Sympathetic nervous system control of anti-influenza CD8+ T cell responses

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Despite the longstanding appreciation of communication between the nervous and the immune systems, the nature and significance of these interactions to immunity remain enigmatic. Here, we show that 6-hydroxydopamine-mediated ablation of the mouse peripheral sympathetic nervous system increases primary CD8+ T cell responses to viral and cellular antigens presented by direct priming or cross-priming. The sympathetic nervous system also suppresses antiviral CD4+ T cell responses, but this is not required for suppressing CD8+ T cell responses. Adoptive transfer experiments indicate that enhanced CD8+ responses do not result from permanent alterations in CD8+ T cell function in sympathectomized mice. Rather, additional findings suggest that the sympathetic nervous system tempers the capacity of antigen-presenting cells to activate naive CD8+ T cells. We also show that antiviral CD8+ T cell responses are enhanced by administration of αβ2 (but not β2 or α) adrenergic antagonist. These findings demonstrate a critical role for the sympathetic nervous system in limiting CD8+ T cell responses and indicate that CD8+ T cell responses may be altered in patients using β-blockers, one of the most widely prescribed classes of drugs.

Viral infections activate innate and adaptive immune mechanisms, whose integrated functions ultimately limit viral replication, pathogenesis, and transmission. Because of its medical and veterinary importance, the interaction of viruses with the immune system has been intensively studied. Remarkably little attention, however, has been paid to the influence of the nervous system on antiviral immunity. It is well-established that the nervous and immune systems communicate through soluble mediators, such as cytokines, hormones, and neurotransmitters (1, 2). Indeed, the definition of molecules as “immune” vs. “neuronal” mediators is frequently an arbitrarily designation based on the chronology of discovery. Many biological mediators have been unwittingly investigated as distinct entities by immunologists and neuroscientists until their molecular identity was revealed by cloning or protein sequencing.

The sympathetic nervous system (SNS), one arm of the autonomic nervous system, is responsible for the “fight or flight” response. The SNS consists of adrenergic nerve fibers that exit the spinal cord to form para-spinal ganglia that innervate peripheral organs, including all primary and secondary lymphoid tissues (3–5). Studies of splenic architecture identified adrenergic nerve fibers in the capsule, trabeculae, and white pulp (6). The close proximity of adrenergic fibers to immune cells in lymphoid organs suggests that the SNS may regulate immune responses. Electron microscopy reveals synapse-like interactions can exist between sympathetic nerves and splenocytes (4). Communication between the SNS and the immune system most likely occurs through the release of neurotransmitters by adrenergic nerves. Stimulation of sympathetic nerves results in the release of preformed granules containing norepinephrine, neuropeptide Y, and ATP. Granule release appears to occur nonsynaptically, so neurotransmitters probably diffuse throughout the tissue (7). Because the utilization half-life of norepinephrine in rats spleen is ~7 h (8), it is likely that norepinephrine can act at considerable distance from its source neurons. Importantly, the cell types in spleen express adrenergic receptors (ARs) and therefore would be able to receive signals from the SNS (9–11).

Numerous studies have reported SNS-mediated immunomodulation, particularly the TH2 polarization of responses to protein immunogens. Interestingly, TH2 CD4+ T cells have been reported to lack ARs. It has been proposed that the SNS reduces secretion of TH2 cytokines, therefore skewing a response toward TH1 (12). Studies by Maestroni (13, 14) suggest that catecholamine-induced TH1 inhibition induced may occur at the level of the dendritic cell (DC).

The SNS function is frequently studied by ablating SNS peripheral nerves by i.p. administration of 6-hydroxydopamine (6-OHDA). Peritoneal 6-OHDA is rapidly distributed by the circulation into tissues, where it destroys sympathetic fibers based on internalization into recycling synaptic vesicles, where it is oxidized to generate neurotoxic free radicals (15). Because 6-OHDA is excluded by the blood–brain barrier, this treatment results in a peripheral “chemical sympathectomy.”

