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Stress-Induced Glucocorticoids at the Earliest Stages of Herpes Simplex Virus-1 Infection Suppress Subsequent Antiviral Immunity, Implicating Impaired Dendritic Cell Function

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The systemic elevation of psychological stress-induced glucocorticoids strongly suppresses CD8⁺ T cell immune responses resulting in diminished antiviral immunity. However, the specific cellular targets of stress/glucocorticoids, the timing of exposure, the chronology of immunological events, and the underlying mechanisms of this impairment are incompletely understood. In this study, we address each of these questions in the context of a murine cutaneous HSV infection. We show that exposure to stress or corticosterone in only the earliest stages of an HSV-1 infection is sufficient to suppress, in a glucocorticoid receptor-dependent manner, the subsequent antiviral immune response after stress/corticosterone has been terminated. This suppression resulted in early onset and delayed resolution of herpetic lesions, reduced viral clearance at the site of infection and draining popliteal lymph nodes (PLNs), and impaired functions of HSV-specific CD8⁺ T cells in PLNs, including granzyme B and IFN-γ production and the ability to degranulate. In knockout mice lacking glucocorticoid receptors only in T cells, we show that these impaired CD8⁺ T cell functions are not due to direct effects of stress/corticosterone on the T cells, but the ability of PLN-derived dendritic cells to prime HSV-1-specific CD8⁺ T cells is functionally impaired. These findings highlight the susceptibility of critical early events in the generation of an antiviral immune response to neuroendocrine modulation and implicate dendritic cells as targets of stress/glucocorticoids in vivo. These findings also provide insight into the mechanisms by which the clinical use of glucocorticoids contributes to altered immune responses in patients with viral infections or tumors. The Journal of Immunology, 2010, 184: 1867–1875.

The mammalian stress response evolved as a means to respond to threats or changes in environmental conditions via physiological adjustments directed at maintaining homeostasis. One pathway by which this response occurs is the activation in the brain of the hypothalamic-pituitary-adrenal axis by the perception of stress, which initiates a hormonal cascade resulting in the systemic release of adrenal glucocorticoids that bind to glucocorticoid receptors (GR) present in essentially all cells (1–3). Brief exposure to stress (measured in minutes to a few hours) can augment immune responses (4–7). However, it has long been both anecdotally and empirically recognized that prolonged systemic elevation of stress-induced glucocorticoids is immunosuppressive and can lead to deterioration of health, including heart disease, cancer, susceptibility to infections, and poor responses to vaccines (2, 3, 8, 9). Just as skin and mucosal surfaces process perceptual challenges that can initiate immune responses, which may be beneficial or not, the brain and nervous system process perceptual challenges that can initiate or modulate immune responses, which may also be beneficial or not. The immune system is indeed structurally and functionally allied with the endocrine and nervous systems through neurotransmitters, hormones, cytokines and the receptors for these mediators that are shared by the cells in each of these systems. Despite the tremendous impact on immunity and human health, many aspects of the intricacies and mechanisms of these interactions, particularly during stress, have yet to be unraveled.

The primary strategy used by the immune system in response to intracellular pathogens and some tumors and vaccines is the activation and deployment of cytotoxic CD8⁺ T cells. Thus, suppression of CD8⁺ T immunity can have global consequences for the host. In efforts to understand the underlying mechanisms of stress-related immunosuppression, it has become well documented that T cell immune responses against a variety of viruses are compromised by glucocorticoids, either stress-induced (corticosterone, or cortisol in humans) or pharmacologically administered (such as dexamethasone or other synthetic analogs) (10–14). Stress/glucocorticoids have been shown to substantially impair CD8⁺ T cell activation, proliferation, cytokine production, trafficking, cytotoxicity, and control of viral replication (14–17). Indeed, stress during infection and the resulting changes in T cell responses have lead to profound increases in mortality from influenza virus, HSV, and Theiler’s murine encephalitis virus infections (12, 17, 18).

Implied in the above studies is that stress/glucocorticoids are acting on the T cells to impair their functions. However, Ag-specific CD8⁺ T cell activation can occur only through instructions supplied to them by APCs, in particular dendritic cells (DCs). DCs are highly specialized for the acquisition, processing, and presentation of Ags.
These cells are the most efficient of all APCs in the mammalian system, are required for cross-priming T cell responses in vivo, and also bridge adaptive and innate immunity (19–21). DCs have been shown to be essential for protection against HSV, as mice that were depleted of DCs prior to footpad HSV infection succumbed with 100% mortality in 3–6 d after infection (22). Thus, although the consequences of prolonged stress-induced neuroendocrine hormones are documented, the initial mechanisms and course of events leading to these outcomes, the role of DCs in particular, have only begun to be examined in detail.

