T Regulatory Cells Contribute to the Attenuated Primary CD8+ and CD4+ T Cell Responses to Herpes Simplex Virus Type 2 in Neonatal Mice


*J Immunol* 2008; 180:1556-1564; doi: 10.4049/jimmunol.180.3.1556

http://www.jimmunol.org/content/180/3/1556

**References**  This article cites 73 articles, 38 of which you can access for free at: http://www.jimmunol.org/content/180/3/1556.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
T Regulatory Cells Contribute to the Attenuated Primary CD8⁺ and CD4⁺ T Cell Responses to Herpes Simplex Virus Type 2 in Neonatal Mice

Marian A. Fernandez,† Franz K. Puttur,‡ Yuan M. Wang,† Wade Howden,‡* Stephen I. Alexander,‡† and Cheryl A. Jones2*†

The first weeks of life are characterized by immune tolerance and increased susceptibility to intracellular pathogens. The neonatal adaptive response to HSV is attenuated compared with adult control models in humans and mice. T Regulatory cells (Tregs) control autoimmunity and excessive immune responses to infection. We therefore compared Treg responses in the draining lymph nodes (LN) of HSV-infected neonatal and adult C57BL/6 mice with the effect of Treg depletion/inactivation by anti-CD25 (PC61) treatment before infection on Ag-specific T cell effector responses at this site. There was a small, but significant increase in the frequency of CD4⁺Foxp3⁺ Tregs at day 3 postinfection (p.i.) in the LN of neonatal and adult mice, compared with age-matched mock-infected controls. Depletion of Tregs before HSV infection significantly enhanced HSV-specific CD8⁺ T cell cytotoxicity in vivo, cell number, activation, and granzyme B expression 4 days p.i. only in neonatal mice, and significantly enhanced CD8⁺ and CD4⁺ T cell IFN-γ responses in both infected adults and neonates. Treg depletion also reduced the titer of infectious virus in the draining LN and nervous system of infected neonates on days 2 and 3 p.i. Treg suppression of the neonatal CTL response p.i. with HSV was associated with increased expression of TGF-β in the draining LN at day 4 p.i. compared with uninfected neonates, but IL-10 was increased in infected adults alone. These experiments support the notion that the newborn primary T cell effector responses to HSV are suppressed by Tregs. The Journal of Immunology, 2008, 180: 1556–1564.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

*Centre for Perinatal Infection Research, †Centre for Kidney Research, The Children's Hospital at Westmead, Westmead, New South Wales, Australia, and ‡Discipline of Paediatrics and Child Health, University of Sydney, Sydney, New South Wales, Australia

Received for publication November 16, 2007. Accepted for publication November 16, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by The Children’s Hospital at Westmead Research Scholar Award (to C.A.J.).

2 Address correspondence and reprint requests to Dr. Cheryl A. Jones, Centre for Perinatal Infection Research, The Children’s Hospital at Westmead, Locked Bag 4001, Westmead, New South Wales 2145, Australia. E-mail address: cherylj@chw.edu.au

3 Abbreviations used in this paper: Treg, T regulatory cell; p.i., postinfection; wt, wild type; LN, lymph node.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
effective model of the human disease (28, 29). The development of CD4+ CD25+ Tregs in the thymus and spleen of mice has been well defined (30–34). Although Tregs have been shown to undergo proliferation in peripheral lymph nodes (LN) of neonatal mice in an IL-2-dependent manner (33), the development of naturally occurring Foxp3+ Tregs at this site is less well described.

We have previously reported that HSV induces an attenuated primary Th1 CD4+ and CD8+ T cell response in neonatal mice at day 5 p.i. (28). More recently we observed that the neonatal murine CD8+ T cell effector response to HSV is of slow onset with a shortened peak response compared with adult controls (35). Given the critical role of Tregs in regulating effector responses, we have used a murine model of neonatal HSV type 2 (HSV-2) infection to determine whether dominant CD4+ CD25+ Treg responses could account for the observed attenuated HSV-specific T cell effector responses in neonates in vivo. Mice were infected with either wild-type (wt) virus or with a nonlethal replication-defective HSV-2 strain, dl5–29 (28, 36–37), allowing analysis beyond 3 days.