Investigators have found both enhanced and repressed immune responses by using 6-OHDA-treated mice, depending on the experimental conditions. Chemical sympathectomy decreases bacterial loads and increases innate immune responses to Listeria monocytogenes (16). However, 6-OHDA-treated mice have also exhibited decreased antibody responses to sheep red blood cells (17), although the extent of inhibition varies between inbred mouse strains (6). Of note, immune responses to antigen and mitogen are dramatically different in chemically sympathectomized mice. The immune response to mitogens seems to be depressed, whereas the response to antigen is enhanced in 6-OHDA-treated mice (6, 18). The seemingly divergent results are most likely due to variations in mouse strain and type of immune stimuli used. However, all of the reports point to the conclusion that the SNS can regulate the magnitude and quality of immune responses.

Influenza A virus (IAV) and other viruses are known to activate both the hypothalamic–pituitary axis (HPA) and the SNS (19). Previous studies by Sheridan et al. have clearly demonstrated that the nervous system can have an impact on IAV responses (reviewed in ref. 20). In the present study, we address the role of the SNS in the generation of CD8+ T cell (TCDS+) responses to viruses by investigating the effects of 6-OHDA treatment on well-defined mouse TCDS+ responses to viral and tumor antigens. Our findings have important basic implications for understanding antipathogen immunity and practical implications for clinical medicine, because SNS agonists and antagonists are routinely used in patients to control physiological disorders, such as blood pressure and asthma.

Results

Chemical Sympathectomy Increases Antiviral T Cell Responses in Vivo

We next examined the influence of the SNS on TCDS+ responses to influenza A virus (IAV) in C57/B16 (B6) mice. Mice were treated


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with 6-OHDA 3 times over a 1-week interval and infected via i.p. or intranasal (i.n.) route with A/Puerto Rico/8/34 (H1N1) (PR8) 3 days after the last 6-OHDA treatment. Antiviral TCD8+ responses were measured in the spleen by intracellular cytokine staining (ICS) for IFN-γ production ex vivo at the peak of primary responses (days 7 and 10, respectively for i.p. vs. i.n. infection). TCD8+ responses were measured against the 3 IAV determinants that top the immunodominance hierarchy in B6 mice (PA224–233, NP366–374, and PB1(F2)62–70) (21), and also against PR8-infected cells as an approximate measure of overall anti-PR8 response.

Remarkably, by using mice infected i.p., 6-OHDA treatment resulted in a 2- to 4-fold increase in the absolute numbers (or percent) of anti-IAV TCD8+ recovered either from spleen or from the peritoneum, the local site of infection (Fig. 1A). The SNS remained depleted in the spleen of 6-OHDA-treated mice at the time of T cell analysis (Fig. 1E). An increase of similar magnitude in splenic and local TCD8+ responses was observed following i.n. infection of 6-OHDA-treated mice. Local responses were monitored by quantitating antigen-specific TCD8+ present in the bronchial lavage (BAL) fluid collected 7 days after infection (Fig. 1B). 6-OHDA treatment did not greatly modify the immunodominance hierarchy following i.p. or i.n. infection in spleen or BAL TCD8+.

The number of virus-induced inflammatory cells in the BAL was not increased by 6-OHDA treatment. We also examined the CD4+ T cell (TCD4+) responses in the spleens of control and 6-OHDA-treated mice 7 days following i.p. infection. UV-inactivated PR8 virus was used to restimulate TCD4+ ex vivo, and the response was quantified via ICS for IFN-γ. Much like what was observed for the TCD8+ response, the TCD4+ response was enhanced by the 6-OHDA treatment (Fig. 1C). Enhanced TCD4+ responses were not, however, required for 6-OHDA-mediated enhancement of TCD8+ responses, because mice lacking CD4+ cells (due to targeted deletion of the CD4 gene or depletion with anti-CD4 antibody) exhibited enhanced 6-OHDA-mediated TCD8+ responses to IAV (Fig. S1A and B).