Previous studies have shown that synthetic pharmacological glucocorticoids suppress the generation, maturation and immunostimulatory properties of DCs in animal models and humans (23–26). We have reported that physiologically relevant stress levels of naturally occurring corticosterone inhibit TLR agonist (LPS, CpG, and polyinosinic-polycytidylic acid) induced maturation of DCs in a GR-dependent manner (27). These effects include reductions in costimulatory molecule expression, intracellular MHC class II trafficking to the cell surface, proinflammatory cytokine production, and priming of an HSV gB-specific CD8+ T cell response in an in vivo DC vaccination model. We also demonstrated that physiologically relevant stress levels of corticosterone, in a GR-dependent manner, suppress the efficiency of presentation of Ag-specific peptide-MHC class I complexes by virus-infected DCs via a mechanism involving the decrease in the rate of production of antigenic peptides (28, 29). These studies provide insight into molecular mechanisms by which stress glucocorticoids suppress the function of DCs and their ability to activate CD8+ T cells.

In this study, we address several salient issues regarding stress/ corticosterone-induced immunosuppression during an in vivo infection. Unlike paradigms previously used, we used a model in which stress was applied only early during a cutaneous HSV infection and terminated before T cell effector function was detected. This strategy allowed us to delineate the early effects of stress/corticosterone on the CD8+ T cell response against HSV and to follow the course of infection and viral pathology after stress was terminated and corticosterone levels returned to baseline. Notably, it has not previously been feasible to differentiate cellular targets of stress or glucocorticoids in vivo. We accomplished this by using knockout mice lacking GRs only in their T cells (GR-TKO) (30). We report in this study: 1) The course and pathology of a cutaneous HSV infection is exacerbated when stress was experienced at the initial stage of infection; 2) even after stress was terminated, subsequent HSV-specific CD8+ T cell responses were still impaired; 3) these impaired T cell responses were mediated via the GR; 4) counter to current presumption, the impaired T cell responses and control of viral replication in vivo were not due to effects of glucocorticoids directly on the T cells; and 5) instead, the DCs from draining lymph nodes (LN)s of infected, stressed mice were significantly inhibited in their ability to prime HSV gB498–505-specific CD8+ T cells. These results demonstrate the vulnerability of DCs to stress/corticosterone in vivo at the earliest stages of a viral infection, which has consequences for CD8+ T cells and the course of infection that persist after stress/corticosterone has been terminated. Moreover, these consequences on T cell responses and pathology occurred despite a lack of direct effects of corticosterone on the T cells themselves. Notably, our findings also place the previously reported molecular mechanisms of glucocorticoid-mediated impairment of DC function (27, 28) into the context of a relevant in vivo viral infection model, showing that impaired DC functions indeed have consequences for disease outcome. This work has important basic implications for understanding immune responses to pathogens, tumors, and vaccines, as well as practical implications for clinical medicine, as glucocorticoid analogs are routinely used in patients to control inflammation and numerous physiological disorders.

Materials and Methods

Mice

Male C57BL/6 mice were purchased from the National Cancer Institute. GR-TKO mice (provided by Dr. L. Muglia, Washington University, St. Louis, MO) possess LoxP sites surrounding a portion of Nestl, the gene encoding GR, and express Cre-recombinase driven by the Lck promoter, thereby inactivating Nestl and preventing GR expression in T lymphocytes (30). T cells of gB-T3 TCR-transgenic mice (provided by Dr. F. Carbone, University of Melbourne, Melbourne, Australia) express a TCR that recognizes gB498–505 presented by H-2Kd (31). All mice were on a C57BL/6 background, housed under specific pathogen-free conditions, and treated in accordance with the National Institutes of Health and Association for Assessment and Accreditation of Laboratory Animal Care International. All animal-related experiments and procedures were approved by the Penn State Hershey Institutional Animal Care and Use Committee.