Materials and Methods

Animals, viruses, and inoculations

Animal experiments were conducted with the approval of The Children’s Hospital at Westmead (Westmead, New South Wales, Australia) and Westmead Hospital Animal Ethics committees. Female C57BL/6 mice, 4–6 wk of age were purchased from the Animal Resource Centre (Perth, Australia), acclimatized, and used as controls or used as breeders for experiments. Neonatal mice were infected at 1 wk of age unless stated otherwise (24). Adult and neonatal mice were infected with either 1 × 107 PFU/mouse of wt HSV-2 stain 186syn-1 (38) or 10 × 106 PFU/mouse of the replication-defective HSV-2 186syn-1 mutant virus, d15–29, (UL5-5/5’ UL7 (39), or mice were mock-infected with an equivalent volume of low endotoxin PBS (Invitrogen Life Technologies) via s.c. inoculation (10 μl) into each hind footpad. Viruses were propagated and stored as previously described (29). The HSV-2 replication-defective virus dl5–29 and the complementary cell line V5–29 (36) were provided by D. Knappe (Harvard Medical School, Boston, MA). Adoptive transfer of splenocytes for the in vivo CTL assay was by i.v. injection (100 μl) into the tail vein of adults and subcutaneously into the inferior vena cava of neonates.

Depletion or inactivation of Tregs

For in vivo depletion or inactivation of CD4+ CD25+ cells, mice were infected with purified rat anti-mouse CD25 IgG1 mAb (PC61; BioEssay) i.p. into adult mice (0.5–1 mg) or 4-day-old mice (100 μg) 3 days before HSV infection. Control mice were given an equivalent dose of a rat IgG isotype Ab. The efficacy of CD25 depletion/inactivation was confirmed by flow cytometry using PE-conjugated anti-mouse CD4 and purified anti-CD25 biotin (7D4) followed by streptavidin-allophycocyanin. Flow cytometry using PE-conjugated anti-mouse CD4 and purified anti-CD25 PE (GK1.5), purified anti-CD25-biotin (7D4) (secondary reagent was streptavidin-allophycocyanin), anti-mouse FcR (J5-16), and anti-CD8-PerCP (53-67), anti-granzyme B allophycocyanin (MHGBOS), and anti-IFN-γ-HITC (XMGI2.1). Surface staining was at 4°C for 20 min. For in vivo CTL assay, surface stained cells were then fixed and permeabilized at 4°C for 6 h, then washed in perm/wash buffer at 4°C for 30 min, all in the dark. For IFN-γ and granzyme B intracellular staining, cells were first restimulated with gB 20–50 peptides (5 μg/ml) for HSV-specific CD8+ T cell cytokine production or UV-inactivated wt HSV-2 (strain 186, multiplicity of infection of 5) for Cd4+ T cell stimulation, at 37°C, 5% CO2 in RPMI 1640 (In-vitrogen Life Technologies and CALife Technologies) containing 10% FCS, 2 mM l-glutamine, 2 × 10−2 mM 2-ME (Sigma-Aldrich), and 2% penicillin-streptomycin. Brefeldin A (GolgiPlug; BD Biosciences) was added to a 1 μg/ml final concentration for the final 4 h of stimulation as previously published (28). Cells were then fixed, permeabilized, and stained for intracellular determination of Foxp3. Data were acquired on a BD FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences).

Virus titration

The titer of HSV-2 in the tissues of both adult and neonate mice was determined by standard plaque assay as reported (29). In brief, the brain, draining LN, and footpad were collected at days 1, 2, and 3 p.i. and frozen on dry ice in 0.5 ml of assay medium (PBS containing 0.1% BSA, 0.1% glucose, and 0.1% CaCl2). Organs were stored at −80°C until assay. For assay, tissues were then thawed on ice, homogenized using a sterile Kontes pestle, and plated on Vero cells in duplicate using serial 10-fold dilutions of 1-ml aliquots per 6-well plates. Infectious titer was determined after 48 h, after fixing the monolayers with methanol, then staining with 1X Giemsa.

RNA isolation, TGF-β RT-PCR, and IL-10 real-time PCR

Total RNA was extracted from the draining popliteal LN of mice using TRIzol reagent (Invitrogen Life Technologies). cDNA was prepared using a known and constant concentration of total RNA and reverse transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies) and random primer (Promega).