Flow cytometric analysis of spleens from uninfected 6-OHDA-treated vs. saline-treated mice did not reveal significant differences in the fractions of TCD8+, TCD4+, CD4+CD25+ regulatory T cells, B cells, macrophages, or DCs. 6-OHDA treatment increased total splenocyte numbers following i.p. (but not i.n.) infection by ~10%. However, the percent of T cells responding, along with the absolute number of IFN-γ+ T cells, was increased in 6-OHDA-treated animals compared with control animals. The increase in cell number was proportionally distributed among the cell types listed above. There were no significant changes in the expression of T cell activation markers CD69, CD62L, CD44, or CD25. Of note, depletion of CD4+CD25+ regulatory T cells did not eliminate the enhanced TCD8+ response in 6-OHDA-treated mice, strongly suggesting that the increased response is not mediated by regulatory T cells (Fig. S1 C and D).

6-OHDA treatment did not enhance serum IgG responses to IAV (Fig. 1D), demonstrating that 6-OHDA does not generally increase adaptive immune responses or lymphocyte proliferation due to potential global alterations in physiology; e.g., blood flow to immune organs. 6-OHDA enhancement of anti-IAV responses cannot be attributed to increased viral replication, because pulmonary viral titers were not increased by 6-OHDA treatment (Fig. S2). Further, as shown below, 6-OHDA also enhances TCD8+ responses.
to noninfectious antigens. Taken together, these data imply that the SNS functions to restrain TCD8+ responses.

6-OHDA Treatment of Host but not TCD8+ Donor Enhances Antiviral TCD8+ Response. We next examined whether the effect of 6-OHDA on TCD8+ responses is due to intrinsic persistent alterations in responding TCD8+ or alterations in the milieu that supports TCD8+ activation. For this, we turned to OT-I TCR transgenic mice, which generate TCD8+ specific for Kb-Ova257–264 complexes. OT-I mice, expressing serologically distinct congeneric CD45 alleles CD45.1 or CD45.2, were treated, respectively, with 6-OHDA or saline. CD8+ T cells were purified from the 2 groups of mice, mixed in a 1:1 ratio, labeled with carboxyfluorescein succinimidyl ester, and transferred into normal mice. Mice were infected i.p. with an IAV genetically engineered to express Ova257–264 in the stalk of the NA glycoprotein, (WSN-OVA), and division of the 2 splenic OT-I populations was analyzed ex vivo by flow cytometry. On day 2 after infection, OT-I T cells from 6-OHDA-treated mice and the OT-I T cells from the control mice had divided to a similar extent (Fig. S3B). By contrast, 6-OHDA treatment of recipients greatly enhanced the division of OT-I transferred from untreated mice (Fig. S3A). This experiment demonstrates first, that OT-I responses to IAV are enhanced by 6-OHDA treatment, and second, that this is due to alterations in the responding environment and not to permanent intrinsic alterations in the TCD8+ themselves.

6-OHDA Augments TCD8+ Response to Both Direct and Cross-Primed Antigens. During an antiviral response, antigens can be presented to TCD8+ by infected professional antigen-presenting cells (pAPCs; “directpriming”) or by pAPCs that acquire exogenous viral antigens (“cross-priming”). To directly examine SNS involvement in cross-priming, we injected KD25V cells [H-2b cells expressing SV40 large T antigen (Tag)] into C57BL/6 (H-2b) mice and measured primary TCD8+ responses ex vivo to 4 defined determinants (22). 6-OHDA-treated mice demonstrated greatly enhanced local (PEC) and splenonic responses to each of the determinants, which maintained their relative status in the well-defined Tag immunodominance hierarchy (Fig. 2A). Overall, Tag-specific TCD8+ responses measured against H-2b cells expressing Tag (C57SV) demonstrated a similar increase.

