁺ T cells in the corneas and spleens of different groups of mice were determined by FACS analysis and compared with results for control mice that were infected without immunization or immunized without infection. The highest level of DC stimulation was observed in mice immunized with Flt3L DNA and infected 3 days postimmunization. Thus, all the experiments described here with regard to Flt3L DNA immunization are based on ocular infection at 3 days postimmunization. As negative control for Flt3L DNA immunization, some mice were similarly injected with vector DNA alone and are referred to here as mock-immunized mice.

Ocular infection. Mice were infected ocularly with 2×10^4 PFU of HSV-1 strain McKrae per eye in 2 μ l of tissue culture medium without corneal scarification (17).

Monitoring of eye disease. The severity of corneal scarring (CS) in surviving mice was scored in a masked fashion by examination with a slit lamp biomicroscope as we described previously (15). Disease was scored on a scale of 0 to 4 (0, no disease; 1, 25% involvement; 2, 50% involvement; 3, 75% involvement; and 4, 100% involvement).

Monitoring of replication and clearance of HSV-1 from the eye. Tear films were collected from days 1 to 10 after ocular infection from both eyes of 30 mice per group from four separate experiments as described previously (15). Each swab was placed in 1 ml tissue culture medium, and the amount of virus in the medium was determined by a standard plaque assay on RS cells in six-well plates. The plates were incubated at 37°C for 2 days and stained with 1% crystal violet, and the viral plaques were counted.

Detection of infectious virus in TGs. BALB/c-DTR mice that were depleted of their DCs, immunized with Flt3L DNA, or mock immunized with vector DNA and sham depleted were infected ocularly with 2×10^4 PFU of McKrae per eye. On day 1, 3, or 5 p.i., the infected mice were euthanized, and the TGs from each mouse were combined. The TGs from each mouse were homogenized, and the debris was removed by centrifugation at 3,000 rpm for 10 min in a Beckman TA10 rotor. The viral titers in the supernatants of the TGs were measured on RS cells as described previously (19).

Isolation of DCs for transfer experiments. Six-weeks-old female BALB/c mice were used as a source of bone marrow for the generation of mouse DCs in cultures. Bone marrow cells were isolated by flushing femurs and tibiae with PBS. Pelleted cells were briefly resuspended in water to lyse red blood cells and stabilized by adding complete medium (RPMI 1640, 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine). The cells were centrifuged and resuspended in complete medium supplemented with either recombinant murine Flt3L (100 ng/ml; Peprotech, NJ) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/ml; Peprotech, NJ) to enhance replication of DCs (20). The cells were plated in plastic petri dishes (one bone per 10-cm dish) for 5 days, after which adherent cells were recovered and counted and each cell population was divided in half. Half of the isolated DCs from each group were treated with 100 μ g of anti-CD8 monoclonal antibody (clone 2.43; NCCC, Minneapolis, MN) per 1×10^7 DCs on ice for 1 h. Following treatment with anti-CD8 monoclonal antibody, DCs were washed three times

with medium containing no growth factor. Following DC depletion with DT, each recipient BALB/c-DTR mouse received 750,000 or 500,000 DCs that were grown in the presence of GM-CSF or Flt3L growth factor, respectively.

Virus replication in DCs. Monolayers of DCs were infected with 10 PFU/cell of HSV-1 strain McKrae. At 1 h after infection at 37°C, virus was removed, the infected cells were washed three times with fresh medium, and fresh medium was added to each well. The monolayers, including medium, were harvested at various times by freezing at -80°C. Virus was harvested by two cycles of freeze-thawing, and infectious virus titers were determined by standard plaque assays on RS cells as we previously described (18).

RNA extraction and cDNA synthesis. TGs from surviving mice on day 30 p.i. were collected, immersed in RNeasy lysis reagent, and stored at -80°C until processing. For RNA preparation, briefly, frozen tissue was resuspended in TRIzol reagent and homogenized, followed by addition of chloroform and subsequent precipitation using isopropanol. The RNA was then treated with DNase I to degrade any contaminating genomic DNA, followed by purification using a Qiagen RNeasy column as described by the manufacturer. RNA yield was determined by spectroscopy (NanoDrop ND-1000; NanoDrop Technologies, Inc., Wilmington, DE). On average, the RNA yield from trigeminal samples was 2.7 to 7.5 μ g. Finally, 1,000 ng of total RNA was retrotranscribed with random hexamer primers and murine leukemia virus reverse transcriptase contained in the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions.

