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Dendritic Cell Activation and Memory Cell Development Are Impaired among Mice Administered Medroxyprogesterone Acetate Prior to Mucosal Herpes Simplex Virus Type 1 Infection

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Epidemiological studies indicate that the exogenous sex steroid medroxyprogesterone acetate (MPA) can impair cell-mediated immunity, but mechanisms responsible for this observation are not well defined. In this study, MPA administered to mice 1 wk prior to HSV type 1 (HSV-1) infection of their corneal mucosa impaired initial expansion of viral-specific effector and memory precursor T cells and reduced the number of virus-specific memory T cells found in latently infected mice. MPA treatment also dampened expression of the costimulatory molecules CD40, CD70, and CD80 by dendritic cells (DC) in lymph nodes draining acute infection, whereas coculture of such DC with T cells from uninfected mice dramatically impaired ex vivo T cell proliferation compared with the use of DC from mice that did not receive MPA prior to HSV-1 infection. In addition, T cell expansion was comparable to that seen in untreated controls if MPA-treated mice were administered recombinant soluble CD154 (CD40L) concomitant with their mucosal infection. In contrast, the immunomodulatory effects of MPA were infection site dependent, because MPA-treated mice exhibited normal expansion of virus-specific T cells when infection was systemic rather than mucosal. Taken together, our results reveal that the administration of MPA prior to viral infection of mucosal tissues impairs DC activation, virus-specific T cell expansion, and development of virus-specific immunological memory. The Journal of Immunology, 2012, 189: 3449–3461.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ACV, acyclovir; B6, C57BL/6J; DLN, draining lymph node; DMPA, depot-medroxyprogesterone acetate; dpi, days postinfection; GzmB, granzyme B; HSV, HSV type 2; iMFI, integrated median fluorescence intensity; MHC-II, MHC class II; ovx, ovariectomized; RIA, radioimmunoassay; rCD154, recombinant soluble CD154; TG, trigeminal ganglia; WT, wild-type.

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**Mice**

WT and ovariectomized (ovx) female C57BL/6J (B6) mice (CD90.2⁺) and female BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). gBT-I.1 mice on a B6.PL background (CD90.1⁺) were a gift from F. Carbone (University of Melbourne, VIC, Australia), and CD154⁻/⁻ mice on a BALB/c background were a gift from Richard Flavell (Yale University, New Haven, CT).

**FIGURE 1.** Pretreatment reduces T cell expansion elicited by HSV-1 corneal infection. ovx B6 mice were corneally infected with 10⁵ PFU HSV-1 7 d after the insertion of 21-d sustained release pellets containing 50 mg MPA or matching placebo. Mice were euthanized at indicated time points, and various tissues were excised to enumerate infiltrating virus-specific T cells by flow cytometry. (A) Absolute numbers of CD4⁺, CD8⁺, gB98-505-specific CD8⁺, and CD127⁺ (memory precursors) T cell subsets in TG 8 dpi. (B) Relative frequency of gB98-505-specific CD8⁺ and CD127⁺ T cells in TG 8 dpi. Data in (A) and (B) were pooled from three independent experiments (n = 18/group). (C) Absolute numbers of gB98-505-specific CD8⁺ T cells in DLN at 5 and 8 dpi (n = 8–13/group). (D) Absolute numbers of NK cells, NKT cells, and macrophages in TG 8 dpi (n = 10 per group). (E) Total number of live CD45⁺ cells (LIVE/DEAD⁺) in TG at 8, 15, and 35 dpi (n = 6–18/group). (F) Absolute numbers of CD4⁺, CD8⁺, and gB98-505-specific CD8⁺ T cells in TG 15 and 35 dpi (pellets removed 14 dpi) (n = 6–18/group). Data in (C)–(F) were pooled from two independent experiments for each panel. Comparisons were made using an unpaired two-tailed Student t test, except for comparisons of the number of gB98-505-specific CD8⁺ and CD4⁺ T cells per TG in (A), which were made using an unpaired Mann–Whitney U test (horizontal bars accordingly indicate mean and median values).
In vivo procedures