Restraint stress

Using a well-established restraint stress procedure (32), mice were placed individually in ventilated chambers that confined their motion without squeezing or compression such that they can move forward or backward but cannot turn around. Mice were restrained for up to four daily sessions, beginning 1 d prior to infection. Restraint was for 16 h beginning 3 h into the dark cycle when mice are typically active. Because food and water are not available to the mice during restraint, food and water were withheld from a time-matched, nonstressed control mice during this time. All mice received food and water ad libitum between sessions. This method of restraint stress increases circulating levels of the glucocorticoid, corticosterone, to between 5- and 10-fold from baseline levels, with no physical injury, whereas the control mice exhibit no signs of stress (33, 34).

HSV-1 challenge

Mice were anesthetized by an i.p. injection of sodium pentobarbital (70 mg/kg body weight) and challenged with 5 × 105 PFU HSV-1 Patton in a volume of 4 μl PBS containing 1% FBS in each hind footpad via the multiple piercing method (35). Virus was applied to the footpad and abraded into the skin by 60 punctures with a 27-gauge needle. This method introduces the virus into the epidermal and dermal skin layers, providing a skin infection with associated herpetic lesions, and effectively introduces the virus into the nervous system.

Exogenous corticosterone administration

In some experiments, mice were provided corticosterone (150 μg/ml, MP Biomedicals, Solon, OH) or vehicle (0.6% 2-hydroxypropyl-β-cyclodextrin, Sigma-Aldrich, St. Louis, MO) in water, ad libitum, for 1 d prior to and 3 d after HSV infection.

GR antagonist administration

In some experiments, mice were treated with the type II GR antagonist, RU-486 (25 mg/kg, Sigma-Aldrich) (36). RU-486 was dissolved in sesame oil (MP Biomedicals) and administered by s.c. injection 24 h prior to the first stress treatment and 3 d after HSV infection.

HSV lesion observation

The hind footpads of restraint-stressed or control mice were photographed daily using a Sony MP digital camera (Sony Electronics, San Diego, CA). Computer-downloaded images were scrambled and blindly scored for HSV lesion severity on a scale of 0 (no lesions), 1 (covering <25% of footpad), 2 (covering 25–50% of footpad), or 3 (covering >50% of footpad).

Quantification of infectious virus

Viral load was measured from homogenates of hind footpads by standard plaque assay on monolayers of Vero cells (35).

Quantitative PCR

DNA was isolated from popliteal LN (PLN)s of HSV-infected mice using the QIAamp DNA Isolation Kit (Qiagen, Valencia, CA). Quantitative real-time PCR was performed using Quantitect SYBR Green PCR Kit (Qagen), primers directed at amplifying a portion of the HSV-1 viral genome, and the ABI 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Primer sequences were 5'-GCCAGTTTACGTACAACCCACATAACAG-3' and 5'-AGCTTGCGGCGCTCTCGT-3' (37).
Western blotting and flow cytometry

Cells analyzed by Western blotting were from whole thymus, DCs from spleens, or Thy-1.2 cells from thymi. Purified cells were obtained using Pan-DC or Thy-1.2 microbeads on an AutoMACS magnetic cell sorter (Miltenyi Biotec, Auburn, CA). Protein from 5 × 10^6 cells was resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed for GR (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Rockland, Gilbertsville, PA) for control loading. Proteins were detected using peroxidase–anti-rabbit F(ab′)2 fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) and ECL reagents (Pierce, Rockford, IL). For flow cytometric analysis, splenocytes from Cre′ and Cre′ littermates were stained, using methods described previously (27), with the fluorescently conjugated Abs FITC-CD11c (N418), PE-CD4 (L3T4), PE-Cy7-CD45R (B20.20), APC-NC-1.1 (NKR-PI/C, Ly55) (from eBioscience, San Diego, CA), Alexa Fluor-750-CD8α (53–6.7), and PerCP-Cy5.5-CD11b (M1/70) (from BD Biosciences, San Jose, CA). The data were acquired using a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Detection of Ag-specific CD8+ T cells

PLN cells were stained with PerCP-Cy5.5 anti-CD8α (BD Biosciences) and PE-labeled H2-Kb-HSV gB 498–505 tetramer (National Institutes of Health Tetramer Facility) to identify HSV-specific CD8+ T cells. Data were acquired using a FACSCanto flow cytometer and analyzed using FlowJo software.