TaqMan real-time PCR. The expression levels of the latency-associated transcript (LAT), CD8, gamma interferon (IFN- γ), Granzyme-A (GzmA), Granzyme-B (GzmB), and CD45 receptor (CD45R) splice variant (CD45RABC and CD45RO) target genes, along with the expression level of the endogenous control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, were evaluated using commercially available TaqMan gene expression assays (Applied Biosystems, Foster City, CA) containing optimized primer and probe concentrations. Primer probe sets consisted of two unlabeled PCR primers and the 6-carboxyfluorescein (FAM) dye-labeled TaqMan MGB probe formulated into a single mixture. Additionally, a probe for CD8 was designed to overlay an intron-exon junction to eliminate signal from any potential genomic DNA contamination. The assays used in this study were as follows: (i) CD8 (alpha chain) ABI assay Mm01182108_m1 (amplicon length, 67 bp), (ii) IFN- γ ABI assay Mm00801778_m1 (amplicon length, 101 bp), (iii) Granzyme-A ABI assay Mm00439190_m1 (amplicon length, 77 bp), (iv) Granzyme-B ABI assay Mm00442834_m1 (amplicon length, 95 bp), and (v) GAPDH ABI assay Mm999999.15_G1 (amplicon length, 107 bp). Additionally, custom-made primer and probe sets were as follows: (i) for HSV-1 LAT, forward primer 5'-GGGTGGGCTCGTGTTCAGTA-3', reverse primer 5'-GGACGGGTAAGTAACAGAGTCTCTA-3', and probe 5'-FAM-ACACCAGCCCGTCTTTT-3' (amplicon length, 81 bp); (ii) for CD45RABC (3/4 exons), forward primer 5'-TTTGTCACAGGGCAACACCTA-3', reverse primer 5'-CAGAGTGGATGGTGTAAAGAGTTGTG-3', and probe 5'-FAM-CACCCAGTGATGGTGCCAG-3' (amplicon length, 69 bp); and (iii) CD45RO (3/7 exons), forward primer 5'-TTTGTCACAGGGCAACACCTA-3', reverse primer 5'-GCAGTCATGTAGCGAAACCTGT-3', and probe, 5'-FAM-CACCCAGTGATGGGACTG-3' (amplicon length, 63 bp).

Quantitative real-time PCR was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) in 384-well plates, and all reactions were performed in a final volume of 20 μ l. Briefly, all mixtures contained 2 μ l of cDNA template, 1 $\times$ TaqMan universal PCR master mix (Applied Biosystems), and 1 $\times$ TaqMan gene expression assay. Universal thermal cycling conditions were as follows: after an initial 2-min hold at 50°C to allow AmpErase-UNG activity and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 1 min. Relative gene expression levels were normalized to the expression of the GAPDH housekeeping gene (endogenous control) and calculated using the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$ method), described in User Bulletin 2 provided with the ABI Prism 7900HT. The $\Delta\Delta C_T$ method utilizes the assumption that the efficiency of the target amplification and the efficiency of the endogenous control amplification are similar. Therefore, prior to utilizing this method, efficiencies were evaluated by generating cDNA dilution curves for each primer set. Plots of C_T values versus log cDNA concentrations were constructed, and the slopes were calculated using linear regression. The primer efficiency was determined by the following formula: efficiency = $10^{(-1/\text{slope})} - 1$. The calculated primer set efficiencies for all genes were between 0.95 and 0.98, indicating that the $\Delta\Delta C_T$ method was valid. Therefore, for a given tissue sample for each animal in the group, real-time PCR was performed in triplicate on the 7900HT system. The C_T value, which represents the PCR cycle at which there is a noticeable increase in the reporter fluorescence above baseline, was obtained using the SDS 2.2 software. In each experiment the average C_T value for the GAPDH reference gene was determined and the

FIG. 1. Role of DCs in virus replication and CS in infected mice. (A) Virus replication in mouse tears. BALB/c-DTR mice were immunized with Flt3L DNA, depleted of their DCs, or mock-immunized with vector DNA and then sham depleted with PBS. Mice were ocularly infected with HSV-1, tear films were collected from day 1 to 10 p.i., and virus titers were determined by standard plaque assays. Each point represents the mean titer for 60 eyes from four separate experiments $\pm$ standard error of the mean. (B) Virus replication in TGs. Mice, infected as described above, were euthanized on the indicated days, and virus titers were determined from TGs from each mouse. Each bar represents the mean $\pm$ standard error of the mean for the TGs, using a total of seven mice from two separate experiments. (C) Effect of depletion or stimulation of DCs on CS in ocularly in-

fective expression level, as the normalized fold change for each target gene (CD8, IFN- γ , GzmA, GzmB, CD45RABC, or CD45RO), was calculated.