The HSV-1 corneal infection model we used is a well-accepted model of mucosal tissue infection (14). Mice were anesthetized by i.p. injection of 1.8 mg ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, IA) and 0.18 mg xylazine (Lloyd Laboratories, Shenandoah, IA) and implanted with 21-d sustained release pellets containing 50 mg MPA or matching placebo (Innovative Research of America, Sarasota, FL) in supraocular s.c. tissue. Elsewhere, 1 or 4 mg DMPA (Pfizer, New York, NY) was injected s.c. These doses were selected to approximate serum progesterone levels detected in human pregnancy and serum MPA levels achieved by Depo-Provera injection for prevention of undesired pregnancy. One week after pellet placement or DMPA injection, mouse corneas were scarified and infected bilaterally with 10^4 PFU WT HSV-1 (RE strain). Other mice were infected conjunctivally with 10^6 PFU or i.v. or i.p. with 10^6 PFU HSV-1. Draining lymph nodes (DLN), spleens, and trigeminal ganglia (TG) were excised at various days postinfection (dpi) to assess the effects of pretreatment on expansion or function of various leukocyte subpopulations. Where indicated, pellets were removed 14 dpi, and DLN, spleens, and TG were excised ≥35 dpi to measure the effects of pretreatment on memory cell development. To assess in vivo T cell proliferation, mice were injected i.v. with 1 mg BrdU (BD Biosciences, San Diego, CA) 4 h before euthanasia. Effects of MPA on HSV-1 replication were assessed in TG, corneal tissue, and tear film collected with WECK-CEL Surgical Spears (Medtronic Xomed, Jacksonville, FL). Where indicated, pretreated mice were administered 5 μg mouse recombinant soluble CD154 (rCD154) i.p. (eBioscience, San Diego, CA) concomitant with infection, and untreated mice were administered 250 μg blocking anti-CD154 Ab i.p. (clone MR1), both 1 d before infection and 2 dpi. As indicated, 0.7 mg acyclovir (ACV) was administered i.v. and 12 h post-infection. In other experiments, mice 3 dpi were treated i.p. with the indicated doses of anti-CD8 mAb (2.43; BioXcell). As indicated, intact mice that had received 50 μg MPA pellets or 1 or 4 mg DMPA were sacrificed 7 d later to measure serum MPA levels with a radioimmunoassay (RIA) that was used in accordance with the manufacturer’s instructions (Immunometrics, London, U.K.).

Ex vivo and in vitro procedures

For T cell stimulation assays, TG were excised 8 or 35 dpi and dissociated into single-cell suspensions with collagenase type I (Sigma-Aldrich, St. Louis, MO). Cells were stimulated for 6 h with B6WT3 fibroblast targets (previously incubated 12 h with HSV-1) in the presence of FITC-conjugated anti-CD107a (1D4B; BD Biosciences) and GolgiPlug (BD Biosciences) (15). Cells were stained as indicated in the flow cytometric analysis section. For cell proliferation assays, BrdU (BD Biosciences) staining of DLN and TG single-cell suspensions was done according to the manufacturer’s instructions. To characterize TG and DLN lymphocytic infiltrates or dendritic cell (DC) subpopulations in DLN of mice, single-cell suspensions were stained as indicated in the flow cytometric analysis section. To measure apoptosis levels in T cells from DLN, CaspaseTag pan-caspase assay kits (Millipore, Billerica, MA) were used according to the manufacturer’s instructions. For T cell proliferation assays, DLN 2 dpi were digested with collagenase D and DNase I (both Sigma-Aldrich), and resultant single-cell suspensions were enriched for CD11c+ cells by MACS (Miltenyi Biotec, Auburn, CA). Splenocytes from gBT-I.1 mice were processed into single-cell suspensions, enriched for CD8+ cells using MACS (Miltenyi Biotec), and labeled using a CellTrace Violet Cell ProLiferation Kit (Invitrogen). A total of 2.5 × 10^6 CD11c+ cells were cocultured with 5 × 10^4 labeled gBT-I.1 cells for 72 h in 200 μl RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, gentamicin, penicillin, and streptomycin. Proliferation of the CD8+/CD90.1+Vα2+ splenocytes was determined using flow cytometry.

Flow cytometric analysis

In ex vivo T cell stimulation assays, cells were stained with Live/Dead fixable aqua dead cell stain (Invitrogen, Carlsbad, CA), PerCP-conjugated anti-CD45 (50-F11; BD Biosciences), and Pacific Blue-conjugated anti-CD8α (53-6.7; BD Biosciences). Following cell fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences) and Perm/Wash buffer (BD Biosciences), intracellular staining with allophycocyanin-conjugated anti-IFN-γ (XMG1.2; BD Biosciences) and PE-conjugated anti-TNF (MP6- X32; BD Biosciences) was performed. To characterize TG and mucosal lymphocytic infiltrates at various days postinfection, single-cell suspensions were stained with Live/Dead fixable aqua dead cell stain, blocked with anti-CD16/32 mAb (2.4G2, BD Biosciences), and incubated with combinations of the following mAb as indicated: FITC-, PE-, PE-Cy7-, PerCP-, or allophycocyanin-conjugated mAb against CD11b (M1/70), CD25 (PC61), CD44 (IM7), CD45 (30-F11), CD62L (MEL-14), CD90.1 (53-6.7), CD122 (TM-1), CD130 (s4B7), CD138 (CXCR3-173), NK1.1 (PK136), and Vε2 TCR (B20.1) (BD Biosciences); PE-, PE-Cy7-, PerCP-Cy5.5-, allophycocyanin-, allophycocyanin–eF750-, AF700-, and eF450-conjugated mAbs against CD3ε (17A2 or eBio500A2), CD4 (GK1.5 or RM4-5), CD8α (53-6.7), CD8β (eBio3H-35-17.2), CD11b (M1/70), CD11c (N418), CD28 (37.51), CD45 (30-F11), CD69 (H.2F3), CD154 (MR1), and MHC class II (MHC-II) (M5/14.15.2) (eBioscience); or allophycocyanin-conjugated anti-CD27 (LG.3A10), FITC- or PerCP-conjugated CD43 (1B11), and PE-Cy7–conjugated CD90.2 (53-2.1) (Bio-Legend, San Diego, CA); allophycocyanin-conjugated F4/80 (BM8) (Invitrogen); or PE-conjugated H-2Kβ tetramers (National Institute of Allergy and Infectious Diseases tetramer facility) that contained gB99-505 (SSIEEPARL) peptide. After incubation with surface mAbs, cells were fixed with Cytofix or Cytofix/Cytoperm reagent (BD Biosciences). As indicated, cells were intracellularly stained with allophycocyanin-conjugated anti-human granzyme B (GzmB) (GB12) (Invitrogen). To examine DC subpopulations in DLN of mice 2 dpi, single-cell suspensions were stained with FITC-, PE-, PE-Cy7-, or allophycocyanin-conjugated mAbs against CD40 (3/23), CD45R (RA3-GB2), CD80 (16-10A1), and MHC-I (H-2Kβ).

