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A Marked Reduction in Priming of Cytotoxic CD8+ T Cells Mediated by Stress-Induced Glucocorticoids Involves Multiple Deficiencies in Cross-Presentation by Dendritic Cells

John T. Hunzeker, Michael D. Elftman, Jennifer C. Mellinger, Michael F. Princiotta,1 Robert H. Bonneau, Mary E. Truckenmiller, and Christopher C. Norbury

Protracted psychological stress elevates circulating glucocorticoids, which can suppress CD8+ T cell-mediated immunity, but the mechanisms are incompletely understood. Dendritic cells (DCs), required for initiating CTL responses, are vulnerable to stress/corticosterone, which can contribute to diminished CTL responses. Cross-priming of CD8+ T cells by DCs is required for initiating CTL responses against many intracellular pathogens that do not infect DCs. We examined the effects of stress/corticosterone on MHC class I (MHC I) cross-presentation and priming and show that stress/corticosterone-exposed DCs have a reduced ability to cross-present OVA and activate MHC I-OVA257-264-specific T cells. Using a murine model of psychological stress and OVA-loaded β2-microglobulin knockout “donor” cells that cannot present Ag, DCs from stressed mice induced markedly less Ag-specific CTL proliferation in a glucocorticoid receptor-dependent manner, and endogenous in vivo T cell cytolytic activity generated by cross-presented Ag was greatly diminished. These deficits in cross-presentation/priming were not due to altered Ag donation, Ag uptake (phagocytosis, receptor-mediated endocytosis, or fluid-phase uptake), or costimulatory molecule expression by DCs. However, proteasome activity in corticosterone-treated DCs or splenic DCs from stressed mice was partially suppressed, which limits formation of antigenic peptide–MHC I complexes. In addition, the lymphoid tissue-resident CD11b+CD24+CD8α− DC subset, which carries out cross-presentation/priming, was preferentially depleted in stressed mice. At the same time, CD11b+CD24-CD8α+ DC precursors were increased, suggesting a block in development of CD8α+ DCs. Therefore, glucocorticoid-induced changes in both the cellular composition of the immune system and intracellular protein degradation contribute to impaired CTL priming in stressed mice. The Journal of Immunology, 2011, 186: 000–000.
derlying mechanisms of neuroendocrine modulation of immunity, particularly during stress.

The anti-inflammatory properties of corticosteroids have been known and exploited clinically for decades. It is now well documented that antiviral T cell immune responses are compromised by glucocorticoids that are either stress induced (corticosterone, or cortisol in humans) or pharmacologically administered (such as dexamethasone or other synthetic analogs) (23–26). Stress suppresses CD8+ T cell activation, proliferation, cytokine production, and trafficking and impairs viral clearance (24, 27–30). Exposure to stress during an infection can have dire consequences for the survival of the host, because stress-induced changes in T cell responses resulted in profound increases in mortality rates from HSV, Theiler’s murine encephalomyelitis virus, and SIV infections (31–33). Although these and other reports have implied that stress/gluocorticoids may act directly on the T cells, Ag-specific CD8+ T cell activation is dependent on instructions from APCs, primarily DCs (34, 35). Previous studies have shown that synthetic pharmacological glucocorticoids suppress the generation, maturation, and immunostimulatory properties of DCs in animal models and humans (36–40). We previously reported that exposing DCs to physiological “stress levels” of corticosterone reduces the ability of DCs to present endogenous viral Ag in the context of MHC I via a mechanism that limits the generation of antigenic peptides, leading to impaired activation of CD8+ T cells via the “classical” direct Ag presentation pathway (41, 42). We also reported that corticosterone inhibits TLR-induced intracellular MHC II trafficking and costimulatory molecule expression in DCs, thus reducing their ability to prime an Ag-specific CD8+ T cell response (40). In further studies using a murine model in which T cells lacked GR, we reported that DCs, and not T cells, are likely to be the primary targets for stress/gluocorticoid-mediated immunosuppression early during a cutaneous HSV infection, resulting in subsequent impairments of HSV-specific CTL responses and exacerbated pathology (30). CTL-mediated immunity to HSV and many other viral infections is reported to require cross-presentation of Ag (12, 43–48). Indeed, a number of systems in which neuroendocrine modulation of T cell activity has been described likely require cross-presentation as the obligatory mechanism for CD8+ T cell priming. Nonetheless, no aspects of the cross-presentation and priming pathway have been examined directly in these systems in the context of neuroendocrine modulation. Moreover, although DCs have been shown to be vulnerable to stress/glucocorticoids, there have been no studies that have addressed any aspect of neuroendocrine modulation of cross-presentation and priming by DCs in any in vitro or in vivo system.

In this study, we show that stress/corticosterone impairs cross-presentation of OVA by DCs, leading to diminished OVA-specific CD8+ T cell responses. We further dissected the effects of stress and glucocorticoids on the steps involved in cross-presentation/priming including Ag donation, Ag uptake by DCs, and proteasomal processing by DCs, as well as the repertoire of DC subsets in stressed and nonstressed animals. We provide evidence that the impairment of cross-presentation and priming that we observed is caused by at least two contributing mechanisms—an intracellular reduction in proteasomal function that diminishes the production of antigenic peptide and a change in the composition of DC subsets that reduces the CD8+ DC population responsible for cross-priming. These findings add new insight into basic mechanisms by which chronic psychological stress and elevated glucocorticoids impair Ag-specific CTL responses by interfering with cross-presentation by DCs—a process with broad implications for immunity to many diseases.