To specifically examine the effect of 6-OHDA on direct priming, we infected mice with a recombinant vaccinia virus (rVV) encoding a chimeric protein consisting of carboxy-terminal Venus fluorescent protein (VFP), ubiquitin (Ub), and Ova257–264. As described by van Endert and colleagues (23), peptide determinants are rapidly presented by DCs. To address this serious concern, we acutely treated mice with drugs that selectively block α- or β-ARs. Nadolol, a β-blocker, were delivered by implanting an osmotic pump 1 day before infection with IAV. The TCD8+ response to IAV was measured 7 days after infection by intracellular cytokine staining for IFN-γ. Phenolamine had no significant effect on TCD8+ responses, indicating that α-adrenergic stimulation does not enhance or suppress TCD8+ responses to IAV. Strikingly, nadolol enhanced both splenic and peritoneal TCD8+ responses (Fig. 3). The magnitude was similar to that induced by 6-OHDA treatment, pointing to an important role in SNS activation of β-ARs. Drugs were again delivered by osmotic pump throughout the course of infection, and anti-IAV TCD8+ response was measured 7 days later by ICS. IAV infection treatment enhanced the TCD8+ response, much like nadolol, whereas the metoprolol had no significant effect. Based on these data, we conclude that the SNS dampens TCD8+ antiviral responses via stimulation of β2 adrenergic receptors.

We therefore tested the capacity of pAPCs from infected 6-OHDA-treated and control animals to activate naïve T cells ex vivo. We tested the stimulatory capabilities of splenic pAPCs obtained 12 h after infecting mice with PR8-OVA i.p. We found that naïve OT-I division was enhanced 2– to 3-fold when stimulator pAPC preparations were derived from 6-OHDA-treated vs. control mice (Fig. 2D). This was not apparently due to increased expression of cognate antigen, because 6-OHDA did not enhance levels of cell surface Kb-Ova257–264 complexes as measured by 25-D1.16 antibody staining (Fig. 2D).

Splenic pAPCs include DCs, which are considered to be the major APCs involved in priming naïve TCD8+ responses in vivo. We sorted DCs from infected mice into CD8a+ and CD11c+ DCs subsets. CD8a+ DCs were better stimulators than CD11c+ DCs. Importantly, 6-OHDA treatment of mice enhanced the capacity of both CD11c+ subsets to activate naïve OT-I compared with controls (Fig. 2E). Although it had been reported previously that exposure of bone marrow-derived DCs to norepinephrine reduces IL-12 secretion while increasing IL-10 secretion (34), this is unlikely to account for the effect of 6-OHDA on DC function we observed.

First, although norepinephrine reduced CD11c+ DC IL-12 secretion, it had little effect on CD8a+ DC IL-12 secretion (Fig. S4A). Second, this is not due to differences in β2 AR expression, at least as determined at the level of mRNA expression (Fig. S4B). Second, direct measurement of splenic IL-10 and IL-12 from 1 to 7 days after infection did not support the hypothesis that 6-OHDA acts by decreasing IL-10 and increasing IL-12; indeed, IL-12 is actually reduced on D1 (Fig. S4C). Taken together, these data demonstrate that 6-OHDA treatment enhances the ability of pAPCs, including DCs, to activate naïve T cells ex vivo, suggesting a potential mechanism for the enhancing effect of 6-OHDA on antiviral TCD8+ responses. It does not appear, however, that this effect is mediated by modulating IL-10 or IL-12.

β- but Not α-ARs Modulate TCD8+ Response in Vivo. Our findings strongly implicate an important role for the SNS in modulating antiviral TCD8+ response. This conclusion is based strictly on 6-OHDA ablation of the peripheral SNS. Although it has not been described, 6-OHDA could affect other cell types in mice that directly or indirectly affect immune responses. Further, we cannot rule out the possibility that the 6-OHDA ablation of the CNS results in alterations in the immune system unrelated or indirectly related to the real-time effect of the SNS on antiviral TCD8+ responses.