The expression of TGF-β was measured by RT-PCR, as previously published (40). CDNA was subjected to nested PCR amplification using external and internal primers and β-actin as control. The PCR conditions were 95°C for 5 min (1 cycle); 95°C for 45 s, 60°C for 45 s, and 72°C for 1 min (35 cycles) with the final cycle at 72°C for 7 min. The following primers were used: TGF-β 5′-TGGAGCCGCAACACCGCATCTAGTG AAAAAAC-3′ and 5′-TGGAGCTGAAGAATGTTGTATCCAGGACTC-3′; and β-actin 5′-TGGTGCAAGAGACTCTTATG-3′ and 5′-CAGCAAGCTGATGCTTCTT-3′. The PCR products were run on 2% agarose gels and visualized under UV light after staining with ethidium bromide (0.5 μg/ml) using Gel Doc 1000 (Bio-Rad).

Real-time PCR for IL-10 was performed on cDNA obtained from the draining LN as described. The PCR master mix contained 0.3 μM primers and 0.05 μM SYBR Green probe and was cycled at 95°C for 10 min (1 cycle), 95°C for 45 cycles of 15 s each, and 60°C for 1 min (1 cycle). IL-10 mRNA expression was quantified by real-time PCR using the ABI PRISM 7700 (PE Applied Biosystems). IL-10 mRNA expression was calculated by comparative threshold method (or ΔΔCt) to determine the change in IL-10 gene expression in relation to 18 S rRNA, which was used as internal control. The following primers and probes were used: 18 S rRNA primer 5′-GTAACCCGTTGAAACCTATG-3′ and 5′-CCATCCAACTCCTGATAGC-3′; and 5′-CCATCCAACTCCTGATAGC-3′ and 5′-CATCCAACTCCTGATAGC-3′. Each probe. IL-10 was expressed relative to the level detected in draining LN of an uninfected age matched sample. Each sample was analyzed in triplicate.

Statistics

The Student’s two-tailed t test or the Mann-Whitney U test were used where appropriate to calculate the statistically significant differences between samples. A value for p < 0.05 was considered significant.

Draining popliteal LN cells from HSV-infected neonatal or adult mice or mock-infected controls were blocked in PBS containing 1% Fc block (2.4G2), 1% human FBS, 1% normal rat serum (Animal Resource Centre, Perth, Australia), and 1% normal goat serum (Siles, Graz, Austria) at 4°C. Abs obtained from BD Biosciences were: anti-CD4-PE (GK1.5), anti-CD8-PerCP (53-67), anti-granzyme B allophycocyanin (MHGBOS), and anti-IFN-γ-HITC (XMGI2.1). Surface staining was at 4°C for 20 min. For in vivo CTL assay, surface stained cells were then fixed and permeabilized at 4°C for 6 h, then washed in perm/wash buffer at 4°C for 30 min, all in the dark. For IFN-γ and granzyme B intracellular staining, cells were first restimulated with gB 20–50 peptides (5 μg/ml) for HSV-specific CD8+ T cell cytokine production or UV-inactivated wt HSV-2 (strain 186, multiplicity of infection of 5) for CD4+ T cell stimulation, at 37°C, 5% CO2 in RPMI 1640 (Invitrogen Life Technologies and CALife Technologies) containing 10% FCS, 2 mM l-glutamine, 2 × 10−2 mM 2-ME (Sigma-Aldrich), and 2% penicillin-streptomycin. Brefeldin A (GolgiPlug; BD Biosciences) was added to a 1 μg/ml final concentration for the final 4 h of stimulation as previously published (28). Cells were then fixed, permeabilized, and stained for intracellular determination of Foxp3. Data were acquired on a BD FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences).

Abs, intracellular cytokine staining, and flow cytometry

The titer of HSV-2 in the tissues of both adult and neonate mice was determined by standard plaque assay as reported (29). In brief, the brain, draining LN, and footpad were collected at days 1, 2, and 3 p.i. and frozen on dry ice in 0.5 ml of assay medium (PBS containing 0.1% BSA, 0.1% glucose, and 0.1% CaCl2). Organs were stored at −80°C until assay. For assay, tissues were then thawed on ice, homogenized using a sterile Kontes pestle, and plated on Vero cells in duplicate using serial 10-fold dilutions of 1-ml aliquots per 6-well plates. Infectious titer was determined after 48 h, after fixing the monolayers with methanol, then staining with 1X Giemsa.