FIGURE 2. Pretreatment impairs expansion of virus-specific CD8+ T cells induced by HSV-1 corneal infection in a dose-dependent manner. (A) Intact B6 mice were implanted with 21-d sustained release pellets containing 50 mg MPA, administered 1 or 4 mg DMPA, or were left untreated. Seven days later, all groups were corneally infected with 10^6 PFU HSV-V1. Mice were euthanized 8 dpi and TG excised to enumerate infiltrating HSV-1–specific T cells by flow cytometry. Absolute numbers of CD8+ T cells per TG are shown (n = 20–25/group) (data shown pooled from three independent experiments). Of note, the numbers of CD8+ and gB99-505–specific CD8+ T cells were similarly reduced by pretreatment (data not shown). (B) Groups of mice administered MPA in exactly the same fashion as in (A) were sacrificed 7 d later to determine serum levels of MPA by RIA (n = 5–8/group) (**p < 0.01, ***p < 0.001 by one-way ANOVA and Tukey’s multiple comparison posttest; horizontal bars indicate mean values).
FIGURE 3. Reduced CD40 expression by pretreated mice inhibits T cell expansion. Untreated B6 mice administered anti-CD154 mAb and pretreated (4 mg DMPA) B6 mice were corneally infected with 10^5 PFU HSV-1 (another group of pretreated mice were also administered rsCD154 concomitant with infection as indicated). Mice were euthanized 8 dpi, and TG were excised to interrogate T cell infiltrates by flow cytometry. (A) Absolute numbers of total CD45+, CD8+, and CD4+ T cells are shown (n = 13–15/group). (B) Panel depicts percentages of CD8+ T cells that expressed GzmB (n = 10/group). (C) Relative expression of CD69 by CD4+ and CD8+ T cells (n = 10/group) (data shown are pooled from two independent experiments). (D–G) To confirm results above using anti-CD154 mAb, we corneally infected untreated BALB/cJ controls, pretreated BALB/cJ mice, and CD154−/− BALB/cJ mice with 10^5 PFU HSV-1 and harvested TG 10 dpi (n = 10/group). (D) Absolute numbers of TG-resident CD45+ cells and CD8+ and CD4+ T cells. (E) Percentage of CD8+ T cells that express GzmB in TG. (F) Relative expression of CD69 by CD8+ and CD4+ T cells in TG 10 dpi (data are pooled from two independent experiments) (*p < 0.05, **p < 0.01, ***p < 0.0001 by one-way ANOVA and Tukey’s multiple comparison posttest; horizontal bars indicate means). (G) Contour plots show CD69 and GzmB expression by CD8+ T cells infiltrating TG during acute infection of untreated BALB/cJ, pretreated BALB/cJ, and CD154−/− BALB/c mice (representative data; numbers indicate percentages in each quadrant).
AF6-88.5) (BD Biosciences); or PE–, PE–Cy7–, PerCP–Cy5.5–, allophycocyanin–, and eF450-conjugated mAbs against CD11b (M1/70), CD11c (N418), CD8α (53-6.7), CD70 (FR70), CD86 (GL1), CD317 (eBio129c), and MHC-II (I-A/I-E, M5/111.15.2) (eBioscience). Samples processed for flow cytometric analysis were run on a FACSAria flow cytometer (BD Biosciences), and data were analyzed using FACS Diva (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR). As previously described, integrated median fluorescence intensity (iMFI) was calculated from percentage of positive cells × MFI (16).

**HSV replication assays**

To determine multistep in vitro growth kinetics, Vero cells infected with HSV-1 at multiplicity of infection of 0.01 were incubated for 4, 12, 24, and 48 h, and cells and supernatants were harvested for quantification of replicating virus by standard plaque assay. To quantify HSV-1 titers in tear film, surgical spear contents were transferred to PBS, and replicating virus were titrated by standard plaque assay. For quantification of corneal HSV-1 transcripts, whole corneas were excised, total RNA was extracted using RNeasy plus mini kit (Qiagen, Germantown, MD), and cDNA was generated with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA), both according to the manufacturers’ instructions. Quantitative real-time PCR assays (Applied Biosystems) for the murine housekeeping gene encoding pyruvate carboxylase (PCX) and HSV-1 α (immediate early) genes ICP0 and ICP4 and γ2 (late) gene gH, and quantification of genomic copies of HSV-1 in infected TG were performed as described previously (17, 18).