In each experiment, an estimated relative copy number for the LAT target gene was calculated using standard curves generated from pGem-LAT5317-8330. Briefly, pGem-LAT5317-8330 DNA template was serially 10-fold diluted to contain from 10^3 to 10^{11} copies of the desired gene in a 5- μ l volume and subjected to TaqMan PCR with the same set of primers as used for test samples. By comparing the normalized C_T value of each sample to the C_T value of the standards, the copy number for each reaction was estimated.

PCR analysis. Individual mouse TGs were homogenized on day 30 p.i., and the cell pellet was used to detect viral DNA by PCR analysis. Briefly, the pellet was washed twice with PBS, and the TG pellets were suspended in 100 μ l of Tris-EDTA containing 0.1% sodium dodecyl sulfate and 100 μ g of proteinase K per ml. The mixture was incubated at 55°C for 16 h. The DNA was extracted, and TaqMan PCR was performed using custom-made HSV-1 gB primers (Applied Biosystems, Foster City, CA). The gB primers and probe used were as follows: forward primer, 5'-AACGCGACGCACATCAAG-3'; reverse primer, 5'-CTGG TACGCGATCAGAAAGC-3'; and probe, 5'-FAM-CAGCCGCAGTACTAC C-3'. The amplicon length for gB was 72 bp. In each experiment, an estimated relative copy number for the gB target gene was calculated using standard curves generated from pAc-gB1.

Statistical analysis. Protective parameters were analyzed by Student's *t* test and Fisher's exact test using Instat (GraphPad, San Diego, CA). Results were considered to be statistically significant when the *P* value was <0.05 .

RESULTS

Role of DCs in viral clearance from the eyes and TGs of mice during primary infection. Groups of 28 to 37 BALB/c-DTR mice from four separate experiments were depleted of their DCs with DT 1 day before ocular infection and 2 and 5 days after ocular infection or immunized with Flt3L DNA 3 days before ocular infection (to increase DCs). Tear films from infected mice were collected daily on days 1 to 10 p.i., and the amount of virus in each eye was determined by standard plaque assays. As negative controls, some mice were mock immunized with vector DNA and sham depleted of their DCs with PBS and are referred to here as the mock-immunized-sham-depleted group. DC-depleted mice, Flt3L-immunized mice, and mock-immunized-sham-depleted mice all had similar patterns of acute virus replication in the eye (Fig. 1A) ($P > 0.05$ by Student's *t* test). These results suggest that altering the level of DCs did not alter HSV-1 replication in mouse eyes.

To determine if altering DC levels would affect virus replication in TGs, 21 mice per group from two separate experiments were depleted of DCs, mock immunized and sham depleted, or immunized with Flt3L DNA as described above. On days 1, 3, and 5 p.i., seven mice per time point were sacrificed and TGs were harvested for analysis of infectious virus. On day 1 p.i., no virus was detected in TGs of any of the groups (Fig. 1B). On days 3 and 5 p.i., the amounts of virus detected in TGs were similar in all three groups (Fig. 1B) ($P > 0.05$). Thus, depletion or stimulation of DCs did not appear to alter virus replication in eyes or TGs during acute HSV-1 infection of mice.

Effect of DC depletion or Flt3L immunization on survival and CS. The survival and CS in ocularly infected mice that were used in the study described in Fig. 1A were measured on

infected mice. The CS score represents the average $\pm$ standard error of the mean from 20, 10, and 20 eyes for mock-immunized-sham-depleted, DC-depleted, and Flt3L-immunized mice, respectively.

day 30 p.i. Ten of 28 mock-immunized–sham-depleted mice (36%) survived the HSV-1 infection. In contrast, only 5 of 37 DC-depleted mice (14%) survived. This was significantly less survival than for the mock-immunized–sham-depleted group ($P = 0.043$ by Fisher's exact test). However, survival of Flt3L DNA-immunized mice (10 of 36 mice [28%]) was not significantly different from that of DC-depleted ($P = 0.16$) or mock-immunized–sham-depleted ($P = 0.59$) mice.

CS was measured in all of the mice that survived until day 30 after ocular infection. CS in DC-depleted and mock-immunized–sham-depleted mice was similar (Fig. 1C) ($P = 0.14$). Although CS appeared lower in mice immunized with Flt3L, this difference did not reach statistical significance compared with either the DC-depleted or mock-immunized–sham-depleted group (Fig. 1C) ($P > 0.14$ by Student's t test). These results suggest that in this study, DCs did not play a major role in survival or protection against CS in mice ocularly infected with HSV-1.