The Journal of Immunology 1557

Downloaded from http://www.jimmunol.org/ by guest on September 25, 2017
Results

Tregs in murine peripheral LN reach adult proportions by day 7 of life

To study the effect of HSV infection on Treg frequency and function in neonatal mice at the site of T cell activation, we characterized the presence of Tregs in peripheral LN and spleen. The transcription factor Foxp3 is the most consistent marker of naturally occurring Tregs in humans and mice (9, 41–44). The percentage and number of Tregs in the peripheral LN and spleen of newborn mice remained low in the first week of life, (Fig. 1, A and C). Representative dot plots for LN (A) and spleen (B) also show the proportion and number of Tregs. Data are from one of two independent experiments.

There is an increased frequency of Tregs in the draining LN of neonatal and adult mice at early times p.i. HSV

The wt HSV-2 strain causes lethal infection even in adult mice (29). Therefore, to assess the effect of HSV infection on the frequency of CD4- Foxp3+ CD25+ Tregs in the LN, female adult and 1-wk-old mice were infected with the nonlethal HSV-2 replication-defective virus dl5–29 (28, 29), and the percentage of Tregs in the draining LN was determined by flow cytometry after gating on CD4+ T cells at selected time points on days 1–14 p.i. (Fig. 2, A and C). HSV infection induced a small, but significant increase in the percentage of CD4- CD25- Foxp3+ Tregs in the LN, female adult and 1-wk-old mice at early times p.i. HSV infection induced a small, but significant increase in the proportion of Foxp3+ Tregs in the draining LN (~3.5% at days 7 and 60 of life), although the number of Tregs was still reduced at this time (Fig. 1, A and C). In contrast, consistent with previously published reports (30, 33), the proportion and number of Tregs in the spleen of newborn mice remained low in the first week of life, (Fig. 1, B and C). Thus, 1-wk-old mice have adult proportions of Foxp3+ Tregs in the draining LN.

FIGURE 1. Tregs in the peripheral LN of naive neonates reach adult proportions by day 7 of life. The percentage and number of Tregs in the popliteal LN and spleens were measured in 1- to 7-day-old C57BL/6 mice and female adult (6 wk of age) mice (n = 4–6 mice/age/time point). Cells were analyzed for surface expression of CD4 and CD25, and intracellular expression of Foxp3 by flow cytometry. Viable cells were gated on lymphocytes. Tregs were defined as the percentage of cells that coexpress CD4- , CD25- , and Foxp3-. The mean percentage ± SEM of Tregs and of CD4- T cells is shown, as well as the absolute number of Tregs ± SEM in the draining LN (A) or spleen (B). C. Representative dot plots for LN (A) and spleen (B) also show the proportion and number of Tregs. Data are from one of two independent experiments.

FIGURE 2. The percentage of Tregs in the draining LN of neonatal and adult mice after HSV infection. Neonatal (1-wk-old) mice and female adult controls (n = 8–12 mice/age group/intervention/time point) were infected s.c. with 2 × 10^5 PFU/mouse of the HSV-2 mutant dl5–29 (HSV) or mock-infected with PBS (uninfected). Draining LN cells were collected at times indicated postinoculation, stained for surface CD4 and CD25 and for intracellular Foxp3, and analyzed by flow cytometry. A. Mean percentage ± SEM of CD4- Foxp3+ cells after gating on live CD4+ T cells from two experiments is represented. *, p < 0.05, by Mann-Whitney U test for HSV dl5–29 strain-infected mice vs age-matched control (n = 18 mice) at day 3 p.i. B. Mean total cell number of Tregs ± SEM is shown. C. Dot plot representation of adult and neonatal samples from A after gating on CD4+ T cells.
FIGURE 3. Treg depletion enhances neonatal HSV-specific CTL response and total and CD8+ T cell number in draining LN at day 4 p.i. Four-day-old mice and female adult mice were depleted of Tregs (−) by i.p. injection with anti-CD25 mAb (PC61) to yield >95% depletion in neonates and >90% in adults by flow cytometry at day 3 postinjection (data not shown) or injected with an isotype control Ab (Treg +). Three days later, mice were infected with 2 × 10^5 PFU/mouse of the HSV-2 mutant strain dl5–29 (HSV+) or mock-infected with PBS (HSV−) (n = 5–11 mice/intervention/age group). CFSE-labeled HSV gB_{498–505} peptide-primed targets (CFSE^{high}) and unprimed controls (CFSE^{low}) were injected i.v. 4 days p.i. In vivo HSV-specific CTL was measured 4 h posttransfer of targets. A. Mean percentage ± SEM of HSV-specific CTL lysis is shown. B. Histograms from A indicating percentage HSV-specific lysis. The effect of Treg depletion on total cell number (C) and CD8+ T cell number (D) was determined in the draining LN of HSV-infected neonates and adults by trypan blue exclusion staining and by flow cytometric analysis, respectively. *, p < 0.01, Mann-Whitney U test in (n = 18 mice) A and (n = 11 mice) C and D. Data are from one of two separate experiments.