**Statistical considerations**

Statistical analyses were performed using Prism 5 software (GraphPad, La Jolla, CA). Normality of the data was tested using the D'Agostino–Pearson

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**FIGURE 4.** Pretreatment diminishes virus-specific CD8⁺ T cell effector function. Intact B6 mice given 4 mg DMPA 7d prior to corneal infection with 10⁵ PFU HSV-1 were euthanized 8 dpi. Excised TG were dispersed into single-cell suspensions and stimulated with HSV-1–infected targets to interrogate T cell effector function by flow cytometry. (A) Depiction of response profiles using Boolean analysis of three canonical CD8⁺ T cell effector functions (IFN-γ and TNF production and lytic activity as measured by CD107a surface expression) (n = 5/group). (B) Pie charts compiled from bar graph data in (A) illustrate percentages of CD8⁺ cells that expressed 3, 2, 1, or 0 effector function markers (beginning at “12 o’clock” and moving clockwise). Data shown in (A) and (B) are representative of two independent experiments (comparisons made using SPICE 5.2 (59); horizontal bars and pie fractions indicate means). (C) Contour plots display expression of IFN-γ, TNF, and CD107a by TG-resident CD8⁺ T cells stimulated ex vivo with infected targets. Data shown are representative results from each experimental group, and numbers indicate percentages within each quadrant.
omnibus test. Differences between two groups were compared by an unpaired Student $t$ test or an unpaired Mann–Whitney $U$ test, depending on distribution of the data. For comparison of multiple groups, depending on distribution of the data, one-way ANOVA and Tukey’s multiple comparison post hoc test or the Kruskal–Wallis test on ranks and Dunn’s multiple comparison post hoc test were used. Two-way ANOVA was used for comparisons of viral growth over time ($p < 0.05$ were considered statistically significant). SPICE (version 5.2) was used for analyses of CD8$^+$ T cell effector function (19).

Results

Antecedent MPA treatment reduced the size of effector and memory CD8$^+$ T cell populations elicited by HSV-1 infection of corneal mucosa

To examine the effects of MPA treatment prior to HSV-1 infection (termed pretreatment) on antiviral immunity, ovx B6 mice were s.c. implanted with 21-d sustained release pellets containing 50 mg MPA or matching placebo pellets 7 d prior to corneal infection. Mice were euthanized 8 dpi, the time point coinciding with peak HSV-specific CD8$^+$ T cell infiltration into TG (20) [in naive mice, sensory ganglia effectively contain no CD8$^+$ T cells (21)]. Flow cytometry analyses that compared absolute numbers of CD4$^+$ and CD8$^+$ T cells, CD127$^+$ T cells (memory precursors), and CD8$^+$ T cells specific for the HSV-1 immunodominant peptide (gB$498$-$505$) (22, 23) saw that pretreatment was associated with significant reductions in all measured T cell subpopulations (Fig. 1A). Although relative frequencies of the memory precursors and gB$498$-$505$-specific CD8$^+$ T cells were similar (Fig. 1B), pretreatment mice was also associated with decreased numbers of gB$498$-$505$-specific CD8$^+$ T cell numbers in the lymph nodes draining infection (DLN) (Fig. 1C). The diminished virus-specific T cell infiltrates were accompanied by reduced numbers of macrophages, NK cells, and NKT cells in TG 8 dpi, which lowered overall numbers of CD4$^+$ cells in TG of pretreated mice (Fig. 1D, 1E). Although impaired expansion and infiltration of virus-specific T cells may have resulted from MPA-mediated defects in T cell activation, no differences in relative expression of the activation markers CD25, CD27, CD28, CD44, CD62L, CD69, and CD154 were seen among gB$498$-$505$-specific CD8$^+$ T cells in TG (8 dpi) or in DLN (5 and 8 dpi) (data not shown). Reduced numbers of memory cell precursors in TG of pretreated mice 8 dpi, in

FIGURE 5. MPA does not affect HSV-1 replication. (A) The ability of MPA to alter in vitro HSV-1 replication was evaluated using multistep viral replication kinetics with Vero cell monolayers treated with MPA (10 $\mu$M) or vehicle (Ctrl) 24 h prior to infection ($n = 9$). Lower MPA concentrations were also tested, and none was altered in vitro HSV-1 replication (data not shown). (B) Untreated or pretreated female B6 mice were infected with $10^3$ PFU HSV-1 per eye, and eye swabs collected at indicated days postinfection to compare viral titers by standard plaque assay ($n = 12$/group). In (A) and (B), viral titers are shown as mean $\pm$ SD; two-way ANOVA was used to compare groups. In other experiments, pretreated or untreated intact B6 mice or untreated B6 mice administered i.v. ACV 1 dpi were infected with $10^3$ PFU HSV-1 per eye. Eye swabs were collected 2 dpi, and mice were directly euthanized for corneal harvest ($n = 18$/group). In (C), data points denote HSV-1 titers from corneal swabs of individual mice as determined by plaque assay (horizontal bars designate mean values). In (D), data bars denote mRNA expression in whole corneas of the immediate-early viral genes ICP0 and ICP4 and the leaky-late viral gene gH relative to expression of the housekeeping gene PCX as measured by quantitative real-time PCR (bars indicate mean $\pm$ SD). In [C] and [D], *** $p < 0.001$ compared with all groups by one-way ANOVA and Tukey’s multiple comparison postest.) In other experiments, pretreated and untreated B6 mice were infected and sacrificed 8 dpi to excise TG. In (E), HSV-1 genome copy number per TG was determined by quantitative real-time PCR ($n = 22$/group). Each data point represents the viral genome copy number from a single TG. Comparison was performed using an unpaired one-tailed Student $t$ test, and horizontal bars indicate mean values. In all experiments, mice were pretreated with 4 mg DMPA (all data shown are pooled results from three independent experiments).
contrast, correlated with reduced numbers of memory T cells in TG of latently infected mice (Fig. 1F). Similar to results seen in acute infection, however, pretreatment did not affect gB498-505-specific CD8+ T cell percentages or relative expression of the activation markers CD27, CD43, CD69, CD62L, CD122, CD127, and CXCR3 in latently infected mice (data not shown).