### Materials and Methods

#### Mice

Male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), B2-microglobulin knockout (B2m−/−) mice, OT-I/RAG transgenic mice (49, 50), and congenic B6.SJL mice expressing CD45.1 (Taconic Farms, Germantown, NY) were maintained in specific pathogen-free conditions. OT-I/RAG and B6.SJL mice were bred to generate F₁ offspring containing OVA257–264-specific transgenic CD8+ T cells that express CD45.1. All animals were maintained in microisolation cages and treated in accordance with the National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care International Care regulations. All animal-related experiments and procedures were approved by the Penn State Hershey Institutional Animal Care and Use Committee.

#### Restraint stress model

Using a well-established restraint stress procedure (51), mice were placed individually in well-ventilated chambers that confine their motion without squeezing or compression such that they can move forward or backward but cannot turn around. This procedure increases circulating levels of corticosterone 5- to 10-fold (10−6 M) (52) with no physical component that could alter or compromise immune responses. Mice were restrained for four daily sessions beginning 3 d prior to immunizations, 16 h/session beginning 3 h into the dark cycle.

#### Cells and viruses

The STBK-1 cell line-deficient in β2m has been described previously (53). The DC line, DC2.4, was provided by Dr. J. Bennink (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD).

#### Corticosterone treatment

Unless otherwise indicated, cells were treated with physiologically relevant “stress levels” (10−6 M) corticosterone (52) or vehicle (0.1% ethanol) in the culture media for 24–48 h and washed free of corticosterone prior to further procedures. 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 up to 4 d. These methods, kinetics, dose responses, and GR dependency for corticosterone effects have been described previously (40, 42).

#### GR antagonist administration

Mifepristone (Sigma-Aldrich) dissolved in sesame oil (MP Biomedicals) was administered by s.c. injections at a dose of 25 mg/kg. Control mice received sesame oil alone (30). Treatment began 1 d prior to the first restraint stress session and was administered each day thereafter, 2 h prior to the start of stress.

#### Osmotic loading of β2m−/− splenocytes with OVA or fluorescent beads

Single-cell suspensions of splenocytes from β2m−/− mice were obtained by collagenase D digestion of the spleens (2 mg/ml; Roche, Basel, Switzerland), homogenization though 70-mm nylon filters (BD Biosciences, San Jose, CA), and RBC lysis with Tris ammonium chloride. The cells were loaded with OVA (Calbiochem, San Diego, CA) via osmotic shock as described previously (10). Briefly, the cells were incubated for 10 min at 37°C with 10 mg/ml OVA in a hypotonic solution (RPMI 1640, 0.5 M sucrose, 20 mM HEPES, and 10% polyethylene glycol 1000 [MP Biomedicals; pH 7.2]), followed by incubation for an additional 2 min in hypotonic solution (60% RPMI 1640 and 40% H2O). Following washes with cold PBS, the cells were gamma irradiated (1350 rad) and injected into recipient mice at a dose of 2–3.5 × 107 cells/mouse. For some experiments, β2m−/− cells were osmotically loaded as above with 0.2-mm-diameter fluorescent beads (Invitrogen, Carlsbad, CA). Prior to loading, the beads were washed in cold PBS and sonicated in hypertonic solution.

#### Flow cytometry

Fluorescently labeled and biotinylated Abs were purchased from e Bioscience (San Diego, CA), BD Biosciences, or BioLegend (San Diego, CA). The Abs used for identifying cell phenotypes were against Vα2 (clone...
DC2.4 cells were treated for 24 h with corticosterone or vehicle, thoroughly washed as described above, and incubated with 10 mg/ml OVA for 60 min at 37°C or OVA257–264 peptide for 40 min at room temperature. Control cells were fixed with 4% paraformaldehyde prior to incubation with OVA or OVA257–264 peptide. After thorough washes to remove OVA, equal numbers of DC2.4 cells (2 x 10^5) were cocultured for 12 h with OVA257–264-specific B3Z T cells, and β-galactosidase was measured colorimetrically using chlorophenol red β-d-galactopyanoside substrate (55).

Ex vivo cross-presentation assay

Spleenic DCs from stressed or control C57BL/6 mice were obtained by collagenase D digestion as above, and DCs were enriched by magnetic cell sorting or noncoated or pan-DC microbeads on an AutoMACs cell sorting system (Miltenyi Biotec). CD8^+ T cells were purified from OT-I SJL spleens using magnetic beads and labeled with CFSE. A total of 10^5 DCs were cocultured with 5 x 10^4 CD8^+ OT-I cells at 37°C, and proliferation of the OT-I cells was measured by flow cytometry.