To further define the particular β-AR modulating the magnitude of the T cell response in vivo, we used drugs specific for either β1 (metoprolol) or β2 (ICI118,551) ARs. Nadolol, a β-blocker, were delivered by implanting an osmotic pump 1 day before infection with IAV. The TCD8+ response to IAV was measured 7 days after infection by intracellular cytokine staining for IFN-γ. Phenolamine had no significant effect on TCD8+ responses, indicating that α-adrenergic stimulation does not enhance or suppress TCD8+ responses to IAV. Strikingly, nadolol enhanced both splenic and peritoneal TCD8+ responses (Fig. 3). The magnitude was similar to that induced by 6-OHDA treatment, pointing to an important role in SNS activation of β-AR receptors in limiting antiviral TCD8+ responses. It is important to note that nadolol is used in humans at a dose equivalent to 25 μg/g per day in mice, and that we observed effects at a 25-fold lower dose, suggesting that at clinical doses nadolol may modify human TCD8+ responses.

One potential explanation for 6-OHDA-enhanced immunogenicity of directly presented, rVV-encoded antigens is that the SNS limits VV infection of pAPCs or the amount of cognate peptide class I complexes expressed by infected pAPCs. To address this possibility, we infected mice with a rVV expressing VFP-Ub-Ova257–264 (or a control expressing VFP-Ub-NP366–374) and measured splenocyte expression of VFP and Kb-Ova257–264 complex surface expression by using the 25-D1.16 mAb. 6-OHDA treatment did not significantly affect expression of either VFP or Kb-Ova257–264 complexes (Fig. 2C).

6-OHDA Increases pAPC Stimulation of Naïve TCD8+. A previous study reported that peritoneal macrophages from chemically sympathectomized mice are able to activate TCD4+ responding to a protein antigen to a greater extent than control macrophages ex vivo (24).
Discussion

We have provided evidence that strongly supports a role for the SNS in limiting T cell responses to viral and cellular antigens. Although we focused on characterizing TCD8+ responses, the TCD4+ response to IAV was also enhanced in 6-OHDA-treated mice. By contrast, antibody responses to IAV were not detectably altered by 6-OHDA treatment. Increased antiviral TCD8+ responses cannot be attributed to alterations in viral replication, number of virus-infected cells in the spleen, or the amount of peptide class I complexes expressed by splenic pAPCs.

In contrast to our findings, Bonneau and colleagues (25) reported that 6-OHDA decreases TCD8+ responses to i.p. infections with herpes simplex virus (HSV). This discrepancy could be explained by numerous differences between the studies, particularly the timing of 6-OHDA treatment, because Bonneau ablated the SNS 24 h after viral infection (25). Because 6-OHDA treatment can greatly modify innate immune responses (13, 16), this could contribute to the differences between our findings and those of Bonneau and colleagues (25). Using a different HSV model system in mice, Carr and colleagues (26) reported that 6-OHDA treatment before ocular
infection does not significantly modify T<sub>CDS+</sub> responses in draining lymph nodes.

Based on adoptive transfer experiments, it appears that T<sub>CDS+</sub> are not lastingly affected by the 6-OHDA treatment. This suggests that the SNS either directly acts on the T<sub>CDS+</sub> during their activation, or it modulates the immediate environment of responding T<sub>CDS+</sub> to dampen their responsiveness. This effect could be subtle. Assuming that 13 divisions occur before the peak response is attained (27), only a 15% decrease in division time is required to observe the 4-fold increase in T<sub>CDS+</sub> we observed following 6-OHDA treatment.

DCs and macrophages are known to express β-ARs and to respond to norepinephrine (13), and we provide evidence that both CD11b<sup>+</sup> CD8α<sup>+</sup> DC subsets express β<sub>2</sub> ARs. We show that splenic pAPCs, including DCs, isolated from 6-OHDA-treated, infected mice stimulate OT-I cells to a greater extent than the pAPCs from control mice. This appears to occur independently of the number of cells expressing K<sup>o</sup>-Ova<sub>335-346</sub> complexes or the number of complexes expressed per cell, suggesting that 6-OHDA treatment alters the stimulatory capacity of DCs. Because T cells are at least an order of magnitude more sensitive than 25-D1.16 antibody staining, however, we cannot rule out the alternative (but not mutually exclusive) possibility that 6-OHDA increases the number of T<sub>CDS+</sub>-activating pAPCs expressing a low number of cognate–peptide class I complexes.