As MPA is administered to women with conserved ovarian function as an injectable formulation (DMPA), we determined whether pretreatment of intact WT female B6 mice similarly inhibited T cell expansion. By comparing the effects of sustained release pellets containing 50 mg MPA, 4 mg DMPA, or 1 mg DMPA in intact mice, we assessed whether MPA suppressed T cell expansion in a dose-dependent manner. Moreover, as differences in MPA metabolism between humans and mice are possible (24, 25), we also measured MPA serum concentrations 7 d after the administration of these treatments. As expected, pellet and injectable MPA treatments suppressed CD8+ T cell expansion, and RIA revealed this effect to be dose dependent (Fig. 2). Although sustained release pellets produced mean MPA serum levels of 64.12 ng/ml (a concentration similar to the levels seen in the second trimester of human pregnancy), the 4- and 1-mg injections of DMPA produced mean serum concentration of 21.37 and 10.14 ng/ml, respectively (levels that approximate the peak and maintenance serum concentrations of MPA in women using Depo-Provera for prevention of undesired pregnancy) (26, 27). To exclude the possibility that MPA-mediated inhibition of T cell expansion was mouse strain specific, WT female BALB/c mice were administered 4 mg DMPA 7 d prior to corneal infection, and such mice showed reduced TG T cell infiltrates comparable to those seen among pretreated ovx and intact B6 mice (Fig. 3D). Taken together, these initial sets of experiments established that MPA inhibits T cell expansion elicited by mucosal HSV-1 infection and greatly reduces the number of memory T cells found in the TG of latently infected mice.

**MPA decreased virus-specific CD8+ T cell effector function during acute infection**

Because MPA administered to mice after HSV latency was established (≥35 dpi) was shown to impair TG-resident CD8+ T cell effector function (15), we also explored the effects of pretreatment on CD8+ T cell virus-specific effector function. At 8 dpi, relative effector molecule expression (MFI) by TG-resident T cells that responded to stimulation with HSV-infected fibroblasts was similar among treated and untreated mice (data not shown), but pretreatment nearly doubled the number of T cells unresponsive to stimulation (Fig. 4). CD8+ T cells from pretreated mice also showed reduced levels of the cytolytic effector molecule GzmB (Fig. 3B). CD8+ T cell effector function was restored in pretreated mice that had undetectable serum levels of MPA by 35 dpi (data not shown), indicating that MPA directly impaired virus-specific CD8+ T cell effector function during acute infection or that T cells less responsive to Ag stimulation had been eliminated during formation of the memory T cell repertoire.

**MPA did not alter HSV-1 replication**

We determined whether the inhibition of T cell expansion and memory cell development among pretreated mice resulted from MPA-mediated suppression of HSV replication (an effect seen with in vitro HIV-1 replication) (28, 29). Consistent with prior reports showing that size of the Ag load regulates T cell expansion and memory cell differentiation (30, 31), we saw that corneal infection with lower HSV-1 titers or treatment with the antiviral drug ACV reduced HSV-specific T cell expansion (data not shown). However, MPA did not affect in vitro HSV-1 growth (Fig. 5A), and pretreatment did not alter HSV-1 titers in the cornea or TG of acutely infected mice (Fig. 5B–E), indicating that reduced viral fitness had not been responsible for impaired T cell expansion. To reconcile similar HSV loads in the TG of pretreated and untreated mice with the previously seen MPA-mediated reduction in HSV-specific CD8+ T cell numbers, we also completed in vivo studies in which TG-resident CD8+ T cells were depleted from HSV-infected mice using three distinct anti-CD8 mAb concentrations. Interestingly, a higher TG of HSV copy numbers was seen only when TG-infiltrating CD8+ T cells were completely eliminated by anti-CD8 mAb treatment; even 90% reduction in CD8+ T cell numbers was associated with viral burdens identical to controls (Fig. 6). Such results were therefore entirely congruent with the observation that a 50% reduction in the number of TG-resident HSV-specific CD8+ T cells among pretreated mice did not alter HSV load in the TG during acute infection (Fig. 5E).