Intracellular cytokine staining

PLN cells were incubated with 1 μM gB 498–505 or a control peptide (OVA 357–366). After 2 h, 5 μg/ml brefeldin A (Sigma-Aldrich) was added to prevent cytokine secretion. Four hours later, cells were stained with PerCP-Cy5.5 anti-CD8α, fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, and stained with FITC anti-IFN-γ (XMG1.2, eBioscience) and Alexa 647 anti-human granzyme B (GB1; BD Biosciences). Data acquisition and analyses were performed by flow cytometry as described previously.

Degranulation assay

PLN cells were incubated with 1 μM gB 498–505 or a control peptide (OVA 357–366) in medium containing FITC anti-CD107a (BD Biosciences). After 1 h, 10 mM NH4Cl was added to prevent endosome acidification. Three hours later, cells were stained with PerCP-Cy5.5 anti-CD8α and PE anti-1A/1B (AF6-120.1, BD Biosciences) and analyzed by flow cytometry as described previously.

DC functional assay

PLN cells were collected from mice 24 h after HSV challenge, and the DCs were enriched by magnetic separation using Pan-DC microbeads on an AutoMACS cell sorter. At the same time, splenocytes were obtained from naive gB− T TCR-transgenic mice. HSV-1 gB-specific CD8+ T cells were sorted using CD8 microbeads (Miltenyi Biotec) and labeled with CFSE (Molecular Probes, Eugene, OR). Varying numbers of DCs were cocultured with 2.5 × 10^6 CFSE-labeled T cells at 37°C, 5% CO2 for 6 h after coculture, cells were stained with PerCP-Cy5.5 anti-CD8α and APC anti-Vα2 TCR (B20.1; eBioscience). T cell proliferation was assessed by dilution of the CFSE signal using flow cytometry. The efficiency of DC enrichment was checked by staining enriched DCs with PE-Cy5 anti-CD11c (N418, eBioscience) and performing flow cytometry as described previously.

Statistical analyses

Comparisons were made using unpaired Student t test or ANOVA. Infectious virus titer data were log_{10} transformed prior to statistical analysis. Statistical significance is p < 0.05.

Results

HSV pathology is exacerbated by stress applied early in infection

Most of the studies examining immune responses of infected hosts undergoing stress have used models in which stress was applied for extended periods before and throughout the entire course of an infection and the resulting immune response. In this study, we examined the initial events of an HSV immune response, when stress is applied over a shorter time frame at the onset of an infection, and the course of infection after stress has been terminated. C57BL/6 mice, which are resistant to HSV as compared with other mouse strains (38) were subjected to one session of restraint stress prior to bilateral footpad challenge with HSV-1. To limit stress to the early stages of infection, mice were subjected to only three additional daily sessions of restraint stress postchallenge. The footpads of stressed or nonstressed control mice were photographed daily for 11 d beginning on the first day after challenge, and images were scored blindly to assess the severity of HSV lesions over the course of the infection. In nonstressed control mice, lesions appeared on day 4 postchallenge, were most severe on days 5 and 6, and began to resolve beginning on day 7. In stressed mice, the viral lesions were detectable on day 3 postchallenge and did not begin to resolve until day 8. Thus, the onset of viral lesions was accelerated and their resolution delayed when stress was applied at the early stages of infection (Fig. 1).

We measured the viral load in the footpads of stressed or nonstressed control mice at various times postchallenge. The footpads of stressed mice contained significantly higher (3-fold) viral titers on the peak of infection (day 5) than the footpads of nonstressed mice, and infectious virus persisted in the footpads of stressed mice for an additional 2 d as compared with control mice (Fig. 2A). Because infectious HSV has not been detectable in draining LNs by plaque assay ([39]; unpublished observations) viral DNA in the draining PLNs was measured by quantitative real-time PCR at daily time intervals postchallenge, revealing a peak at 3 d (data not shown). Comparing stressed mice with nonstressed mice on day 3 postchallenge, there was a 4-fold increase in HSV DNA in the PLNs of the stressed animals (Fig. 2B).