Treg depletion significantly enhances neonatal HSV-specific CD8+ T cell cytotoxicity, cell number, and activation at day 4 p.i.

We have previously observed that the primary CD8+ CTL response to HSV in neonatal mice is associated with reduced CD8+ T cell expansion and short duration of effector activity compared with adult controls (28, 35). Depletion of Tregs before wt HSV-1 infection of adult mice has been reported to enhance CD8+ T cell expansion, activation, and effector function (16). To assess the effect of Treg depletion on the primary HSV-specific CTL response in neonatal mice (at day 7 of life), 4-day-old mice and adult controls were injected i.p. with the anti-CD25 Ab (PC61) 3 days before HSV infection. A separate group of mice were given a rat IgG isotype Ab as a control. PC61 treatment resulted in a >95% depletion in the percentage of CD4+ CD8− CD25+ cells in adults and a >95% in neonates by flow cytometry at 3 days postinjection (i.e., just before HSV infection) (data not shown). Mice were infected s.c. in the footpad with 2 × 10^3 PFU/mouse of HSV-2 strain dl5–29 3 days after depletion or mock-infected with low endotoxin PBS. The cytoplastic capacity of HSV-specific CD8+ T cells was determined in draining LN at day 4 p.i. using an in vivo CTL assay that assesses the recovery of CFSE-labeled HSV peptide-pulsed (gB_{498–505}) targets in the LN injected 4 h before assay (Fig. 3, A and B) (35, 39). Treg depletion was observed to significantly increase HSV-specific CD8+ T cell cytotoxicity in neonatal mice at day 4 p.i. (p < 0.01, by Mann-Whitney U test, n = 18 mice).

To test whether this robust increase in HSV-specific CTL response after Treg depletion in HSV-infected neonates was due to enhanced CD8+ T cell expansion or recruitment, the total cell number in the LN and CD8+ T cell number was measured by flow cytometry in the draining LN at day 4 p.i. (Fig. 3, C and D). Both cell counts were observed to be significantly increased at this site in Treg-depleted HSV-infected neonatal mice compared with age-matched HSV-infected controls (p < 0.01, Mann-Whitney U test, n = 11 mice). Nonsignificant increases in both parameters were also observed in Treg-depleted infected adults (p > 0.05, Mann-Whitney U test, n = 11 mice) (Fig. 3, C and D).

Expression of stable activation markers in HSV-infected adult mice has been shown to closely correlate with CTL effector function (45). Therefore CD25 expression on CD8+ T cells from the draining LN was measured at day 4 p.i. (Fig. 4). In addition to the positive effects on CD8+ T cell cytotoxicity and number, depletion of Tregs also resulted in an ~60% increase in the proportion of CD8+ T cells that expressed CD25 in HSV-infected neonatal mice compared with nondepleted infected neonatal mice (p < 0.01, Mann-Whitney U test, n = 11 mice). Treg depletion also induced a nonsignificant increase in the proportion of CD8+ CD25+ T cells in HSV-infected adult mice. Thus, Tregs contribute to the suppression of HSV-specific CD8+ cytotoxicity and reduced CD8+ T cell expansion or recruitment into the draining LN in neonatal mice.

FIGURE 4. Treg depletion enhances CD25 expression by CD8+ T cells after primary HSV-2 infection in neonatal mice. Neonatal (1-wk-old) and female adult mice were injected i.p. with anti CD25 mAb (PC61) (Treg −) or with an irrelevant isotype control Ab (Treg +) 3 days before infection with HSV-2 dl5–29 (HSV+) or mock infection with PBS (HSV−). Draining LN cells were collected day 4 p.i. (n = 5–8 mice/age/intervention) and stained for CD8 and the activation marker CD25, then analyzed by flow cytometry after gating on viable cells. A. Mean percentage ± SEM of CD8+ T cells that coexpress CD25 is shown. B. Representative dot plots from A indicate proportion of CD8+ CD25+ T cells expressed. *, p < 0.05, neonates infected vs neonates depleted and infected by Mann-Whitney U test (n = 12 mice). Data are from one of four separate experiments.
Depletion of Tregs increases neonatal T cell IFN-γ and granzyme B expression after acute HSV infection