In vivo cross-presentation assay and in vivo killing assays

In methods described by us previously (57), OT-I splenocytes were labeled with CFSE and adoptively transferred via the tail vein into recipient C57BL/6 mice. One day later, the mice were immunized by ip injection with 10^6 STBMK-1 cells that were infected with rVV-OVA and treated with UV-C and psoralen to prevent viral replication (58). Three days after immunization, spleens were harvested to obtain single-cell suspensions. The adoptively transferred OT-I cells were identified by either Vα2 (OT-I/ RAG) or CD45.1 (OT-I SJL). expression, and their proliferation was measured by assessing the decrease in CFSE fluorescence intensity by flow cytometry.

The in vivo killing assay was performed as described previously (59, 60). Briefly, “target” cells were prepared from splenocytes from B6.SJL (CD45.1) mice by pulsing with 1 μM either OVA257–264 peptide or HSV glycoprotein B (gB)748–505 control peptide and labeled with 5 or 0.5 mM CFSE. Equivalent cell numbers of each were adoptively transferred into stressed or nonstressed control C57BL/6 mice that had been immunized with rVV-OVA and treated with UV-C and psoralen. The adoptively transferred OT-I cells were identified by either Vα2 (OT-I/ RAG) or CD45.1 (OT-I SJL) expression, and their proliferation was measured by flow cytometric analysis of the loss of CFSE fluorescence intensity.

Endocytosis by DCs

BMDCs purified by CD11c magnetic bead sorting were incubated in prewarmed (37°C) phenol red-free media. To measure fluid-phase endocytosis, Lucifer Yellow (Molecular Probes) was added (300 μg/ml), and the cells were incubated at 37°C. Cells removed at 20-min intervals were transferred to ice-cold PBS/1% FBS in chilled, black 96-well plates (Dynex Technologies, Chantilly, VA), washed extensively in cold buffer, and lysed in PBS containing 0.3% Triton X-100. Lysates were analyzed for fluorescence (excitation at 428 nm and emission at 544 nm wavelengths) using a XFLuor Safire II plate reader (Tecan, Männedorf, Switzerland). Phagocytosis of fluorescent beads (Molecular Probes) by BMDCs was measured after 10-min incubation at either 37°C and visualized by fluorescence microscopy on an Olympus IX81 Deconvolution microscope (Olympus America, Center Valley, PA) with Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO).

Proteasome activity

DC2.4 cells were assayed for live-cell proteasome activity essentially as described previously (61). Briefly, cells were harvested, washed in PBS, and resuspended in 50 mM Tris (pH 7.4) containing 5 mM MgCl_2 and 2 mg/ml digitonin. Cells were transferred to a black 96-well flat-bottom plate in six replicate wells per treatment at a final concentration of 2 x 10^6 cells in 100 μl/well. The proteasome inhibitor lactacystin was used at 10 μM as a control. The fluorogenic substrate Suc-LLVY-AMC was added to wells at a final concentration of 100 nM. At timed intervals, fluorescence was measured at 380-nm excitation and 465-nm emission on a Victor model 1420 multilabel counter (UKB-Wallac, Gaithersburg, MD). Proteasome function was assessed in splenic DCs from control or stressed mice using the Proteasome-Glo Cell-based assay system (Promega, Madison, WI). DCs from control or stressed mice were purified using pan-DC microbeads. Briefly, 2.5 x 10^6 DCs in 100 ml were incubated for 10 min in a black 96-well plate with 100 ml of the appropriate substrate to measure the trypsin, chymotrypsin, and caspase-like activities of the proteasome. Samples were read on a Synergy HT reader with KC4 software (Bio-Tek Instruments, Winooski, VT).

Statistical analysis

Statistical significance was determined by unpaired Student t test using StatView 5.0.1 software (SAS Institute, Cary, NC), and p < 0.05 was considered significant.

Results

Corticosterone suppresses cross-presentation in vitro

To examine the cross-presentation of MHC I-restricted exogenous Ag, we used DC2.4 cells, because these cells were the first clonal DC cell line developed and were described for their ability to cross-present Ag (54). Cells were pretreated for 24 h with 10^-5 M corticosterone or vehicle (0.1% ethanol) as described previously (42). This concentration of corticosterone is comparable to stress-induced levels present in the circulation (52). The corticosterone was removed by washing prior to incubating the cells with soluble OVA, and excess OVA was removed prior to coculture with Ag-specific T cells. Thus, neither corticosterone nor OVA was present in the assay conditions. Activation of the OVA257–264-specific CD8^+ T cell hybridoma B3Z was reduced by >50% in cultures containing corticosterone-treated DC2.4 as compared with vehicle-treated DC2.4 (Fig. 1A). The B3Z cells required active uptake and processing of OVA, because DC2.4 cells that had been fixed prior to OVA incubation did not activate the T cells. Because exogenously applied peptide can bind to MHC I molecules on fixed cells, incubating fixed DC2.4 cells with OVA also ensured that no contaminating OVA257–264 peptide was present in the OVA preparation that could activate the B3Z T cells. The decreased ability of corticosterone-treated DC2.4 to activate B3Z T cells was not due to a reduction of bulk MHC I expression by the DCs, because both vehicle- and corticosterone-treated DCs that were fixed and pulsed with OVA257–264 peptide activated the B3Z T cells equally. Moreover, we previously demonstrated that corticosterone treatment of DCs does not reduce the overall expression of bulk surface MHC I molecules (42).