Stress terminated early after viral challenge is sufficient to impair HSV-specific CD8+ T cell function in draining LNs

CD8+ T cells are important for providing protection during cutaneous HSV infection (35, 40). Previous studies have demonstrated impaired CD8+ T cell responses against viral pathogens when the animals experienced stress throughout the entire course of T cell expansion (14–17, 41). After bilateral cutaneous footpad infection with HSV-1, we examined the draining PLNs for Ag-specific CD8+ T cell responses that were generated after stress was terminated. As described previously, stress was administered 1 d prior to HSV-1 challenge and terminated 3 d

![FIGURE 1](http://www.jimmunol.org) Early onset and delayed resolution of footpad viral lesions in restraint stressed mice. A, Digital photographs of footpads from control and stressed HSV-infected mice were blind-coded, scrambled, and scored for the severity of lesions. Data are mean ± SEM; n = 15 mice/group. *p < 0.05. B, Representative photographs of footpads on day 7 postchallenge. White arrowheads indicate herpetic blisters.
postchallenge. On day 5 postchallenge, a time after which corticosterone levels have returned to baseline (12), and the time of peak of T cell activation in the PLNs (42), the PLNs were removed and the CD8+ T cell responses were measured. The number of activated CD8+ T cells was measured by intracellular staining for granzyme B. HSV-1 gB498–505-specific CD8+ T cells were identified by tetramer staining, and the functional properties of these cells were determined by measuring Ag-specific degranulation and the production of IFN-γ.

Stressed animals as compared with nonstressed controls exhibited a 3.9-fold reduction in the total numbers of CD8+ T cells producing granzyme B and a 2.4-fold reduction in HSV-1 gB498–505-specific CD8+ T cells (Fig. 3A,3B). Functional measures of HSV-1 gB498–505-specific CD8+ T cells in stressed animals showed a 2.9-fold reduction of cells that were able to degranulate and a 5-fold reduction of cells producing IFN-γ in response to specific peptide (Fig. 3C,3D). Thus, 2 d after stress had been terminated, the PLNs of stressed mice contained fewer activated CD8+ T cells and HSV gB498–505-specific cells at the time of peak T cell activation, and specific functional abilities of the Ag-specific CD8+ T cells were impaired.

**Stress impairs antiviral CD8+ T cell responses via GR**

Mice were administered either vehicle alone or the GR antagonist RU-486, which blocks corticosterone binding. The antagonist was administered 24 h prior to the first session of stress and then daily, 2 h prior to each stress session. Mice were subjected to stress and HSV infection as described previously (restraint stress administered 1 d prior to footpad challenge and terminated 3 d postchallenge). After 5 d postchallenge, PLNs were removed to measure CD8+ T cell responses. Similar to the responses shown in Fig. 3, the T cell responses in stressed mice that did not receive the GR antagonist were reduced (Fig. 4A), and the numbers of HSV-1 gB498–505-specific CD8+ T cells that were able to degranulate (Fig. 4C) or synthesize IFN-γ in response to specific peptide (Fig. 4D).

**Impaired CD8+ T cell responses are not due to direct effects of stress/corticosterone on the T cells**

Previous studies have implicated T cells as the direct targets of stress-mediated antiviral immunosuppression. However, because all cell types express GR, no distinction has been made in vivo between the
action of stress and/or corticosterone on T cells versus cells required for their activation, particularly in the context of an Ag-specific infection. To address this issue, we used a targeted KO mouse strain (GR-TKO) in which T cells are genetically deleted for the GR (30). These mice have no differences in thymus cellularity, T cell development, and subset distribution compared with control mice, as well as normal TCR-β and CD24 expression and T cell distribution in spleen and LNs as compared with control mice (30). We confirmed the lack of GR expression in Thy-1+ thymocytes from Cre+ mice by Western blots. Cre− littermates, which are phenotypically identical to wild-type C57BL/6 mice, were used as controls. We detected GR in CD11c-enriched splenic DCs from Cre+ mice and Cre− littermate controls and found equivalent expression of GR in both groups of mice (Fig. 5A). Thymic tissue from every mouse was screened to confirm the expression of GR in Cre− mice and the absence of GR in T cells of GR-TKO mice for all subsequent experiments described in this study. We also determined by flow cytometry that the splenocyte composition in Cre+ and Cre− control mice (Cre+, n = 4; Cre−, n = 6) was essentially the same, as there were no statistically significant differences in the percentages of total DCs (CD11c+), DC subsets (CD11c+/CD11b+/CD8α− and CD11c+/CD8α+/CD11b−), B cells (B220+), T cells (CD4+ and CD8+ subsets), macrophages/neutrophils (CD11b+), and NK cells (NK1.1+) (data not shown).