Effect of DC depletion or Flt3L immunization on latency in BALB/c-DTR mice. To determine if altering DC levels affected the amount of latency, BALB/c-DTR mice were depleted of DCs or immunized with Flt3L DNA as described above and the amount of LAT in individual mouse TGs was determined on day 30 p.i. (Fig. 2). The level of LAT RNA in TGs of Flt3L-immunized mice was approximately 60% higher than that in mock-immunized–sham-depleted mice, but this difference did not reach statistical significance (Fig. 2) ($P > 0.05$). However, as shown below (see Fig. 3), Flt3L immunization did significantly increase LAT in wild-type (wt) C57BL/6 mice. In DC-depleted mice LAT was decreased more than threefold compared with that in mock-immunized–sham-depleted mice and fivefold compared to that in Flt3L-immunized mice (Fig. 2) ($P < 0.05$). Thus, decreasing the number of DCs appeared to decrease the amount of latency in BALB/c-DTR mice.

Effect of Flt3L immunization on latency in CD4^{-/-}, CD8^{-/-}, IFN- γ ^{-/-}, and Flt3L^{-/-} mice. Recently, it was shown that CD8⁺ T cells (28), by expressing IFN- γ (27), can block ex vivo HSV-1 reactivation from latency in TGs and that CD8⁺ T cells provide active surveillance of HSV-1 gene expression in latently infected sensory neurons (24). Although in the studies described above we were focusing on the amount of latency rather than HSV-1 reactivation, since DCs are involved in cross-presenting antigens to T cells (2), it was of interest to examine the roles of CD4, CD8, and IFN- γ in the amount of latency.

CD4^{-/-}, CD8^{-/-}, and IFN- γ ^{-/-} mice and parental wt C57BL/6 mice were immunized with Flt3L DNA or mock immunized with vector DNA as described above. Flt3L immunization of wt C57BL/6 mice increased LAT in TGs of latently infected mice by more than threefold (Fig. 3) ($P < 0.05$ compared to mock-immunized C57BL/6 mice). LAT levels in mock-immunized IFN- γ ^{-/-} mice were similar to those in wt C57BL/6 mice (Fig. 3) ($P = 0.8$), and Flt3L immunization of IFN- γ ^{-/-} mice increased LAT levels during latency (Fig. 3) ($P < 0.05$). The level of LAT in mock-immunized CD4^{-/-} mice was significantly higher than that in mock-immunized wt or IFN- γ ^{-/-} mice (Fig. 3) ($P < 0.01$). Flt3L immunization of CD4^{-/-} mice appeared to further increase LAT levels by approximately 70%; however, this was not statistically significant (Fig. 3) ($P > 0.05$). Finally, the level of LAT in mock-immu-

FIG. 2. Detection of LAT transcript in TGs of latently infected BALB/c-DTR mice. BALB/c-DTR mice depleted of DCs, immunized with Flt3L, or mock immunized with vector DNA and sham depleted were ocularly infected with HSV-1. TGs from individual mice were isolated at 30 days p.i., and quantitative reverse transcription-PCR was performed. Each point represents the mean $\pm$ standard error of the mean from 10 mice.

nized CD8^{-/-} mice was significantly lower than that in mock-immunized wt, CD4^{-/-}, or IFN- γ ^{-/-} mice (Fig. 3) ($P < 0.05$) (note that the y axis scales for the CD8^{-/-} mice is different from that for the wt, IFN- γ ^{-/-}, and CD4^{-/-} mice). In addition, the amount of LAT in CD8^{-/-} mice immunized with Flt3L was not changed compared to that in mock-immunized CD8^{-/-} mice (Fig. 3) ($P = 0.9$). These results suggest that the amount of latency is unaltered in IFN- γ ^{-/-} mice, decreased in CD8^{-/-} mice, and increased in CD4^{-/-} mice.

Previously it was reported that Flt3L-deficient mice have reduced levels of both myeloid-related (CD11c⁺ CD8 α ⁻) and lymphoid-related (CD11c⁺ CD8 α ⁺) DCs (32). Since the results described above suggest that Flt3L immunization was associated with an increase in the amount of latency in wt mice, it was of interest to look at latency levels in Flt3L^{-/-} mice. Flt3L^{-/-} mice were immunized with Flt3L or mock immunized as described above. Mock-immunized Flt3L^{-/-} mice had significantly lower levels of LAT than CD4^{-/-}, CD8^{-/-}, IFN- γ ^{-/-}, and wt C57BL/6 mice (Fig. 3) ($P < 0.05$). As expected, Flt3L immunization of Flt3L^{-/-} mice significantly increased LAT levels (Fig. 3) ($P < 0.01$). Thus, the absence of Flt3L in Flt3L-deficient mice appeared to reduce latency.