**MPA decreased T cell proliferation in the lymph nodes draining acute infection**

Because pretreatment did not impair HSV-1 replication, we next determined whether increased cell death or decreased cell proliferation contributed to MPA-mediated inhibition of T cell expansion. Levels of apoptosis in CD4+ and CD8+ T cells in DLN of pretreated B6 and BALB/cJ mice and untreated controls revealed no significant between-group differences (Supplemental Fig. 1A, 1B), but as assessed by BrdU incorporation, T cell proliferation was greatly inhibited in DLN of pretreated mice (Fig. 7). At 5 dpi, a time point of peak T cell priming (32), reduced proliferation was seen in CD4+, CD8+, and gB498-505-specific CD8+ T cells of pretreated B6 mice (Fig. 7), and T cell proliferation was similarly suppressed in pretreated BALB/cJ and ovx B6 mice (Supplemental Fig. 2A, 2B). No defects in proliferation, however, were seen in T cells in the DLN or TG of pretreated B6 mice 8 dpi (data not shown), indicating that MPA preferentially suppressed T cell expansion at earlier time points postinfection.

**Pretreatment decreased DC expression of CD40, CD70, and CD45R0 in lymph nodes draining acute mucosal tissue infection**

Given the well-established role of DC in T cell priming (33, 34), we next explored the effects of pretreatment on DC activation.

**FIGURE 6.** HSV burden in the acutely infected TG is increased only when TG-infiltrating CD8+ T cells are fully eliminated. Female B6 mice were corneally infected with 103 PFU HSV-1 and at 3 dpi were administered the indicated amounts of anti-CD8 mAb. Mice were euthanized 8 dpi, and TG was excised to enumerate virus-specific T cells by flow cytometry and to determine viral genome copy number per TG by quantitative real-time PCR (n = 5/group). Each data point denotes the absolute number of CD8+ T cells or the HSV-1 genome copy number per TG (*p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA and Tukey’s multiple comparison posttest; horizontal bars indicate the mean values). Data shown are results from one of two independent experiments.
Among conventional (CD11c<sup>hi</sup>) lymph node-resident DC subsets, CD11c<sup>hi</sup>CD8<sup>a</sup>+CD11b<sup>2</sup>CD45R<sup>2</sup> (CD8<sup>+</sup>) DC prime virus-specific CD8<sup>+</sup> T cells (35) and CD11c<sup>hi</sup>CD8<sup>a</sup>CD11b+CD45R<sup>2</sup> (CD8<sup>+</sup>) DC prime virus-specific CD4<sup>+</sup> T cells (36), whereas among the more recently identified tissue-derived migratory DC subsets, CD11c<sup>int</sup>MHC-II<sup>hi</sup> tissue DC and CD11c<sup>int</sup>MHC-II<sup>int</sup> inflammatory DC prime virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells (33, 37). Because earlier results revealed pervasive MPA-mediated suppression of T cell expansion (Fig. 1), we used flow cytometry to measure the expression of MHC molecules and the costimulatory molecules CD40, CD70, CD80, CD86, and TNFSF4 (OX40 ligand) in all four of these DC subsets in the DLN of pretreated and untreated mice 2 dpi (Fig. 8A). Although similar expression of MHC-I, MHC-II, CD86, and TNFSF4 was observed, CD40, CD70, and CD80 levels were greatly diminished in pretreated mice among all DC subsets examined (Fig. 8A). Of note, CD40 expression was the activation molecule most dramatically decreased by pretreatment, whereas the highest levels of CD70 expression were in tissue DC.

**Pretreatment decreased ability of DC to elicit T cell proliferation**

Although pretreatment decreased DC expression of multiple costimulatory molecules, it remained to be resolved whether T cell expansion was suppressed by MPA-mediated effects on DC activation or a more direct effect on T cell proliferation. To distinguish between these two possibilities, ex vivo T cell proliferation assays were performed with DC and CD8<sup>+</sup> T cells from pretreated and untreated mice. Because priming of unstimulated HSV-specific T cells begins 6 h postinfection and peaks 2 dpi (32), CD11c<sup>+</sup> cells from the DLN of pretreated and untreated B6 mice were isolated 2 dpi. Of note, no between-group differences in the frequencies of CD11c<sup>hi</sup> cells or any DC subset were detected (data not shown). gB<sub>498-505</sub>-specific splenic CD8<sup>+</sup> T cells were next isolated from pretreated or untreated naive HSV-1 gB<sub>498-505</sub>-specific TCR transgenic (gBT-I.1) mice, labeled with a cell tracing reagent, and cocultured directly ex vivo for 72 h with the CD11c<sup>+</sup> cells from pretreated or untreated infected mice. Compared with...
FIGURE 8. Pretreatment impairs DC activation and T cell priming. (A) Pretreated (4 mg DMPA) and untreated female B6 mice were corneally infected with 10^5 PFU HSV-1. Mice were euthanized 2 dpi. DLN were excised and rendered into single-cell suspensions to determine expression levels for MHC and several costimulatory molecules in DC and B cells by flow cytometry. CD70, CD80, CD86, TNFSF4 (OX40 ligand), MHC-I, MHC-II, and CD40 iMFI are shown as a relative measure of the amount of each protein expressed by the indicated DC subsets (n = 10/group; data pooled (Figure legend continues).
untreated controls, cultures that contained CD11c+ cells from pretreated mice showed a 4-fold reduction in the proliferation of gB498–505-specific CD8+ T cells (Fig. 8C). However, CD40, CD80, or CD86 blockade with mAb reduced T cell proliferation only 2-fold less than controls, results that suggested pretreatment suppressed proliferation via more than one costimulatory pathway. Conversely, CD8+ T cells from gBT-L1 mice treated with MPA 7 d prior to selection and cocultured with DC from untreated controls exhibited T cell proliferation similar to controls (Fig. 8C). Taken together, these results established that MPA-mediated inhibition of DC activation was responsible for the impaired T cell expansion seen among pretreated mice.