GR-TKO mice or Cre+ littermates received drinking water supplemented with vehicle or corticosterone at a dose that results in serum concentrations of corticosterone that are comparable to those measured in vivo in stressed animals (34, 43). Similar to the timing used in our restraint stress paradigm, mice received corticosterone or vehicle for 1 d prior to and 3 d after HSV-1 challenge, and then were returned to normal drinking water. At 5 d post-challenge (2 d after termination of corticosterone treatment), PLNs were removed to measure gB498–505-specific and functional CD8+ T cells, as described for Figs. 3 and 4. Fig. 5B−D shows that in Cre− mice that received corticosterone, the numbers of HSV-1 gB498−505-specific CD8+ T cells were significantly reduced, and activated functional CD8+ T cells were suppressed as assessed by granzyme B synthesis, IFN-γ production, and degranulation. Similarly, CD8+ T cell responses were also suppressed in Cre− GR-TKO littermates that received corticosterone. To determine the effects of stress in these mice, Cre− GR-TKO mice or Cre− littermates were subjected to restraint stress and HSV-1 infection as described previously. At 5 d postchallenge, PLNs were removed and HSV-1–specific CD8+ T cell functions were assessed as described previously. Similar to the results seen with corticosterone, restraint stress had a significant suppressive effect on the generation of Ag-specific and functional CD8+ T cells in Cre− mice, and in the Cre− GR-TKO mice the CD8+ T cell responses were not restored (Fig. 6A−D). Together, these results demonstrate that the suppression of CD8+ T cells during an HSV-1 infection is not due to direct effects of stress or corticosterone on the T cells.

FIGURE 5. Corticosterone impairs CD8+ T cell responses independently of direct effects on T cells. A, Western blot for GR in sorted Thy-1+ thymocytes or sorted splenic DCs from Cre+ and Cre− mice. CD8+ T cell responses in cells from PLNs on day 5 postchallenge in corticosterone- or vehicle-treated mice were measured for (B) granzyme B expression, (C) the number of gB498−505-specific CD8+ T cells, and numbers of functional HSV-1 gB498−505-specific CD8+ T cells as assessed by (D) degranulation, and (E) IFN-γ production. Data are mean ± SEM; n = 2−7 mice/group. *p < 0.05.

FIGURE 6. Stress suppresses CD8+ T cell responses and limits control of viral replication independently of direct effects on T cells. CD8+ T cell responses in cells from PLNs on day 5 postchallenge in stressed or control (nonstressed) Cre+ GR-TKO mice (that lack GR in their T cells) or Cre+ littermates (that possess GR in their T cells) were measured for (A) granzyme B expression, (B) the number of HSV-1 gB498−505-specific CD8+ T cells, and numbers of functional HSV-1 gB498−505-specific CD8+ T cells as assessed by (C) degranulation, and (D) IFN-γ production. E, Infectious virus was quantified by plaque assay on footpads from stressed or control mice on day 5 postchallenge. Data are mean ± SEM; n = 5−8 mice/group. *p < 0.05.
Impaired control of viral replication is not due to direct effects of stress/corticosterone on T cells

We have determined that stress-induced corticosterone at the time of initial infection prolonged the time course of lesions in footpads, increased HSV load in footpads and PLNs, and reduced HSV-specific CD8+ T cell numbers and function via a mechanism in which corticosterone did not affect the T cells directly (Figs. 5, 6A–D). We next measured the amount of infectious virus in the footpads from the GR-T KO Cre+ and Cre− mice used in the previous experiment. Stressed Cre− mice had a 3.7-fold increase in viral PFU/footpad compared with nonstressed Cre− mice (Fig. 6E). Cre+ GR-TKO littermates that were stressed also had significantly more virus in their footpads, a 6.9-fold increase compared with nonstressed Cre+ GR-TKO mice (Fig. 6E). These data show that the presence or absence of GR in T cells had no impact on the amount of infectious virus in the footpads of stressed mice. Therefore, although T cell responses are diminished by stress by an indirect mechanism as shown previously, control of viral infection was also not due to effects of stress-induced corticosterone directly on T cells.