Stress impairs cross-priming in a GR-dependent manner in vivo

To extend our findings on corticosterone-induced impairment of cross-presentation in vitro, we examined the effect of psychological stress on cross-priming in vivo using a system in which Ag can only be cross-presented. Mice received four overnight sessions of restraint stress, a procedure that is well documented to increase circulating levels of corticosterone 5- to 10-fold (to 10^-6 M) with no physical injury or components that could skew immune responses (52). One hour before the start of the final stress session, stressed or control mice received i.v. injections of OVA-loaded β2m^- spleenocytes, which have no stable surface MHC I molecules and are unable to directly present Ag. Twelve hours later, splenic DCs were purified, and equal numbers were cocultured with freshly isolated OVA257–264-specific OT-I SJL CD8^+ T cells (OT-I cells). DCs from rVV-OVA-infected or naive mice served as positive and negative controls, respectively, for OT-I cell proliferation. DCs from stressed mice induced ~50% less proliferation of the OT-I cells as compared with equivalent numbers of DCs from nonstressed mice (Fig. 1B, 1C).
Mice that were stressed and also given the GR antagonist mifepristone or vehicle were then immunized with OVA-loaded β2m−/− splenocytes, and the induction of OT-I cell proliferation by splenic DCs isolated from the mice was measured as described above. Similar to the results shown above, proliferation of OT-I cells induced by DCs from stressed mice that received vehicle was reduced by ~50% as compared with DCs from vehicle-treated nonstressed mice (Fig. 1D). However, blockade of the GR in the stressed mice restored cross-presentation to the levels observed in the vehicle-treated nonstressed mice. Mifepristone treatment alone had no significant effect on cross-presentation. These results indicate that stress-induced suppression of cross-presentation is mediated in a GR-dependent manner.

Stress incapacitates the in vivo cytotoxicity of endogenous CD8+ T cells

The ultimate outcome of cross-presentation and priming of CD8+ T cells is the generation of an endogenous CTL response against target cells bearing the specific cross-presented Ag in vivo. To determine the impact of stress on this outcome, mice received rVV-OVA–infected β2m−/− STBKM-1 cells, UV/psoralen treated to prevent further viral replication (58), after the third of four sessions of stress, as described above. Direct infection of mice with rVV-OVA, or naive mice that were undisturbed, served as positive and negative controls, respectively. One week after the last stress session, OVA257–264 or gB498–505-pulsed, CSFE-labeled target cells were adoptively transferred by i.v. injection into all groups, and the endogenous CTL response was measured 4 h later. Notably, this method allows for distinguishing the effect of stress on cross-priming by DCs without exposing the target cells to stress or glucocorticoids. We found that mice exposed to stress at the time of immunization exhibited an 85% reduction in the killing of the Ag-specific target cells as compared with killing of target cells in nonstressed mice (Fig. 2). No killing of target cells bearing the control Ag HSV gB498–505 was detected. These results demonstrate that the stress-induced impairment in the ability of DCs to cross-present Ag results in substantially deficient Ag-specific T cell priming and killing of target cells in vivo.

Corticosterone pretreatment does not alter Ag donation in vivo

The results presented above show that physiological “stress levels” of corticosterone suppressed cross-presentation of soluble exogenous OVA in vitro, that psychological stress suppressed cross-presentation in vivo in a GR-dependent fashion, and that the Ag-specific cytolytic activity of endogenous CD8+ T cells is greatly reduced in stressed mice receiving Ag that can only be cross-presented. To address potential mechanisms for these observations, we first assessed the effect of corticosterone on the ability of Ag donor cells (cells that cannot present Ag or prime T cells) to transfer Ag to APCs, which then cross-prime T cells in vivo. STBKM-1 (β2m-deficient) cells were cultured for 48 h with either 10−6 M corticosterone or vehicle and then infected with rVV-OVA. Viral replication was then stopped with UV-C and psoralen, and the cells were injected into mice that had received CFSE-labeled OT-I cells via i.v. injection 1 d prior. We have previously determined that corticosterone treatment of cells does not alter the efficiency of rVV-OVA infection or the amount of virally produced Ag within the cells (Ref. 42 and our unpublished data); thus, Ag load in the donor cells was equivalent. Three days later, spleens
were removed, and the in vivo proliferation of the adoptively transferred OT-I cells was measured. Fig. 3A and 3B shows that similar levels of OT-I cell proliferation occurred whether or not the donor STBKM-1 cells had been subjected to corticosterone. Direct infection of mice with rVV-OVA (positive control) induced more than twice the amount of proliferation of OT-I cells as that induced by cross-presented Ag; as expected, minimal proliferation occurred in naive mice. Corticosterone also had no effect on Ag donation using STBKM-1 cells into which OVA was loaded by electroporation prior to immunization (data not shown). These results demonstrate that corticosterone pretreatment of Ag-donating cells does not affect their ability to transfer Ag to the cross-priming cells.