**Reduced CD40 expression among pretreated mice inhibited T cell expansion**

Because CD40 was the activation molecule most affected by pretreatment, we hypothesized that in vivo blockade of CD40–CD154 interaction with anti-CD154 mAb (clone MR1) would recapitulate MPA-mediated inhibition of T cell expansion. Although this mAb treatment did not affect T cell activation markers expression (Fig. 3C), T cell expansion was similarly suppressed among pretreated mice or mice administered anti-CD154 mAb (Fig. 3A). In addition, CD154+ mice on a BALB/c background showed inhibition of T cell expansion similar to that seen among pretreated BALB/c mice (Fig. 3D). However, neither CD154 Ab blockade nor absence of CD154 produced the decrease in CD8+ T cell GzmB expression seen among untreated mice (Fig. 3B, 3E), suggesting that MPA effects on T cell function were not solely a result of diminished DC CD40 expression. Interestingly, pretreated mice administered rCD154 concomitant with infection showed levels of virus-specific CD8+ T cell expansion and GzmB expression comparable to that seen among untreated controls (Fig. 3A, 3B). Combined, these results confirm and extend earlier studies that found CD40–CD154 interactions regulate the CD8+ T cell expansion produced by viral infection of mucosal tissue (38, 39).

**MPA-mediated reduction of T cell expansion is site dependent**

To determine whether the ability of MPA to dampen antiviral immunity was affected by route of infection, we assessed the proliferation and expansion of gB498–505-specific CD8+ T cells among pretreated and untreated mice systemically (i.p. or i.v.) infected with HSV-1. No between-group differences in HSV-specific CD8+ T cell numbers or BrdU incorporation were seen (Fig. 9), indicating that compared with primary viral infection of mucosal tissue, systemic viral infection may be less susceptible to MPA-mediated inhibition of DC activation and T cell expansion.

**Discussion**

Adaptive immunity evolved to provide appropriate host responses to commensal and pathogenic microorganisms. Activation of naive T cells requires Ag recognition and costimulatory signaling (40); thus, non-specific activation is inhibited, and tolerance to self-Ag is promoted in uninfected tissue, where resting APC express very few costimulatory molecules (41). With active infection, Ag binding and increases in IFN-γ stimulate APC to upregulate CD80 and CD86 (42), whereas T cells activated by Ag recognition and CD28 signaling upregulate CD154 expression (43). Engagement between activated APC, which also upregulate CD40 expression, and the activated T cells potentiates APC expression of CD70, CD80, CD86, and TNFSF4 and increases APC secretion of multiple T cell-activating cytokines (e.g., IL-12 and TNF) (44–46). As APC-mediated activation of T cells authorizes additional APC to participate in the ongoing response to infection, the reciprocal activation of the B7 and CD40 pathways has been termed “APC licensing.”
impaired generation of memory virus-specific CD8+ T cells (50), expansion among pretreated mice was unaffected if HSV infection
CD154 gene mutation, showed enhanced susceptibility to nu-
viduals with X-linked hyper-IgM syndrome, a disease linked to
responses against intracellular pathogens (64–67), whereas indi-
CD154 interactions were needed to acquire protective immune
adenovirus (62, 63); several murine models showed that CD40–
impaired CTL responses against vesicular stomatitis virus and
expression, migration of DC to regional lymph nodes, and virus-
specific T cell expansion (47, 48).

Our conclusions are supported by in vitro studies that showed DC
stimulated with CD154-transfected cells exhibit enhanced survival,
increased IL-12 production, and higher CD80 expression (45, 49),
and in vivo studies that show CD40+ mice display impaired early
expansion of Mycobacterium tuberculosis-specific T cells and
impaired generation of memory virus-specific CD8+ T cells (50, 51).
Although prior investigations reported that progestin-
containing compounds do not affect DC expression of CD80 or
CD40 (52, 53), the DC in these earlier studies were matured in vitro
from blood precursors, whereas DC in our investigation were ac-
tivated in vivo by viral infection of mucosal tissue. In addition to
impaired T cell expansion, the inhibition of DC activation among
pretreated mice in our study was associated with reduced numbers
of other leukocyte subpopulations in acutely infected TG; results
predicted by reports that DC control NK cell infiltration into
infected tissues (54, 55) and that migration and accumulation of
inflammatory cells to infected tissue is T cell dependent (56, 57).
Because CD154 plays a role in the development of bone marrow
precursor cells (58), it remains a possibility that reduced expression
of CD40 in pretreated mice may have contributed to the observed
decrease in the numbers of cell lineage precursors. However, we
found no differences in the frequencies of any DC subset or any
lymphoid or myeloid cell lineage in lymphoid organs or peripheral
blood of uninfected mice administered DMPA (data not shown),
results that suggest it is unlikely that a direct effect of DMPA on
immune cell development was responsible for the reduced expan-
ion of virus-specific lymphocytes in our study. In further support of
this conclusion, earlier investigations have shown that progestrone
nutrition treatment does not impair DC differentiation but instead acts to
promote DC differentiation and tissue accumulation (59, 60).
Effect T cell function (e.g., GzmB expression) was also im-
paired among pretreated mice, and reduced DC expression of CD40
may have contributed to this finding as optimal CTL function
requires CD4+ T cell help responses regulated by CD40 signaling
(61). In support of this conclusion, CD154−/− mice developed
impaired CTL responses against vesicular stomatitis virus and
adenovirus (62, 63); several murine models showed that CD40–
CD154 interactions were needed to acquire protective immune
responses against intracellular pathogens (64–67), whereas indi-
viduals with X-linked hyper-IgM syndrome, a disease linked to
CD154 gene mutation, showed enhanced susceptibility to nu-
merous microbial pathogens (68). In the current study, T cell
expansion among pretreated mice was unaffected if HSV infection
was initiated through a nonmucosal route, a finding in agreement
with earlier work that showed mucosal infection is more depen-
dent than systemic infection on CD40–CD154 interaction for
expansion (but not differentiation) of the CTL response (38, 39).
Taken together, these data highlight the central role of CD40–
CD154 interaction during acute infection of mucosal tissue while
also indicating that MPA-mediated suppression of CD40 expres-
sion contributed to the impaired effector T cell function observed
among pretreated mice in our investigation.