DC function is impaired by stress in vivo

Previous studies in our laboratory have demonstrated that corticosterone impairs DC maturation and Ag presentation, such that corticosterone-treated DCs are less able to prime naive or Ag-specific CD8+ T cells (27, 28). In the current study, we have determined that suppression of HSV-1–specific CD8+ T cell responses and control of viral replication in vivo were not due to direct effects of stress-induced corticosterone on T cells. We therefore asked whether stress impairs the function of DCs from HSV-infected mice, as DCs are critical for Ag-specific CD8+ T cell activation (19, 44–46). For these experiments, mice were subjected to stress for only 1 d prior to and 1 d after footpad HSV-1 challenge. DCs were enriched from the PLNs of stressed or nonstressed mice 24 h after challenge, and equivalent numbers of DCs were cocultured with CFSE-labeled HSV-specific CD8+ T cells from TCR transgenic HSV gB 498–505-T mice. T cell proliferation was assessed by the dilution of CFSE signal. Because the efficiency of DC enrichment from lymph organs can vary, we determined the precise percentage of DCs in each preparation to ensure the same number of DCs were present in each coculture. Naïve DCs alone or DCs pulsed with a control peptide (OVA 257–264) did not induce proliferation of CD8+ HSV-1 gB T cells (Fig. 7A, 7B). However, DCs from the PLNs of stressed, HSV-infected mice were substantially less efficient (~63% reduction) at inducing proliferation as compared with DCs from nonstressed mice (Fig. 7C–E), demonstrating that stress had impaired the ability of these DCs to prime Ag-specific CD8+ T cells. These data provide strong evidence that the observed suppression of Ag-specific CD8+ T cell responses in animals undergoing psychological stress is due to impairments in DC function that occur at the earliest stages of infection.

Discussion

From our results, we can begin to construct a chronology of the suppressive effects of stress/glucocorticoids on the immune response to a cutaneous footpad HSV infection and the early role of DCs in this response. We have demonstrated in vivo that stress experienced at the onset of infection impaired DC function. After stress had been terminated, HSV-specific CD8+ T cell priming and functions, which are dependent on signals from DCs, were subsequently impaired. Stress at the initiation of infection also resulted in an increased amount of virus in the draining LNs early postinfection and later in the footpads, and promoted a more aggressive pathology indicated by earlier onset and delayed resolution of skin lesions. The timing and limited duration of the restraint stress used in this study, the results from GR-TKO mice lacking GR on T cells, and the blockade of the suppressive effects by the GR antagonist together support our hypothesis that elevated glucocorticoids acting early in an infection on the function of DCs can have lasting effects on adaptive immunity and viral pathology.

Previous reports documenting the effects of psychological stress on adaptive immunity used daily sessions of stress over a prolonged period, beginning up to 4 d prior to infection and continuing for as long as 8 d postinfection spanning the T cell expansion phase of the cellular immune response (12–15, 41). In the current study, exposure to stress was limited to one session 24 h prior to viral challenge and either one or three daily sessions postchallenge, ending prior to detectable infection-induced T cell expansion. Serum corticosterone levels are increased 5- to 10-fold during...
stress and then return to baseline within 4 h of cessation of stress (47). Thus our results delineate the impact of stress/glucocorticoids on the earliest stages of the generation of an antiviral immune response and the consequences after stress has been stopped and serum corticosterone levels reduced.

Clearance of HSV from the footpad is delayed when CD8+ T cells are depleted in mice (40). This observation is similar to our findings of increased viral loads, indicating reduced HSV clearance, and more aggressive pathology in mice that had been stressed early during infection. The requirement for DCs in the initial stages of an antiviral response is illustrated by the report of 100% mortality of mice depleted of DCs prior to footpad HSV infection (22). Thus, it is clear that DCs and CD8+ T cells play major roles in protection against and clearance of HSV. Although it has been established that chronic psychological stress substantially suppresses CD8+ T cell responses and exacerbates infection, no distinctions have been made regarding the extent to which this suppression is due to direct effects of stress-induced glucocorticoids on T cells themselves versus effects on DCs required for T cell activation. One cannot readily make that distinction in mice subjected to stress or receiving exogenous corticosterone or pharmacologic analogs, as all mammalian cells bear GR, including DCs (1–3). Even if one compares responses of T cells isolated from stressed versus nonstressed mice, or T cell preparations treated ex vivo with glucocorticoids, the unavoidable presence of contaminating APCs could confound interpretation of the results. The use of GR-TKO mice in our studies circumvented these issues. The GR-TKOCre+ littermates used as controls assured that all mice were otherwise genetically identical, and each mouse for every experiment was doubly screened for controls assured that all mice were otherwise genetically identical, and each mouse for every experiment was doubly screened. Using just one 16 h session of stress prior to HSV infection, using a psychological stressor with no physical injury to mice, the priming of CD8+ T cells by conventional CD11b+/CD8+ DCs in cutaneous HSV infections (56, 57). Recent ongoing work in our laboratory indicates that both the CD8α+ DCs and plasmacytoid DC subsets are preferentially highly vulnerable to the effects of stress-induced corticosterone, and that cross-priming of Ag-specific CD8+ T cells is significantly impaired in stressed mice in a GR-dependent manner (manuscripts in preparation).