Neither corticosterone nor psychological stress alters Ag uptake in vitro or in vivo

As we determined that corticosterone does not alter Ag donation in vivo, we examined the uptake of Ag by DCs. A combination of in vitro and in vivo experiments were used to measure three of the primary active metabolic modes of endocytosis by DCs—fluid-phase uptake, fluid-phase/receptor-mediated uptake, and phagocytosis. We have previously reported that endocytosis of soluble Ag (FITC-OVA) occurs with essentially identical kinetics in both corticosterone- and vehicle-treated BMDCs (40). Similar results were obtained with the DC2.4 cell line (data not shown). Internalization of FITC-OVA likely involves both receptor-mediated and fluid-phase uptake (62). To directly assess fluid-phase uptake, which predominantly involves macropinocytosis, we measured uptake of Lucifer Yellow at 37°C in vehicle or corticosterone-treated BMDCs (63). We found that fluid-phase uptake was not affected by pretreatment with corticosterone. No uptake occurred at 4°C under either treatment condition as expected for an active metabolic process (Fig. 4A). Quantification of phagocytosis of particulate Ag (fluorescent beads) by BMDCs (Fig. 4C) showed that ~50% of both the corticosterone- and vehicle-treated BMDCs...
and injected into normal mice just prior to the last of four restraint
splenocytes from approx. 10% had two beads per cell. The bead-positive BMDCs contained three or more beads per cell, whereas no beads were present in the cells maintained at 4˚C. Corticosterone also did not affect the number of beads that were taken up on a per cell basis. In both corticosterone- and vehicle-treated BMDCs, the majority (60%) of the bead-positive BMDCs contained three or more beads per cell, whereas ~30% of bead-positive BMDCs contained one bead, whereas ~10% had two beads per cell.

To confirm and extend these observations in our stress model, splenocytes from β2m<sup>−/−</sup> mice were loaded with fluorescent beads and injected into normal mice just prior to the last of four restraint stress sessions. Bead loading of the β2m<sup>−/−</sup> cells was confirmed by flow cytometry (data not shown). Fourteen hours later, the percentage of bead-positive fluorescent DCs from the spleens of recipient mice was determined by flow cytometry. To ensure that the bead-positive DCs were from the recipient mice and not the donor cells, we analyzed only MHC I (H-2K<sup>b</sup>/H-2D<sup>b</sup>),CD11c<sup>+</sup>, pDC, and CD8<sup>+</sup> subsets, with an increased percentage in the subsets obtained from stressed mice (Fig. 5C). Taken together, these in vitro and in vivo results demonstrate that the corticosterone- or stress-mediated suppression of cross-priming is not due to decreased Ag uptake by Ag-presenting DCs. In fact, in vivo phagocytosis by DCs appears to be stimulated by stress.

**Stress does not affect the expression of costimulatory molecules**

Differences in costimulation of T cells by DCs could contribute to altered T cell responses in vivo (65). Using the method described above and in Fig. 5, costimulatory molecule and MHC II (I-Ab) expression by the DCs that had ingested fluorescent beads “donated” from β2m<sup>−/−</sup> splenocytes in vivo was measured. In these experiments, DCs that ingest the beads serve as a proxy for DCs that could potentially cross-present Ag. The resident splenic DCs were identified using flow cytometry by excluding CD90.2<sup>+</sup>, CD11b<sup>+</sup>, and NK1.1<sup>+</sup> cells and gating on CD11c<sup>+</sup>MHC I<sup>+</sup> cells. The expression level of costimulatory/maturity molecules (CD80, CD86, CD40, and MHC II I-Ab) was then assessed on the bead-positive population. Bead-negative CD11c<sup>+</sup>CD90.2<sup>+</sup> T cells in the same samples (which do not express I-Ab or costimulatory markers), stained for each marker, served as the negative staining control for the assay. We found that the expression levels of each of the markers in bead-positive DCs were comparable between stressed and nonstressed control mice (Fig. 5D, 5E). Thus, the observed impairment of T cell cross-priming in stressed animals is not due to altered costimulatory molecule or I-Ab expression by the cross-presenting DCs.

**Corticosterone or restraint stress impairs proteasome function**

The results described above eliminate changes in Ag donation by donor cells, as well as Ag uptake and costimulatory molecule expression by the Ag-presenting DCs, as explanations for the suppression of cross-presentation and priming that we observed. Previous data from our laboratory suggests that corticosterone impairs the generation of antigenic peptides prior to TAP transport in the direct presentation pathway by virus-infected DCs, implicating reduced protein proteolysis as a potential mechanism (42). Cross-presentation of OVA has been reported to be proteasome and TAP dependent (66, 67), and peptide generation is the rate-limiting step in the direct-presentation pathway (68). Therefore, we assessed chymotrypsin-like proteasome activity in vehicle- or corticosterone-treated DC2.4 cells. Using a live-cell assay (61), we found that corticosterone treatment of the cells suppressed the chymotrypsin-like proteasome activity ~20% compared with the vehicle-treated controls (Fig. 6A). The slopes are constant from the time of substrate addition for both corticosterone- and vehicle-treated cells, indicating the rates of substrate degradation reflect real differences in proteasome activity for the two populations, rather than nonspecific death of the corticosterone-treated cells. To confirm and extend these studies, we measured proteasome activity ex vivo in splenic DCs from control or stressed mice. As shown in Fig. 6B, the trypsin-, chymotrypsin-, and caspase-like activities of the proteasome were decreased by ~20% in DCs obtained from stressed mice compared with the nonstressed controls.
CD8+ DCs are preferentially decreased in stressed mice in a GR-dependent manner