Effect of DC depletion or Flt3L immunization on the level of gB DNA in latently infected mice TG. To confirm that the higher level of LAT RNA transcript in latently infected mice correlates with a higher level of viral DNA, Flt3L^{-/-}, wt C57BL/6, and BALB/c-DTR mice were immunized with Flt3L DNA as described above. In addition, some of the BALB/c-DTR mice were depleted of DCs, as described in Materials and Methods. The mock-immunized or mock-immunized–sham-depleted mice were used as controls for Flt3L DNA-

FIG. 3. Detection of LAT in knockout mice following Flt3L immunization. CD4 $^{-/-}$, CD8 $^{-/-}$, IFN- $\gamma^{-/-}$, Flt3L $^{-/-}$, and their parental wt C57BL/6 mice were immunized with Flt3L DNA or mock immunized with vector DNA as for Fig. 2. Mice were ocularly infected with HSV-1, and quantitative reverse transcription-PCR was performed to assay LAT expression. Each point represents the mean $\pm$ standard error of the mean from 5 mice (for the CD4 $^{-/-}$, CD8 $^{-/-}$, and IFN- $\gamma^{-/-}$ groups), 10 mice (for the Flt3L $^{-/-}$ group), or 15 mice (for the C57BL/6 group).

immunized or DC depleted mice, respectively. Following Flt3L immunization or DC depletion, the mice were ocularly infected with HSV-1 strain McKrae, and TGs from surviving mice were isolated individually on day 30 after ocular infection. TaqMan PCR was performed on the total individual TG DNA, and the level of gB DNA for each group was determined as described in Materials and Methods. The data for the Flt3L-immunized and DC-depleted groups were calculated as the fold increase or decrease in relation to that of their respective control group (Fig. 4). The Flt3L $^{-/-}$, wt C57BL/6, and BALB/c-DTR mice that were immunized with Flt3L DNA had approximately 0.8- to 2.2-fold-higher levels of gB DNA than their control counterparts (Fig. 4). Conversely, the level of gB DNA was approximately twofold lower in DC-depleted mice than in their control group (Fig. 4). These results suggest that the expansion of DCs following immunization with Flt3L DNA increased the amount of HSV-1 DNA in TGs of latently infected mice, while DC depletion significantly reduced the amount of latency in latently infected TGs. These results are in agreement with the above data regarding the level of LAT expression in TGs of latently infected mice.

Effect of CD11 $^{+}$ CD8 α^{-} and CD11 $^{+}$ CD8 α^{+} cells on the expression of LAT transcript in ocularly infected mice. DCs are divided into myeloid-related (CD11c $^{+}$ CD8 α^{-}) DCs and lymphoid-related (CD11c $^{+}$ CD8 α^{+}) DCs. Since in the experiment described above CD8 $^{-/-}$ mice had reduced LAT levels, it was of interest to determine if the increased LAT levels following Flt3L immunization were due to increased lymphoid-related (i.e., CD8 α^{+}) DCs rather than CD8 α^{-} DCs. Mice were depleted once of their DCs, and then either CD11c $^{+}$ CD8 α^{+} or CD11c $^{+}$ CD8 α^{-} DCs were adoptively transferred as described in Materials and Methods. The level of LAT RNA in mice that received CD11c $^{+}$ CD8 α^{+} cells was at least 100-fold higher than the LAT RNA level in mice that received CD11c $^{+}$

FIG. 4. Effect of DC stimulation or depletion on the level of gB DNA in TGs of latently infected mice. Flt3L $^{-/-}$ and their parental wt C57BL/6 mice or BALB/c-DTR mice were immunized with Flt3L DNA or mock immunized with vector DNA as described for Fig. 2. Some of the mock-immunized BALB/c-DTR mice were depleted of DCs or sham depleted as described in Materials and Methods. Mice were ocularly infected with HSV-1, and TGs from infected mice were removed individually at autopsy on day 30 p.i. TaqMan PCR of total DNA isolated from each mouse TG was performed as described in Materials and Methods using gB primers. GAPDH expression was used to normalize the relative expression of gB DNA in TGs. The levels of gB DNA in Flt3L-immunized mice or DC-depleted mice are shown relative to the level of gB DNA in the mock-immunized mice (horizontal line). Each point represents the mean $\pm$ standard error of the mean from 10 TGs.