In conclusion, our study newly reveals that MPA impairs de-
velopment of immunological memory. Although prior in vitro
investigations demonstrated that progestrone or MPA inhibited DC
activation, this effect was shown at progesterone concentrations much
higher than those achieved by pregnancy or Depo–Provera injec-
tion for prevention of undesired pregnancy (69, 70). As clinical
studies indicate that steady-state serum concentrations of MPA
range between 0.7 and 10 ng/ml (26, 27, 71–73), further study is
needed to learn whether DC activation is impaired at concen-
trations of the drug lower than we examined. In addition to the
effects of MPA on virus-specific immunological memory, our
study also provides novel elucidation of a mechanism by which
progesterone may simultaneously promote tolerance and suppress
nonspecific T cell activation in mucosal tissue. As female sex
steroids help maintain tolerance to commensal organisms and
allogenic sperm and modulate defense against microbial patho-
gens, our results highlight the complexities of a system in which
estrogen and progesterone are involved in the regulation of these
seemingly divergent activities. Our results thus emphasize the
need for studies that are able to reveal the extent to which men-
strual cycle changes, pregnancy, and exogenous sex steroids alter
host susceptibility to infection by perturbing the balance between
tolerance and immunity. Although MPA binds to the glucocorti-
coid receptor with much greater affinity than progesterone (74),
there is little direct evidence to support that pharmacologically
relevant doses of MPA modulate host immunity via glucocorticoid
receptor binding. Therefore, it is also necessary to delineate the
molecular mechanisms responsible for MPA’s immunomodulatory
properties to facilitate both development and more confident se-
lection of progestrin-containing contraceptives that can prevent
pregnancy without inhibiting host defense mechanisms or en-
hancing susceptibility to mucosal tissue infection.

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**Supplemental Figure 1.** Pretreatment does not increase apoptosis of T cells in DLN during acute HSV-1 infection. Ovx B6 (A) pretreated with sustained-release 50 mg MPA pellets, and intact Balb/cJ (B) mice pretreated with 4 mg of DMPA, as well as their matched controls, were corneally infected with $10^5$ PFU HSV-1. 5 dpi, mice were euthanized and DLN excised for CaspaTag pan-caspase staining. Figures depict the percentage of CD4$^+$ and CD8$^+$ T cells present in DLN that were positive for caspase activity. Data shown are from one representative experiment of two independent experiments performed for each condition (n = 6-12 per group). Comparisons were performed using unpaired one-tailed Student t test; horizontal bars indicate the mean values.
Supplemental Figure 1
**Supplemental Figure 2.** Pretreatment reduces proliferation of virus-specific T cells in DLN. (A) Intact Balb/cJ treated with 4 mg of DMPA or left untreated were corneally infected with 10^5 PFU HSV-1. 5 dpi, mice were administered BrdU intravenously, and then euthanized 4 h later to excise DLN for BrdU staining. Figures show the percentages of CD4^+ and CD8^+ T cells that incorporated BrdU. (B) Ovx B6 mice treated with 21-day sustained release pellets containing 50 mg MPA or matching placebo were infected and sacrificed in the same way as in (A). Percentages of CD8^+ T cells and gB_{498-505}-specific CD8^+ T cells that incorporated BrdU present in DLN 5 dpi are shown. Data are from one representative experiment of two separate experiments performed (n = 5-12 per group). Comparisons were performed using unpaired one-tailed Student t test; horizontal bars indicate the mean values.
Supplemental Figure 2

A

% BrdU^+CD4^+ T cells

p=0.0100

% BrdU^+CD8^+ T cells

p=0.0117

Ctrl MPA

B

% BrdU^+CD8^+ T cells

p=0.0033

% BrdU^+K^b^+9^b^+50^b^+50^b^+CD8^+ T cells

p=0.0044

Ctrl MPA