The major lymphoid organ-resident DC subsets are characterized as CD11b+CD8α2 (hereafter CD11b+) and CD11b2CD8α+ (hereafter CD8+), with the CD8+ DC subset reported to be the primary phenotype capable of cross-presentation/priming (7–13). We examined these DC subsets from the spleens of stressed and nonstressed mice by flow cytometry. DCs were identified as CD11c+CD45RA2B2202NK1.12CD902CD192 to exclude both pDC and non-DC cell types that express CD11c. Subsets within this population were further identified using additional cell surface markers. In numerous independent experiments, we found that the CD8+ DC subset was routinely decreased by 40–60% of the total DCs, whereas the CD11b+ DC subset was typically unchanged (Fig. 7A, 7B). This preferential reduction of CD8+ DCs was reproduced in mice that were administered corticosterone in their drinking water at a concentration that leads to “stress levels” of serum corticosterone for 2 d (data not shown). We confirmed that the stress-induced reduction of the CD8+ DC population was GR dependent, because the percentages of this subset in stressed mice treated with the GR antagonist mifepristone did not differ from control mice (Fig. 7C).

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from the same samples stained for the respective markers (negative control). E, Combined data (mean ± SEM of four mice); *p < 0.05.
Stressed mice have differential levels of CD8⁺ DC precursors as compared with control mice

An immediate precursor to the CD8⁺ DC subset recently has been characterized by the expression of CD24 and lack of CD8α (CD24⁺CD8⁻) (69). We examined the prevalence of these DC subsets in spleens by flow cytometry and found that splenic DCs in nonstressed mice were composed of ~7% CD24⁺CD8⁻ DC precursors and ~15% CD24⁺CD8⁺ DCs. However, splenic DCs from stressed mice consisted of significantly greater percentages of the CD24⁺CD8⁻ precursor DCs (~18%) than nonstressed mice with a concomitant decrease in the CD24⁺CD8⁺ DC population (~7%), representing ~2-fold change in the distribution between CD24⁺CD8⁻ precursors and CD24⁺CD8⁺ DCs in control versus stressed mice (Fig. 8). Thus, in terms of the CD24⁺ DC population, 68% of these cells were CD8⁻ in nonstressed mice, but only 28% of these cells expressed CD8 in stressed mice, with a nearly reciprocal redistribution of CD8⁻ DCs. These results suggest that stress may hinder the development of CD8⁺ DCs from their CD24⁺CD8⁻ precursors in spleen.

Discussion

The complex interactions and mechanisms involved in neuroendocrine regulation of immune responses continue to be unraveled. A growing body of in vitro and in vivo evidence demonstrates that DCs are vulnerable to glucocorticoids, whether stress induced or pharmacologically administered (36–42), and that this vulnerability of DCs, apart from any direct effect of glucocorticoids on T cells, can have an adverse impact on antiviral T cell responses and disease pathology (30). In the current study, we tested the relevance and underlying mechanisms of these observations with respect to the efficacy of MHC I cross-presentation and CD8⁺ T cell priming—a process that is required to generate CD8⁺ T cell immune responses against a variety of viral infections and other disease conditions. Using systems in which Ag can only be cross-presented, this study demonstrates that corticosterone and psychological stress-induced glucocorticoids substantially impair the cross-presentation of OVA and priming of OVA257–264-specific CD8⁺ T cells, ultimately incapacitating the Ag-specific killing of target cells by endogenous CTL in vivo. These impairments were prevented in mice that were administered GR antagonist, indicating that the impairment is mediated via stress-induced HPA axis activation. By isolating and testing effects of stress/corticosterone on steps of the cross-presentation pathway, we found that the DCs involved in the final step of cross-presenting Ag and priming CTLs are targets of stress and stress-induced glucocorticoids. We tested several possible mechanisms that could contribute to impaired cross-presentation by DCs and found that these defects were likely mediated, at least in part, by decreases in proteasome activity within DCs as well as a substantial deficit in splenic CD8⁺ DCs that may have resulted from a blockade in their differentiation from precursors. Such stress-induced
changes in Ag processing, Ag presentation, and DC subset repertoire early during an infection would have consequences for the development of T cell-mediated immunity, as has been shown in this paper and described previously (30). In this study, we demonstrate the vulnerability of DCs to stress and stress-induced glucocorticoids, illustrate the consequences of this vulnerability with respect to immune responses involving cross-presentation and CD8+ T cell priming, and provide new insight into the underlying mechanisms involved.