T cell-derived IFN-γ production plays a critical role in the immune response to HSV by limiting spread of infectious virus and activating other immune effectors (46). Therefore, we tested whether depletion of Tregs enhanced T cell IFN-γ expression in the draining LN after HSV infection, using intracellular cytokine staining and flow cytometry (Fig. 5). We observed an increase in the percentage of CD8^+ T cells that express IFN-γ by day 4 p.i. in the depleted infected neonatal mice compared with nondepleted infected controls (3.6 ± 0.82% vs. 1.0 ± 0.27%, p = 0.01, Mann-Whitney U test, n = 12 mice). A 2- to 3-fold increase in IFN-γ CD8^+ T cell response was also observed in the depleted infected adult mice compared with nondepleted controls (p < 0.05, Mann-Whitney U test, n = 12 mice). Similarly, Treg depletion induced increased IFN-γ expression by CD4^+ T cells at day 4 p.i. in HSV-infected neonatal mice (5.0 ± 0.18% vs. 4.1 ± 0.13%, p < 0.05, Mann-Whitney U test, n = 17 mice) and in adult mice compared with nondepleted infected controls (6.1 ± 0.5% vs. 4.4 ± 0.4%, p < 0.05, Mann-Whitney U test, n = 15 mice). Thus, Treg depletion enhances CD8^+ and CD4^+ T cell IFN-γ responses shortly after primary HSV infection of neonatal and adult mice.

Granzyme B is one of the cytolytic granules secreted by activated CD8^+ T cells for CTL-mediated killing of virus-infected targets (47). We therefore measured the effect of Treg depletion on CD8^+ T cell granzyme B expression after HSV infection. Adult and neonates were depleted of Tregs before infection with dl5–29, cells were collected from the draining LN at day 4 p.i. and analyzed by flow cytometry. A, Mean percentage ± SEM of CD8^+ T cells or CD4^+ T cells that express IFN-γ is shown. B, Representative dot plots from A indicating proportion of cells that express IFN-γ and either CD8^+ or CD4^+. *, p < 0.05; **, p < 0.01 by Mann-Whitney U test (n = 12 mice) or (n = 15–17 mice/intervention/age) for CD8^+ T cells and CD4^+ T cells, respectively. Data are from one of two separate experiments with similar results.

Treg depletion before wt HSV-2 infection reduces the titer of infectious HSV in the LN and CNS but not the site of infection

Having shown that Treg depletion enhances neonatal CD8^+ T cell and CD4^+ T cell function, we next wanted to define the effect of Treg depletion on the titer of infectious HSV from the site of infection, nervous system and organs. For this investigation, neonatal and adult mice were infected with a high dose of wt HSV-2. The site of infection (footpad), LN, and brain were collected at days 1, 2, and 3 p.i., and the titer of infectious HSV was determined by standard plaque assay (Fig. 7). Later time points were not studied due to the rapid onset of lethal encephalitis in HSV-infected neonatal mice. We observed that Treg depletion did not significantly alter the titer of infectious HSV in the footpad of neonatal mice, or at any of the measured sites in adult mice. In contrast, Treg depletion induced a significant reduction in the titer.
of infectious virus in both the LN and brain of neonatal mice compared with nondepleted virus-infected controls at days 2 and 3 p.i. (p < 0.0001, days 2 and 3 p.i. in LN and day 3 p.i. in brain; p < 0.001 day 2 p.i. in brain, by Student’s two-tail t test). Development of lethal HSV disease was not used as an endpoint of the study for ethical reasons. However, despite the reduction in virus titer, signs of lethal encephalitis were observed in both Treg-depleted and nondepleted infected neonates by day 3 p.i. Thus, depletion of Tregs in neonatal mice improves host responses against infectious HSV systemically and in the brain.

**HSV induces increased expression of TGF-β but not IL-10 in the draining LN of neonatal mice**

The role of immunosuppressive cytokines (IL-10, TGF-β) in the suppression of CD8+ CTL effector activity by Tregs was also evaluated at day 4 p.i. with the replication-defective HSV-2 mutant dl5–29. Neonatal mice showed increased TGF-β expression in the draining popliteal LN by RT-PCR compared with uninfected neonates (Fig. 8A). There was also a smaller increase in TGF-β expression after HSV infection in adult mice compared with age- and sex-matched mock-infected controls. In contrast, IL-10 expression determined by real-time PCR was significantly increased in the draining LN of infected adults alone (p < 0.05, Student’s two-tailed t test) (Fig. 8B). Thus, Treg suppression is associated with enhanced TGF-β, but not IL-10 expression in the draining LN of neonatal mice after HSV infection.