We show that it is unlikely that the SNS modulates DC stimulatory capacity by altering IL-10 or IL-12 secretion in DCs or other splenic cells. Little is known about the effect of catecholamines on costimulatory molecule expression on DCs, which could also contribute to the magnitude of the T cell response. We failed, however, to find 6-OHDA-induced differences in splenic DC expression of defined costimulatory molecules 41BB, B7-1, CD80, CD86, CTL-A4, and OX40L. Our working hypothesis is that norepinephrine released from the SNS upon infection interacts with β<sub>2</sub> ARs on DCs and modifies them to dampen their costimulatory capacity mediated by yet-to-be-defined surface molecules or secreted cytokines.

Sympathetic nerves release a number of characterized messenger molecules, including norepinephrine, neuropeptide Y, and adenosine. The findings that nadolol and IC1118,551, pan β- and β<sub>2</sub>- blockers, respectively, enhance antiviral T<sub>CDS+</sub> responses suggest that SNS suppression of T<sub>CDS+</sub> responses is largely due to the effects of released norepinephrine. Although we favor the idea that the β-blockers act directly on immune cells, we cannot eliminate the potential contribution of more global effects of norepinephrine on mouse physiology (e.g., blood flow to immune tissues).

β-Adrenergic agonists and antagonists are among the most widely used drugs in clinical practice, and they are used for a number of distinct diseases. Our findings raise the possible contribution of altered T<sub>CDS+</sub> responses to the clinical effects of β-adrenergic modifiers in patients with ongoing T<sub>CDS+</sub> responses to microbial and tumor antigens. Given the vagaries of the mechanisms of drug action in humans, it is also possible that the effects of β-adrenergic modifiers on cellular immune responses unknowingly contribute to their therapeutic or side effects in “nonimmune” diseases.

Materials and Methods

Mice. Female 6- to 8-week-old C57BL/6 OT-I Rag<sup>−/−</sup>, OT-I CD45.1 Rag<sup>−/−</sup>, and CD4<sup>−/−</sup> mice were obtained from Taconic Farms. Mice were housed under specific pathogen-free conditions.

Viruses and Immunizations. A Puerto Rico/8/34 (PR8), PR8-OVA (28), and WSN-OVA (29) were used as infectious allantoic fluid. Virus titers were determined by tissue culture 50% infective dose (TCID<sub>50</sub>) in Madin Darby canine kidney (MDCK) cells and by LD<sub>50</sub> in 8-week-old B6 mice. Mice were injected i.p. with PR8 at 2 × 10<sup>6</sup> TCID<sub>50</sub> or 1 mL of PR8-OVA at 6 × 10<sup>6</sup> TCID<sub>50</sub>. Mice were i.n. infected with 0.1 LD<sub>50</sub> PR8 in 25 μL of PBS. Recombinant vaccinia virus inoculations were made as described previously (30). Mice were infected i.p. with 5 × 10<sup>3</sup> pfu of VV. Mice were depleted of CD4<sup>+</sup> cells by injecting 200 μg of GK1.5 i.p. on days 3, 2, 1, and 4. Mice were depleted of CD25<sup>+</sup> cells by injecting 500 μg of PC61 i.p. on day 3–4.

Chemical Sympathectomy and Antagonists. Mice were treated with 100 μg/kg 6-OHDA (Sigma) in 0.9% NaCl and 10<sup>−7</sup> M ascorbic acid on day −7 and day −5 and 200 μg/kg on day −3. Control mice received injections of 0.9% NaCl and 10<sup>−7</sup> M ascorbic acid. Sympathectomy was confirmed by staining frozen splenic sections with tyrosine hydroxylase Abs or the sucrose-phosphate-glucose oxidase (SPG) reaction (31). Nadolol, phentolamine, metoprolol, and IC1118,551 (Sigma) were administered continuously by using Alzet osmotic pumps (Alzet) implanted s.c.