Our observation that presentation of exogenous OVA by DCs in vitro was reduced by >50% after exposure to corticosterone indicates that a substantial intrinsic defect exists within a subcellular process or processes involved in this pathway of Ag presentation. We have previously reported that corticosterone modulates surface expression of TLR ligand-induced costimulatory molecules on BMDCs (40). In the current study, however, we examined cross-presentation using DC2.4 cells, which are not susceptible to corticosterone-mediated modulation of costimulatory molecule expression (unpublished observations). Moreover, we measured cross-presentation by these cells via activation of the B3Z cell line, an exquisitely sensitive OVA257–264-specific T cell hybridoma that does not require costimulation or other activation signals. Thus, corticosterone-mediated changes in costimulatory molecule expression on DC2.4 cells are unlikely to account for the reduction of B3Z T cell activation that we measured. An overall reduction in cell surface expression of MHC I or changes in the stability of OVA257–264–MHC I complexes could also account for the reduction in Ag presentation that was observed, but neither of these factors are affected by corticosterone exposure (42). The subcellular mechanisms involved for the processing and presentation of exogenous OVA via the cross-presentation pathway have been extensively studied in vitro and have revealed that OVA is processed via the cytosolic pathway that is TAP and proteasome dependent (66, 67). Internalization of OVA involves fluid-phase pinocytosis and receptor-mediated endocytosis (70, 71), neither of which was affected by exposure to corticosterone. Following internalization, OVA is recycled from endosomal compartments to the cytosol in a process that may use components of the ER-associated degradation pathway (72). However, none of the assays for measuring these processes was of sufficient sensitivity to reliably quantify OVA release into the cytosol of DCs (data not shown).

Once released into the cytosol, OVA is processed into peptides via the action of the proteasome (66, 67). We previously described that corticosterone-induced impairment of MHC I direct presentation of virally produced OVA peptide occurred via effects on protein processing, with no effects on TAP transport of peptides into the endoplasmic reticulum or further loading, transport, or stability of peptide–MHC I complexes (42). In the current study, we expanded on those studies to show that proteasome activity is reduced in corticosterone-treated DC2.4 cells as well as in splenic DCs from stressed mice. Ag presentation is sensitive to changes in proteasomal activity. This is because the processing of protein into antigenic peptides is the rate-limiting step in the generation of MHC I–peptide complexes in the direct-presentation pathway (73); thus, any reduction in proteasome activity will reduce the number of peptide–MHC I complexes reaching the cell surface. Substantial or complete inhibition of proteasomes results in rapid cell death (61), whereas neither high concentrations of corticosterone nor stress resulted in apoptosis or death of DCs (Ref. 40 and our unpublished observations). The efficiency of peptide generation from endogenous proteins is low, conservatively calculated to be approximately one peptide produced for every 3000 substrate proteins (74). By these calculations, a 20% block in proteasome activity could result in ~5.2 × 10^8 substrate molecules/day that would not get degraded and, therefore, would be unavailable as sources of peptide for presentation by MHC I. Moreover, different peptides are liberated from the proteasome with vastly different efficiencies (i.e., some peptides are generated at a much lower frequency than others on a per-protein molecule basis [75]). This difference can affect a response such that some or even many peptide determinants may drop below a detectable threshold with even just a 20% drop in overall proteasome function. The efficiency of cross-presentation is even lower than that of direct presentation (e.g., incubating cells with concentrations of soluble OVA as high as 10 mg/ml produced far fewer surface OVA257–264–MHC I complexes than those generated by direct infection of the cells with OVA-producing viral constructs, as evidenced by the ability to detect surface complexes produced by direct presentation [42] but not cross-presentation with a Kb-OVA257–264-specific Ab). Thus, cross-presentation of exogenous Ags may be even more sensitive to changes in proteasomal activity. It has also been suggested that proteasomes directly associated with endosomal compartments, such as macropinosomes or phagosomes, process Ag that is released into the cytosol, and the resulting peptides are shuttled back into the original compartment via the action of the TAP complex (76, 77). It is possible that only the subpopulation of proteasomes that are endosome-associated participate in cross-presentation, thereby further magnifying the effects of lowered proteasome activity. We also wish to point out that proteasome activity was reduced after corticosterone or stress was removed, indicating a relatively long-lasting effect. This lingering suppression of proteasome activity could reduce the duration of Ag presentation and/or the availability of surface complexes on DCs to levels that are below the threshold required for T cell activation. Because degradation of Ag by the proteasome can occur within minutes of Ag internalization by DCs, and because both the availability and duration of Ag presentation are critical factors that regulate the magnitude and potency of a CD8+ T cell response (78), a sublethal reduction in proteasomal activity within DCs during an infection could sufficiently reduce antigenic surface complex formation to impair or delay the priming of T cells.

We further expanded our studies to examine the effects of stress and glucocorticoids on the induction of CD8+ T cell responses via the cross-priming pathway in vivo. Using a system in which Ag can only be cross-presented, we examined the effect of stress on cross-presentation by DCs ex vivo and found that splenic DCs from stressed animals induced ~50% less Ag-specific T cell proliferation in a GR-dependent manner, compared with proliferation induced by equivalent numbers of DCs from nonstressed controls. The most important biological outcome of cross-presented Ag is the efficient induction of a primary Ag-specific CTL response within the host. When we examined this outcome, we found a marked 85% reduction in endogenous Ag-specific killing of target cells in stressed mice as compared with nonstressed mice. The method we used to measure in vivo CTL killing capacity distinguishes the effect of stress on the resident cross-presenting DCs and killing by CTL without subjecting the target cells to stress/glucocorticoids. These results are similar to our observations of the effects of stress on the generation of a CD8+ T cell response during a cutaneous footpad HSV infection (30), a process that has been shown to involve cross-presentation (44). In the HSV system, we ruled out a direct effect of stress-induced corticosterone on T cells (30), so a suppressive effect on DC-mediated cross-presentation stands as a likely mechanism for stress-mediated reduction of T cell priming in vivo.