**Discussion**

HSV causes severe disease in the newborn period associated with reduced primary and memory adaptive response (48). Given the critical role of Tregs in modulating effector responses, we investigated whether Tregs suppress the primary T cell responses to HSV in neonatal mice. We observed an increased frequency of Tregs in the draining LN of neonatal mice shortly after HSV infection, associated with increased expression of Foxp3 at this site. Furthermore, depletion of Tregs from neonatal mice before HSV infection significantly enhanced the magnitude of HSV-specific CD8+ T cell effector function, activation, and cell number and CD4+ T cell IFN-γ expression more profoundly than depletion from adult controls, and decreased the titer of infectious virus from the periphery and nervous system. HSV was also observed to increase the expression of TGF-β but not IL-10 in neonatal LN. Together these data suggest that Tregs suppress neonatal T cell effector responses to acute viral infection, which may contribute to increased virulence of intracellular pathogens in the newborn period.

Age affects both the number and function of Tregs in humans and mice (10, 30–34, 49–51). The murine thymus contains a low frequency of naturally occurring Tregs at birth that increases significantly over the first week of life to approach adult levels by 3 wk of age in the thymus (9) and 6 wk of age in the spleen (49). Foxp3 expressing CD4+CD25+ splenocytes can be detected in 3-day-old mice, but at levels considerably lower than levels in adult mice (32). The human fetus and newborn display similar patterns of Treg development at these sites (50), although the frequency of CD4+CD25+ Tregs in human cord blood approximates the proportion found in the blood of healthy adults (51). CD4+CD25+ Tregs are present in the LN of 6-day-old mice in approximate adult proportions (33). However the ontology of Foxp3-expressing Tregs in the murine LN in the first week of life remains poorly defined. This study demonstrates that Foxp3-expressing Tregs are first present in detectable levels in the peripheral lymphoid tissue of newborn mice from day 3 of life, and reach
adult proportions at ~day 7 of life. The low number of Tregs in the LN on days 1–3 of life could be due to the presence of a large number of precursor cells or a limitation in detection due to small size of newborn LN and the very low number of T cells.

Having established that the proportion of Tregs in the peripheral LN of 1-wk-old mice was similar to adults, we next compared the effect of HSV on the proportion of Tregs at that site. We show that HSV induces a small but significant increase in the proportion of Tregs in the draining LN of neonatal mice shortly after infection. A number of microbial pathogens including viruses have been previously shown to increase the frequency of Tregs in infected tissues or lymphoid organs of adult mice and humans (12, 52–56). CD4+CD25+ Tregs have been shown to suppress CD8+ T effector function and activation to viruses including HSV and to viral vaccines in adult humans ex vivo, and in adult mice in vitro and in vivo (12, 20, 53–60). Enhanced Treg responses have been detected in the cord blood of human neonates born to women with Plasmodium falciparum infection compared with unexposed infants or infants born to mothers who had been treated for malaria (22).

Increased proportions of Tregs with weak HIV-1 specific T cell responses have also been detected in the blood of HIV-1-exposed uninfected infants, compared with unexposed controls (23), suggesting that persistence of in utero Treg responses have suppressed postnatal HIV-specific effector T cell responses. The report speculated that the enhanced Treg response may have been beneficial, protecting infants against vertical infection by reducing T cell activation and thereby lower the pool of activated CD4+ targets and hence HIV-1 replication.

In this study, we show that depletion of Tregs before HSV infection significantly increased neonatal HSV-specific CD8+ T cell cytotoxicity, suggesting that enhanced Treg responses to HSV in the draining LN contribute to the attenuated neonatal CTL cell response to HSV that we have previously observed (29, 35). Tregs have been previously shown to suppress HSV-specific CD8+ and CD4+ T cell responses to wt HSV-1 (16) and to HSV DNA vaccines (59) in adult mice and to wt HSV-2 in humans (61). In this study, we did not observe enhanced HSV-specific CTL lysis after Treg depletion in adult mice. The likely explanation for the discrepancy is the timing of the analyses. Our experiments were performed day 4 p.i. when HSV-specific lysis is close to the peak response in adults (i.e., at ~70%), thus limiting detection of minor augmentation of CTL responses by Treg depletion. The study by Suvas et al. (16) analyzed adult CTL responses at day 7 p.i. when the CTL response in HSV-infected adults has waned to 10%. When we perform our analyses at day 7 p.i., we find a similar augmentation of adult HSV-specific CTL activity after Treg depletion, in addition to a smaller but significant augmentation in neonates (data not shown).