FIG. 5. LAT expression in latently infected mice following adoptive transfer of CD11⁺CD8α⁺ or CD11⁺CD8α⁻ cells. Quantitative reverse transcription-PCR to determine the copy number of the LAT transcript was performed on RNA isolated from the TGs of surviving BALB/c-DTR mice that had been adoptively transferred with DCs grown in the presence of GM-CSF or Flt3L. Mock-transferred mice were used as controls. Each point represents the mean $\pm$ standard error of the mean from experiments with seven mice.

CD8α⁻ cells and approximately 10-fold higher than that in sham-depleted control mice (Fig. 5). Thus, adoptive transfer of CD11c⁺ CD8α⁺ cells appeared to significantly increase latency, while transfer of CD11c⁺ CD8α⁻ cells appeared to reduce latency.

HSV-1 does not replicate in DCs. DCs were isolated from BALB/c mice, expanded by culturing in the presence of recombinant Flt3L or GM-CSF growth factor, and infected with 10 PFU/cell of HSV-1 strain McKrae, as described in Materials and Methods. The kinetics of virus replication were measured by determining the amount of infectious virus at various times p.i. using a plaque assay as described above. The amount of HSV-1 recovered from DCs expanded with Flt3L declined between all time points examined (Fig. 6). The amount of HSV-1 recovered from DCs expanded with GM-CSF remained constant for the first 24 h and then declined (Fig. 6). These results suggest that HSV-1 did not replicate efficiently in DCs. This is consistent with previous studies showing that human DCs are nonpermissive for HSV-1 infection (1, 46, 51). In addition, the lack of HSV-1 replication in DCs suggests that increased infectivity of DCs is not responsible for the increased latency seen in any of the experiments described above.

Effect of macrophage depletion on the establishment of latent infection. BALB/c-DTR mice were depleted of their macrophages on days -5, -2, +1, +3, and +6 relative to ocular infection with HSV-1, and LAT levels were determined as described above. Although LAT appeared to increase about twofold in macrophage-depleted mice (Fig. 7), the differences

FIG. 6. Replication of HSV-1 in DCs isolated from BALB/c mice. Subconfluent monolayers of DCs grown in the presence of recombinant GM-CSF or Flt3L as a growth factor were infected with 10 PFU per cell of McKrae, and virus allowed to attach for 1 h at 37°C. Monolayers were washed three times, and the total virus remaining associated at each time for each cell type was determined by plaque assay as described in Materials and Methods. Each point represents the mean $\pm$ standard error of the mean ($n = 16$) from two separate experiments.

did not reach statistical significance ($P > 0.05$). Thus, in contrast to DCs, macrophages did not appear to play a major role in decreasing latency.

Expression of GzmA, GzmB, IFN-γ, CD8, and CD45 mRNAs in TGs of latently infected mice. In vitro experiments showed that compared to the CD11c⁺ CD8α⁻ subset of DCs, the CD11c⁺ CD8α⁺ subset induces stronger IFN-γ and apoptosis responses and has an inhibitory effect on CD8⁺ T-cell cytokine production (25, 36, 42, 50). To determine whether the amounts of CD8, IFN-γ, GzmA, and GzmB mRNAs in TGs were affected by altering the number of DCs, we performed real-time PCR analysis of TG RNA in mice that were depleted of their DCs with DT, immunized with Flt3L DNA to increase the number of DCs, or received CD11c⁺ CD8α⁻ or CD11c⁺ CD8α⁺ by adoptive transfer experiments. We also determined the effect of these treatments on the relative levels of

FIG. 7. Effect of macrophage depletion on the levels of LAT transcript in TGs of latently infected mice. BALB/c-DTR mice were depleted of their macrophages and ocularly infected with HSV-1. TGs from individual mice were isolated on day 30 p.i., and quantitative reverse transcription-PCR was performed on total RNA. Each point represents the mean $\pm$ standard error of the mean from five mice.

CD45RABC and CD45RO to estimate the CD8 T-cell activation state (Fig. 8).