In addition to the evidence for altered intracellular effects on DCs that we have shown in this paper and previously described (40, 42), we further considered the added complexity of the mechanisms required for cross-presentation and priming in vivo and the
effects of stress and corticosterone on these mechanisms. The donation of Ag, the uptake of Ag by the cross-presenting DCs, and expression of costimulatory molecules occur shortly postinfection in vivo, as reports have shown that DCs can activate T cells within 6 h after vaccinia or HSV infection (79, 80). Thus, stress at the time of an infection could alter any or all of these processes, with substantial consequences for T cell priming. We have already shown that experiencing stress early during an HSV infection impairs the subsequent HSV-specific T cell response even days after stress has been terminated and ruled out direct effects of stress/glucocorticoids on the T cells as an underlying mechanism (30). In the current study, we ruled out the possibility that exposure to corticosterone of the β2m-deficient cells we used as Ag donors in vivo alters the ability of these cells to donate Ag. We also demonstrated that DC-mediated uptake of Ag from these cells was not impaired in stressed mice and may actually be enhanced in some cases. The modulation of expression of costimulatory molecules on the surface of DCs is a potential mode of action of stress that is independent of the generation and display of peptide-MHC I complexes. We previously demonstrated that exposure of DCs to corticosterone interferes with TLR-mediated upregulation of costimulatory molecules in vitro, which resulted in reduced priming of CD8+ T cells in vivo upon immunization with peptide-pulsed DCs (40). However, we did not find an effect of stress on the expression of costimulatory molecules by splenic DCs in vivo in the current study. This apparent discrepancy likely results from the fact that corticosterone treatment does not alter costimulatory molecule expression by DCs in the absence of TLR ligation (40), and TLR ligands were not administered in our in vivo cross-priming experiments. Our cumulative evidence described in this paper indicates that direct effects of stress or corticosterone on donation of Ag to the DCs, uptake of Ag by the DCs, costimulatory molecule expression by the DCs, or the target cells of CTL killing are not responsible for the impairment of cross-presentation of Ag, priming of CD8+ T cells, and CTL killing that we observed.

DCs are a heterogeneous population of cells composed of various subsets with different developmental lineages and complex functions in both adaptive and innate immunity (81, 82). The most important APCs for cross-presentation are CD8+ DCs, which are required for protective immunity in certain viral infections (12, 43, 48, 83). We have shown in this study that stressed mice or mice given corticosterone in their drinking water have a marked depletion in the proportion of splenic CD11b+ CD8α DCs, whereas the proportion of CD11b+CD8α DC subset remains relatively stable. Moreover, although the CD8α DCs are decreased, we found that CD24+CD8α DCs, which have been recently characterized as the immediate precursors of terminally differentiated CD8α DCs (69), are increased in stressed mice. Because the CD24+CD8α precursor pool was reported to develop into CD8α DCs without dividing, one explanation for our results is that stress may be blocking the differentiation of CD8α DCs from their precursors, thus reducing the efficiency of cross priming in vivo. Although the precursors appear to have some ability to induce a primary Ag-specific T cell response, this response was less efficient than that produced by CD8α DCs.

Our in vivo results do not rule out potential modulation of DCs or Ag presentation by other stress-induced neuroendocrine or central and sympathetic nervous system products that are known to be immunomodulatory and can suppress T cell responses (17, 84–87). However, few studies have segregated the specific cellular targets of these stress-associated products or have examined DCs in particular. Furthermore, to our knowledge, this study is the first to examine the multiple steps involved in cross-presentation and priming. Sympathetic nervous system stress responses are typically acute “fight-or-flight” responses, and brief exposure to stress can boost certain immune responses (18–20, 88). The restraint stress model used in these studies results in sustained elevated systemic glucocorticoids, and we have shown in this paper and previously that the effects on DCs and CTL responses are mimicked by the administration of physiologically relevant levels of corticosterone and blocked by GR antagonist, demonstrating an HPA axis and glucocorticoid-mediated mechanism. Our results also do not rule out potential effects of stress/glucocorticoids on other cell types that could contribute to stress-induced impaired cross-presentation or modulate the cross-presentation/priming or developmental capacity of DCs. Thus, although our findings do not likely include all possible mechanisms for reduced cross-presentation by glucocorticoids, our results strongly support a direct influence of stress/corticosterone on DC functions. These effects include impaired Ag processing and presentation via modulation of proteasome activity and impaired differentiation of CD8α DCs in vivo, which cumulatively could contribute to the profound impairment of in vivo CTL responses to cross-presented Ag that we observed.

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Disclosures

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References