Treg depletion before HSV-2 infection enhanced CD4+ and CD8+ T cell IFN-γ expression in adult mice, with similar sized increases observed in neonatal mice, consistent with previous reports of a variety of viral agents after functional blockade or Treg depletion in adult mice (12, 16, 58). Tregs have been shown to suppress granzyme B expression by Friend retrovirus-stimulated CD8+ T cells in vitro (62). We show that Treg depletion significantly increased granzyme B expression in HSV-infected neonatal mice alone. The reason for the age-associated difference is not clear. Acquisition of granzyme B expression by CD8+ T cells after influenza infection has been tightly associated with cell division (63). Thus, it is possible that the age-associated augmentation of CD8+ T cell granzyme B expression was related to the larger increase in CD8+ T cell number induced by Treg depletion in infected neonates.

Treg depletion was also associated with a reduced titer of infectious HSV in the LN and brain shortly after infection in neonates, suggesting that suppression of neonatal immune effectors by Tregs may contribute to the enhanced virulence of this virus in newborn animals and humans. Future studies will determine whether this represents delayed dissemination of infectious virus to these sites or enhanced clearance by the innate and adaptive effectors.

We used PC61 mAb to reduce the frequency of naturally occurring Tregs for our functional studies on HSV immunobiology. Recent reports have indicated that PC61 depletes only a subset of Tregs (particularly those that are CD69lowFoxp3low) and functionally inactivates the remaining naturally occurring Tregs (64, 65). These studies have also shown that although PC61 treatment significantly lowers the CD4+CD25+ population, it results in only a small change in the number of CD4+Foxp3+ cells. In this study we report >90% reduction in the number of CD4+CD25+ Tregs in adult mice treated with high-dose PC61 (500 μg/adult mouse Ab). Furthermore, we observed a 50% reduction in Foxp3 expression (by real-time PCR) in the LN of mock-infected PC61-treated mice (data not shown), consistent with these studies.

Treg suppression of T cell effectors has been reported to occur by both cytokine-dependent (IL-10 and TGF-β) and cell contact-dependent mechanisms (66–69). Naturally occurring Tregs in the conjunctiva of rabbits after ocular HSV-1 infection have been shown to suppress T cell effectors by cell contact, and not by the production of suppressive cytokines (69). The mechanisms by which Tregs suppress T cell effectors after cutaneous HSV infection have not been fully defined. In this study we show that there is increased expression of TGF-β, but not IL-10, in the neonatal murine LN shortly after cutaneous HSV infection. The neonatal cytokine microenvironment is generally thought to be Th2-biased (24). We were therefore surprised that the reduced neonatal CTL response to HSV was not associated with a greater increase in the neonatal IL-10 response. However, this observation is consistent with previous observations by our group and by other groups that there is a general paucity of both Th1 and Th2 cytokine production by T cells compared with infected adult controls (27, 28, 70).

TGF-β has been reported to exert an inhibitory effect on T cell differentiation and proliferation. It has also been shown to be required for the conversion of CD4+CD25+ T cells into Tregs in vitro, and to be necessary for the maintenance of Tregs in the periphery (71). Whether the enhanced expression of TGF-β in HSV-infected neonates directly contributes to the impaired HSV-specific CTL response and whether Tregs are the source of the TGF-β is yet to be determined.

Tregs have also been reported to have bidirectional interactions with dendritic cells (72, 73). Neonatal dendritic cells are functionally immature compared with adult controls (74). Future work will therefore also determine the role of age-associated differences in the interaction of Tregs and dendritic cells in the generation of antiviral responses. Demonstration of the mechanisms by which Tregs expand locally in the neonate and suppress Ag-induced effector responses in the newborn period may give insight into enhanced therapeutic strategies while limiting potential autoimmune side effects.

Acknowledgments
We thank Eddy Hassan, Christine Victoire, Ingrid Evans, Mary Sartor, and Sanda Lum for technical assistance. We also thank Megan Cameron for dedicated animal care and Nick Taylor for assistance with the graphics.

Disclosures
The authors have no financial conflict of interest.
References