Mock-immunized–sham-depleted mice, Flt3L-immunized mice, DC-depleted mice, and DC depleted mice that received CD11c⁺ CD8 α ⁺ cells had similar levels of CD8 mRNA in their TGs, while DC-depleted mice that received CD11c⁺ CD8 α [−] cells had significantly reduced levels of CD8 mRNA (Fig. 8A). Mock-immunized–sham-depleted and Flt3L DNA-immunized mice had similar levels of IFN- γ mRNA (Fig. 8B). DC depletion appeared to decrease IFN- γ mRNA levels, and these levels may have been further reduced by transfer of CD11c⁺ CD8 α [−] cells to the DC-depleted mice (Fig. 8B). In contrast, transfer of CD11c⁺ CD8 α ⁺ cells increased IFN- γ mRNA levels by 2.5-fold compared to those in the mock-immunized–sham-depleted group (Fig. 8B). Similar to the results with the CD8 and IFN- γ mRNA transcripts, the levels of GzmA (Fig. 8C) and GzmB (Fig. 8D) mRNA transcripts appeared to be reduced in TGs from the DC-depleted mice and were lowest in the DC-depleted mice that received CD11c⁺ CD8 α [−] cells. Compared to mock-immunization–sham-depletion treatment, neither GzmA nor GzmB mRNA levels appeared to be altered by Flt3L immunization or by DC depletion followed by transfer of CD11c⁺ CD8 α ⁺ cells. The level of CD45RABC mRNA appeared slightly higher in the CD11c⁺ CD8 α ⁺-treated group than in the mock-immunized–sham-depleted or Flt3L-immunized group. DC depletion or DC depletion followed by CD11c⁺ CD8 α [−] transfer appeared to slightly decrease CD45 RABC mRNA (Fig. 8E). For CD45RO mRNA, the DC-depleted mice that received CD11c⁺ CD8 α ⁺ cells had very high levels of LAT compared to all the other groups (Fig. 8F). The transfer experiments shown in Fig. 8 were done using DCs expanded ex vivo by GM-CSF. Similar results were obtained with DCs expanded ex vivo using Flt3L (data not shown). Finally, we did not find any significant changes to CD62L, CCR7, major histocompatibility complex (MHC) class I, MHC class II α , or MHC class II β mRNA transcripts in mice treated as described above (data not shown). Thus, our results suggest that a decrease in the level of LAT RNA following adoptive transfer of CD11c⁺ CD8 α [−] cells correlated with a decrease in the expression of CD8, IFN- γ , GzmA, GzmB, and CD45RABC mRNAs but not CD45RO mRNA.

DISCUSSION

Using signals that are known to activate DCs to control various pathogens is gaining widespread interest due to the critical role that DCs play in orchestrating the immune response. Both Flt3L and GM-CSF have been used extensively as molecular adjuvants to increase and stimulate DC subsets in vivo (29–31, 40, 47, 48). However, the negative aspects of DC proliferation/stimulation have received little scrutiny in favor of reports of the positive roles that DCs may play when they are used immunotherapeutically. Unlike recovery from infection due to viruses such as measles virus or poliovirus, primary infection with HSV-1 does not prevent recurrent infection at the original site (11, 37, 39). In an individual with a history of ocular HSV-1 infection, eye disease is induced mainly by reactivation of virus from latency (5, 10). The lack of a completely protective immune response following HSV-1 infection complicates vaccine development. Therefore, understanding

immune factors that contribute to virus clearance from TGs of infected individuals during HSV-1 infection may help reduce the incidence of eye disease resulting from reactivation of latent virus. As sentinels of the immune system, DCs are at the crossroads of innate and adaptive immunity. In this study, we investigated the effect of altering DCs on the amount of latent HSV-1 as judged by relative levels of LAT RNA and gB DNA in TGs of latently infected mice.

We found that DC depletion appeared to reduce the establishment of latency in TGs of infected mice, while increasing DCs by Flt3L immunization increased latency. DC alterations did not significantly alter eye disease or virus replication in the eye or TGs during primary HSV-1 infection, suggesting that changes in the level of latency were not due to altered virus replication during the acute infection. Previously it was shown that DCs can play a deleterious role in HIV-1 (14, 22), dengue virus (56), and vaccinia virus (12) infections. Although we did not find that DCs played a negative role during acute HSV-1 infection, DCs appeared to have a negative impact on HSV-1 infection because they increased latency. In mice, CD11c⁺ DCs are classified as CD11c⁺ CD8 α ⁺ or CD11c⁺ CD8 α [−] (3, 6, 53). These cells have different functions in terms of T-cell stimulation (21, 35, 52), their requirement for different cytokines for propagation (38), their ability to induce T_H1 or T_H2 responses (36), and their anatomical distribution (33). In this study, transfer of CD11c⁺ CD8 α ⁺ cells to recipient mice that had been depleted of their DCs significantly enhanced latency in TGs of infected mice, while transfer of CD11c⁺ CD8 α [−] cells reduced latency in infected mice. It has been reported that CD8 α [−] DCs capture antigens more efficiently than CD8 α ⁺ DCs (9, 41, 44). Thus, compared to CD11c⁺ CD8 α ⁺ DCs, CD11c⁺ CD8 α [−] DCs may induce a more robust T_H1 response in TGs, leading to faster clearance of virus and reduced latency, while CD11c⁺ CD8 α ⁺ DCs may induce a weaker immune response and thus reduced virus clearance and increased latency.