Our earlier studies provide insight into the cellular and molecular mechanisms by which stress-induced glucocorticoids suppress DC function (27–29). These previous studies demonstrated that corticosterone renders DCs less efficient for priming Ag-specific CD8+ T cells in vivo and impairs Ag presentation by virus-infected DCs. Impaired Ag presentation by DCs is not due to altered expression, loading, or trafficking of MHC class I molecules or MHC class I-peptide complexes, but is due to a reduced efficiency in the generation of antigenic peptides prior to their entry into the endoplasmic reticulum, implicating a defect or alteration in protein processing. We have also shown that corticosterone impedes the ability of DCs to produce proinflammatory cytokines (IL-6, IL-12, and TNF-α) in response to TLR stimulation (27).

Our results do not preclude direct effects of stress on T cells or modulation of DCs by other neuroendocrine-derived products. Stress-induced opioids and products of the central and sympathetic nervous systems are immunomodulatory (58–61) and can suppress antiviral T cell responses (62). However, most studies have not segregated the effects of these neurochemicals on specific immune target cells, and little attention has been given to DCs. Moreover, the sympathetic nervous system is typically associated with acute fight or flight stress responses, whereas our model results in sustained elevation of systemic glucocorticoids. Notably, the results of our study show that the stress-induced suppression of the CD8+ T cell responses was mimicked by corticosterone and blocked by the administration of the GR antagonist, illustrating a hypothalamic-pituitary-adrenal axis and glucocorticoid-mediated mechanism. Our previous reports also demonstrate that the impairments of Ag presentation and other DC functions by corticosterone were also blocked by the GR antagonist (27, 28), further supporting a glucocorticoid-mediated mechanism underlying our current observations. Glucocorticoid-induced apoptosis has been reported for certain lymphoid cells (63), which could account for some aspects of stress-impaired immune responses. However, despite the presence of GR in DCs, we have observed no apoptosis, loss of viability or a reduction in numbers of DCs that were treated with high concentrations of corticosterone for up to 48 h in vitro (27). Furthermore, DCs from stressed mice in our current studies, in equivalent numbers as those from nonstressed mice, were clearly deficient in their ability to prime Ag-specific CD8+ T cells. Our results also do not rule out a role for potential indirect effects of glucocorticoids on DCs via other cell types, such as monocytes/macrophages, or the effects of stress on cells other than DCs, that could contribute to stress-induced immunosuppression. It is likely that impairment of DCs by stress/glucocorticoids in vivo is influenced by both direct and indirect mechanisms(s). However, the evidence for impaired DC function that we show here as well as previous studies mentioned previously illustrating direct glucocorticoid responsiveness of DCs strongly support that DCs in vivo are directly impaired by stress/glucocorticoids.

Overall, this study highlights the critical importance of early events in the generation of an antiviral immune response and the susceptibility of these events to stress-induced immunosuppression. Impaired DC function in stressed individuals could increase...
their susceptibility to disease or weaken their ability to respond to vaccination. Stress during exposure to a pathogen could result in a prolonged course of illness with more severe symptoms. Furthermore, the timing of the stress is relevant in considering the effects of stress on recovery from disease. Individuals who temporarily experience stress and simultaneously become infected by a pathogen may compromise their ability to mount an effective immune response, even after the stressor is removed. This immunosuppression could buy time for a pathogen to replicate and spread, making it more difficult to clear when the immune system is able to respond.

As glucocorticoids are widely and routinely used clinically for a variety of medical conditions, these findings are relevant to the basic understanding of the mechanisms by which these agents may contribute to altered CD8+ T cell responses in patients with viral infections or tumors.

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Disclosures
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References