Nadolol, phentolamine, and metoprolol were administered in saline, whereas

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**Fig. 3.** Blocking β-ARs but not α-ARs enhances anti-IAV CD8<sup>+</sup> T cell responses. (A) Mice were treated with the β-adrenergic blocker nadolol (black bars), the α-blocker phentolamine (hatched bars), or saline (gray bars). Mice were infected i.p. with PR8, and anti-IAV T<sub>CDS+</sub> responses were measured on day 7 after infection. T<sub>CDS+</sub> responses were measured against PR8-infected cells (PR8 EL4) and viral peptides [NP 366, PA 224, and PB1(F2) 62]. Uninfected EL4 and OVA 257 peptides were used as controls. Spleens were analyzed individually from 3 mice per group. PEC samples were pooled from 3 mice per group for analysis. The experiment was performed independently 3 times. (B) Mice were treated with the β<sub>2</sub>-adrenergic blocker IC1118,551 (black bars) or vehicle (gray bars), and T<sub>CDS+</sub> response was measured as in A. (C) Mice were treated with the β<sub>1</sub>-adrenergic blocker metoprolol (black bars) or vehicle (gray bars), and T<sub>CDS+</sub> response was measured as in A. Experiments in both A and C were repeated independently 3 times. * P < 0.05.
IC118,551 was administered in 50% saline/50% DMSO. Control mice received pumps administering vehicle alone. Nadolol was given at a dose of 1 mg/kg per day, and phentolamine, metoprolol, and IC118,551 were given at a dose of 10 mg/kg per day.

Flow Cytometry 25D-1.16 Staining of in Vivo-Infected Cells. Mice were infected i.v. with 1 × 10⁶ pfu of rTV or i.p. with 6 × 10⁶ TCD₅₀ IAIV. Twelve hours after infection, spleens were digested with type I collagenase (Miltenyi Biotech) and analyzed with FlowJo software (TreeStar). ICS was performed as described previously (21).

Preparation of pAPC Populations. Spleens were digested with type I collagenase for 1 h at 37 °C. For pAPC preparations, the first step of the CD8⁺ DC isolation kit (Miltenyi Biotech) was used. This preparation consisted of about 10% CD11c⁺ cells. For sorting of DC populations, spleens were treated with FC-receptor-blocking antibody (clone 2.4G2) and stained with the monoclonal antibody 25D-1.16 (which recognizes Kb-SIINFEKL complexes). Flow cytometry was performed with an LSR-II (BD) and analyzed with FlowJo software (TreeStar). ICS was performed as described previously (21).

DC Stimulation and Cytokine Measurement. Sorted splenic DCs were cultured at 1 × 10⁶/mL with and without 100 ng/mL LPS. No norepinephrine or 10⁻⁵ M norepinephrine was added to the cultures, and they were incubated for 24 h. Cells were spun down, and the supernatant was collected and analyzed for IL-12p40 by cytokine bead array assay (Bio-Rad) according to the manufacturer’s specifications.

RT-PCR for β₂-AR Expression. RNA from sorted splenic DC populations was prepared by using an RNeasy mini kit (Qiagen). β₂-AR and GAPDH expressions were measured by using Taqman Gene Expression Assays (Applied Biosystems). The β₂-AR probe was labeled with FAM, and GAPDH was labeled with VIC to allow for multiplexing of the 2 assays.

Cytokine Measurements in Spleen. Spleens were harvested on days 1, 2, 3, 5, and 7 after infection and placed in 1.5 mL of PBS containing complete, EDTA-free protease inhibitor mixture (Roche). Spleens were homogenized, and lysate was spun down to collect supernatant. Cytokines were measured by using a Bio-Plex cytokine bead array assay (Bio-Rad) according to the manufacturer’s specifications. Assay was read out on a Bio-Plex 200 Suspension Array System (Bio-Rad) and analyzed with Bio-Plex manager software (Bio-Rad).

Statistical Analysis. Statistical analysis was performed where appropriate with Prism 4 software (Graphpad Software) using an unpaired t test (for 2 groups) or 1-way ANOVA (for 3 groups). P values less than 0.05 were considered significant.