We also found the following. (i) Decreased CD8 correlated with decreased LAT, since both CD8 and LAT decreased following adoptive transfer of CD8 α [−] DCs and since latently infected CD8^{−/−} mice had decreased LAT compared to wt mice. (ii) Increased IFN- γ appeared to correlate with increased LAT in DC transfer experiments, and decreased IFN- γ appeared to correlate with decreased LAT in DC depletion experiments. However, since latently infected IFN- γ ^{−/−} mice had LAT levels indistinguishable from those in their wt counterparts, it is likely that IFN- γ was not involved but just happened to change in the same direction as LAT levels following DC depletion and transfer experiments. (iii) Decreased GzmA and GzmB mRNA levels correlated with decreased LAT RNA levels following DC depletion and CD8 α [−] DC transfer.

Overall, in these studies, decreased LAT expression in TGs and hence decreased latency appeared to be associated with depletion of DCs and adoptive transfer of CD8 α [−] DCs, while adoptive transfer of CD8 α ⁺ DCs increased latency. Generally, persistent or latent infections are often characterized by various degrees of functional impairment of virus-specific T-cell responses, and this defect is a main factor in the inability of the host to eliminate the pathogen. Recently, it was reported that exhaustion of CD8⁺ T cells is the main factor leading to per-

FIG. 8. Effect of treatments with DCs on the levels of CD8, IFN- γ , GzmA, GzmB, CD45RABC, and CD45RO transcripts in TGs of latently infected mice. TGs of surviving mice from the experiments described for Fig. 2 and 5 were isolated individually on day 30 p.i., and quantitative reverse transcription-PCR was performed on total RNA. CD8 (A), IFN- γ (B), GzmA (C), GzmB (D), CD45RABC (E), and CD45RO (F) expression in naive mice was used to estimate the relative expression of each transcript in TGs of treated mice. Each point represents the mean $\pm$ standard error of the mean from five mice.

sistent viral infection (4). The CD8⁺ T-cell impairment was associated with upregulation of PD-1 (programmed death 1; also known as Pdc1) gene expression. In addition, in vivo administration of antibodies that blocked the interaction of this inhibitory receptor with its ligand, PD-L1 (also known as B7-H1), enhanced T-cell responses and viral clearance (4). Similarly, in this study we have demonstrated that increased latency is associated with higher expression of transcripts associated with granule-mediated cytotoxicity, including abundant GzmA, GzmB, and IFN- γ . Consequently, lower levels of these transcripts may serve as an important autoprotective mechanism to preserve the integrity of T cells, leading to a more efficient surveillance capacity and ability of T cells to clear infectious virus more efficiently, thus leading to a reduction in latency. This is consistent with our observations that CD11c⁺ CD8 α ⁺ cells appeared to play a role in increasing latency in ocularly infected mice, whereas CD11c⁺ CD8 α ⁻ cells appeared to reduce latency. Thus, any vaccination strategy that stimulates the wrong subpopulation of DCs may hinder rather than improve overall vaccine efficacy against HSV. Previously, it has been shown that polyethylene glycol (PEG)-modified GM-CSF [PEG-(GM-CSF)] expands the CD8 α ⁻ DC population but not the CD8 α ⁺ DC population in vivo (9, 42). We are therefore pursuing studies to determine if PEG-(GM-CSF) will reduce latency.

In conclusion, our results suggest that CD11c⁺ CD8 α ⁺ DCs either directly or indirectly result in increased latency, while CD11c⁺ CD8 α ⁻ DCs either directly or indirectly result in decreased latency. One possible mechanism is that during the acute infection, CD11c⁺ CD8 α ⁺ DCs suppress or interfere with one or more immune factors. This results in decreased virus clearance from TGs and increased latency. In contrast, CD11c⁺ CD8 α ⁻ DCs stimulate or enhance one or more immune factors, which results in more efficient virus clearance from the TG and decreased latency.

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