T Cell Proliferation Assays. OT-1 TCD8⁺ T cells were purified (~99% purity) from spleen and lymph nodes of either OT-1 Rag⁻/⁻ or OT-1 CD45.1 Rag⁻⁻ mice by using CD8⁺ T cell isolation kit (Miltenyi Biotech) with the AutoMACs purification system (Miltenyi Biotech). Cells were labeled with 2μM CFSE (Molecular Probes), and 5 × 10⁵ cells were transferred into C57Bl/6 mice by i.v. injection. Two days following virus challenge, spleens were harvested, stained for anti-Vß5 PE (BD) and anti-CD45.1 PE-Q7 (eBioscience), and analyzed on a LSRII (BD). Division was calculated using FlowJo software (Treestar). To measure T cell division ex vivo, OT-1 T cells were plated at 1 × 10⁵ cells per well in a 96-well plate. Irradiated (40°C) splenocytes were added at a ratio of 1:1. After 48 h of stimulation, 0.25μM [³²P]thymidine was added to each well. Twenty-four h later, incorporation of ³²P into DNA was measured using a FilterMate Harvester (Perkin-Elmer) and β-scintillation counting by using a 1450 TriLux Microbeta Counter (Perkin-Elmer).

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Fig. S1. 6-OHDA-mediated enhancement of CD8^+ T cell responses does not require CD4^+ or CD4^-CD25^+ cells. Mice were infected with PR8 i.p., and the anti-IAV CD8^+ T cell response was measured in the spleen and PEC 7 days after infection. Spleens were analyzed individually from 3 mice per group. *, P < 0.05; **, P < 0.005. PEC samples were pooled from 3 mice per group for analysis. (A) CD8^+ T cell response of 6-OHDA (black bars) or saline-treated (gray bars) mice, which were depleted of CD4 T cells by treatment with GK1.5. (B) CD8^+ T cell response of CD4^- mice that were treated with 6-OHDA (black bars) or saline (gray bars). (C) CD8^+ T cell response of mice that were treated with saline (gray bars), saline and PC61 (white bars), 6-OHDA (black bars), and 6-OHDA and PC61 (hatched bars). Statistical analysis was performed comparing 6-OHDA-treated and untreated mice for each group. *, Where the 6-OHDA samples were statistically different from the control samples. (D) FoxP3 and CD25 expression of spleen cells from saline or 6-OHDA-treated mice with and without PC61 treatment.
Fig. S2. 6-OHDA does not enhance IAV viral replication or clearance. Solid black lines represent control mice, and dashed black lines represent 6-OHDA-treated mice. Viral titers were measured by TCID$_{50}$ (A) and by RT-PCR for matrix expression (B) following infection with 1 LD$_{50}$ PR8 i.n. Error bars represent the standard deviation between the 3 mice analyzed per point per group.
Fig. S3. 6-OHDA treatment of host but not donor cells increases OT-I proliferation. (A). Purified CFSE-labeled OT-I T cells were adoptively transferred into 6-OHDA-treated or saline-treated mice. Mice were subsequently infected with WSN-OVA i.p., and OT-I division was observed. Each transfer was repeated independently 3 times with 3 mice per group. (B) T cells were purified from 6-OHDA-treated (CD45.1; black line) and saline-treated (CD45.2; gray filled) OT-I mice. T cells were mixed together in a 1:1 ratio, labeled with CFSE, and adoptively transferred into wild-type hosts and infected with WSN-OVA i.p. The transfer was repeated independently 2 times with 2–3 mice per group.
Fig. S4. SNS influence on DC cytokine production. (A) Sorted CD8α+ and CD11b+ splenic DCs were stimulated with 100 ng/mL LPS in the presence (black bars) or absence (gray bars) of 10^{-5}M norepinephrine for 24 h. IL-12p40 was measured in supernatants from DC cultures. (B) β2-AR expression in was measured in sorted CD8α+ and CD11b+ splenic DCs by RT-PCR and normalized to GAPDH expression. (C) IL-10 and IL-12p40 levels were measured in spleen lysates following i.p. IAV infection. Solid line represents control animals, and dotted line represents 6-OHDA-treated animals.